THE EFFECTS OF BIOACTIVE COMPOUNDS FROM THE MARINE MOLLUSC *Dicathais orbita* ON HUMAN REPRODUCTIVE CELLS AND HUMAN REPRODUCTIVE CANCER CELLS

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‘A book Must be the AXE for the frozen SEA within us’

Franz Kafka

For Simon

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ABSTRACT

Marine compounds which constitute the ancient purple dye, Tyrian purple have gained specific interest over the past few years for their biological specificity and cytotoxicity towards human cancer cells in comparison to primary cells. In particular, the indole-based compounds, 6-bromoisatin and tyrindoleninone from the Australia whelk, *Dicathais orbita*, are compounds of interest because of their anti-cancer activity and ability to induce apoptosis in several cancer cell lines, both *in vitro* and *in vivo*. Interestingly, the secretions from muricid marine molluscs are the source of a natural homeopathic remedy, 'Murex purpurea', sold for the treatment of a range of gynaecological disorders, including cancer of the uterus. However, to date the effects of these indole-based compounds on human reproductive cells is unknown. The objective of this project was to examine the effects of these natural indole compounds derived from *D. orbita*, on primary-derived female human granulosa cells, along with a series of female human reproductive cancer cells. The hypothesis of this research was that the novel bioactive compounds from *D. orbita* would exert selective cytotoxicity towards the reproductive cancerous cells while having minimal, or no effect on the reproductive primary cells.

In vitro cell screening assays are beneficial for examining the immediate cytotoxicity of novel compounds however an added benefit of screening on reproductive cells is that the effects of these compounds on certain cell functions, such as hormone synthesis can also be examined. Furthermore, the manipulation of steroid hormone synthesis using human chorionic gonadotrophin (hCG) can be induced to mimic the *in vivo* response of these cells. Therefore, a second aim of this thesis was to determine the effects of the muricid compounds on hormone synthesis by female reproductive cells.

To address the aims of this research initially the *in vitro* cell culture conditions were optimised and characterised using the choriocarcinoma JAr cell line. Cell metabolic activity and cell viability were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide (MTT), the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetazolium (MTS) and the crystal violet assays. Measurement techniques for hormone synthesis comparing the radioimmunoassay (RIA) and enzyme-linked immuno-sorbent (ELISA) assay were also examined, to establish the most reliable and reproducible assay. The optimised assays were then used to determine the effects of the bioactive compounds from *D. orbita* and several synthetic analogues of these indole-based compounds on human reproductive cells. Mechanisms of cell death were further investigated using apoptotic and necrotic assays along with examining changes in nuclear fragmentation and cell morphology, as key features of cell death mechanisms.

The optimised cell culture assay for the JAr cell line was a seeding density of 20,000 JAr cells per well and 2h cell adherence period, followed by incubation with0.5mg/ml MTT for 1h. The intra- and inter-assay coefficients of variation were 11.3% and 10.9% respectively. Human choronic gonadotrophin (1,000mIU/ml) significantly increased progesterone synthesis after 2h ($n=4$; $p< 0.05$) as determined by the RIA.

The synthetic compounds, indirubin and 6'6-dibromoindirubin, were not cytotoxic to the human choriocarcinoma JAr cell line, whereas, 5-bromoisatin (100µg/ml) significantly reduced cell viability by 50% (IC_{50}; 442µM) after 4h (n=3; $p<0.01$) and caused a significant reduction in JAr cell numbers at ≤ 100µg/ml for 6, 8 10 and 24h incubation ($p < 0.001$) as determined by the MTT assay. In a similar manner, indirubin had no effect on primary granulosa cell viability, whereas 5-bromoisatin significantly reduced cell numbers at concentrations greater than 10µg/ml. Both indirubin and 5-bromoisatin did, however, stimulate progesterone synthesis at low concentrations (0.01µg/ml) after 48h in granulosa cells derived from women with normal reproductive physiology. Indirubin and 5-bromoisatin (100µg/ml) did however inhibit estradiol synthesis by granulosa cells derived from women with abnormal reproductive physiology after 24h exposure. The results of this research therefore do not support the use of indirubin, 6'6-dibromoindirubin and 5-bromoisatin for the treatment of female reproductive cancers.

The semi-purified compounds tyrindoleninone and 6-bromoisatin, extracted from the muricid whelk, *D. orbita*, significantly decreased all three reproductive cancer cell lines, KGN, JAr and OVCAR-3 at a concentration at least 100-fold lower than in the primary reproductive cells although, hCG and cAMP afforded some protection against the cytotoxic effects of the compounds. Furthermore, this research confirmed that tyrindoleninone and 6-bromoisatin activated cell death in the KGN cancer cell line by apoptosis rather than necrosis. Progesterone secretion was either inhibited or not affected in primary granulosa cells from women with abnormal reproductive physiology when treated with these natural compounds both in the presence and absence of hCG. If these results are at all indicative of the *in vivo* response, the results of this study would not support the use of these compounds for women while

pregnant, or trying to conceive. Conversely though, the stimulatory effect on estradiol synthesis by primary granulosa cells (albeit only cells from women with normal reproductive physiology), along with the inhibitory and anti-proliferative effect on reproductive cancer cells, could potentially be advantageous for women during menopause as an alternative to hormone replacement therapy.

In conclusion, this study highlights the importance of *in vitro* cell culture assays for assessing the effects of both natural and synthetic compounds on reproductive cytotoxicity and hormone synthesis, providing essential data which can support current *in vivo* animal assays.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma at the university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Vicki S Edwards

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ABBREVIATIONS

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
AC	After Christ
ADME/Tox	Absorption, distribution, metabolism and elimination toxicity
AIHW	Australian Institute of Health and Welfare
ARAC	1-beta-D-arabinofuranosylcytosine
ART	Assisted Reproductive Technology
BC	Before Christ
BSA	Bovine serum albumin
CAD	Caspases activated DNase enzyme
CAM	Complementary and alternative therapy
cAMP	Adenosine 3',5'-cyclic monophosphate
CL	Corpus Luteum
COX	Cyclooxygenase
CDK	Cyclin-dependent kinase
CSIRO	Commonwealth Scientific and Industrial Research
DAG	Donkey anti-goat
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DBI	Diazepam binding inhibitor
DCM	Dichloromethane
DHA	trans-dehydroandrosterone
dbcAMP	Dibutyryl adenosine 3',5'-cyclic monophosphate
DIM	Diidoylmethane
DMEM	Dulbecco's minimum essential (or modified Eagle) medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonucleic acid enzyme dUTP 2'-Deoxyuridine 5'-Triphosphate
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor

ESI	Electro-spray ionisation mass spectrometry
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FSH	Follicle stimulating hormone
GC	Gas chromatography
GnRH	Gonadotrophin-releasing hormone
G-protein	Guanine nucleotide binding protein
GSK-3	Glycogen synthase kinase-3
hCG	Human choronic gonadotrophin
HGC	Human granulosa cells
HCl	Hydrochloric acid
HIV	Human immunodeficiency syndrome
HPLC	High performance (pressure) liquid chromatography
IC3	Indole-3-carbinol
IGF-I	Insulin-like growth factor I
ITS	Insulin,transferrnin apo-human and selenium sodium selenite
3βHSD	3-β-Hydroxysteroid dehydrogenase enzyme
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
LH	Luteinising hormone
LOX	Loxogenase
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetazolium
m/z	Mass to charge ratio
NAD	β-Nictoinamide adenine dinucleotide
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium hydrogen orthophosphate
$NaH_2PO_4.2H_2O$	Sodium dihydrogen orthophosphate
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance

NRS	Normal rabbit serum
NSAID	Non-steroidal inflammatory drug
OECD	Organisation for Economic Co-operation and Development
OTR	Oxytocin receptors
PBR	Peripheral-type benzodiazepine receptor
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PEG	Polyethylene glycol
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PMS	Premenstrual syndrome
PR	Progesterone receptor
P450	Family of oxidase cytochrome enzymes
P450scc	Side chain cleavage enzyme
RIA	Radioimmunoassay
ROS	Reactive oxygen species
rTdT	Recombinant terminal deoxynucleotidyl transferase enzyme
t_R	Retention time
SAP	Steroidogenesis activator polypeptid
SAR	Sheep anti-rabbit antibody
SCP_2	Sterol carrier protein 2
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride and sodium citrate solution
StAR	Steroidogenic acute regulatory protein
TLC	Thin layer chromatography
TUNEL	Terminal dUTP Nick End-Labelling
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Chapter 1 Review of the Literature

1.1 Introduction

Gynaecological diseases including cancers of the female human reproductive system, have been to known to humans since the ancient Egyptians and Greeks (Ricci, 1943). Over history and throughout different cultures there have been numerous descriptions of remedies and cures for different gynaecological diseases. Despite this cervical cancer today is the second most common gynaecological cancer among women worldwide (World Health Organisation, 2006). According to the World Health Organisation (WHO) approximately half a million new cases of cervical cancer are diagnosed each year (World Health Organisation, 2006). In 2003, the Australian Institute of Health and Welfare declared that 11,778 Australian women were diagnosed with breast cancer and that the incidence of other gynaecological cancers were, endometrial cancer = 1,613, ovarian cancer = 1,273 and cervical cancer = 725 (Australian Institute of Health and Welfare, 2006).

Besides cancer, there are numerous other gynaecological disorders that, although not necessarily life-threatening, increase the risk of the sufferer developing more chronic diseases. Polycystic ovary syndrome (PCOS) increases the sufferers risk of developing hypertension, obesity, diabetes and arteriosclerotic heart disease (Dursun, *et al.*, 2006). Furthermore, sufferers of PCOS often endure menstrual irregularities, infertility, acne and superfluous hair growth (hirsutism), which can adversely disrupt and affect a woman's quality of life (Kocak and Üstün, 2006). Women who suffer from the debilitating disease endometriosis, in which the endometrium tissue is found on the outside of the uterine cavity, often suffer multiple gynaecological

disorders including infertility and chronic pain of the pelvic region (Dawood, 1993; Olive and Schwartz, 1993; van Kaam, *et al.*, 2007).

Sufferers of more common gynaecological disorders including menorrhagia (irregular menstrual cycles) and dysmenorrhoea (painful menstruation) often go undiagnosed and untreated (Jia, *et al.*, 2006; Proctor and Farquhar, 2006). It is not uncommon for women suffering these conditions to seek unconventional and alternative therapies including homeopathic treatments, traditional Chinese medicines and nutraceuticals, such as vitamin and mineral supplements (Jia, *et al.*, 2006; Proctor and Farquhar, 2006). These alternative or complementary therapies and medications are predominately sourced from nature (Jia, *et al.*, 2006).

Long before Fleming's discovery of penicillin, ancient civilisations such as the Chinese, were using remedies derived from natural products to treat numerous diseases including gynaecological disorders and cancers (Needham and Gwei-Djen, 1962). Many of the drugs developed and sold today were originally discovered from natural species in fact, 30% of drugs on the market are derived from natural compounds or are synthetic analogues (Grabley and Thiericke, 1999). Furthermore, the biodiversity, bioavailability and the low-molecular weight of these compounds, makes them very desirable targets (Grabley and Thiericke, 1999; Bindseil, *et al.*, 2001). Until recently the majority of drugs sourced from nature have been developed from the terrestrial environment and the marine environment had been left relatively untouched, however this was mainly due to accessibility (Nuijen, *et al.*, 2000). In the last 50-years compounds sourced from the marine environment have yielded a large variety of highly complex chemical structures many of which have been of interest in

pharmaceutical research and complementary and alternative medicine (CAM; Grabley and Thiericke, 1999).

1.2 Benefits of Marine Organisms as Potential Source of New Compounds

Natural products have always played a major role in the discovery and development of new drugs, however sources from the marine environment have only just began to emerge (Nuijen, *et al.*, 2000). To date hundreds of compounds have been isolated from various marine species, including algae, microorganisms, sponges, bryozoans, tunicates and molluscs (Blunt, *et al.*, 2008; Mayer, *et al.*, 2009). In 2006 alone, over 779 novel compounds were documented (Blunt, *et al.*, 2008). Jimeno, *et al.* (2004) reported that over 2,500 different metabolites isolated from the marine environment in the past decade have demonstrated anti-proliferation activity.

Both pharmaceuticals and nutraceuticals have been successfully developed for a range of diseases from species in the marine environment (Chin, *et al.*, 2006; Blunt, *et al.*, 2008; Mayer, *et al.*, 2009). These drugs have either come directly from the bioactive compounds and/or their metabolites, or have been developed from synthetic analogues of these compounds (Newman and Cragg, 2004b; Blunt, *et al.*, 2008). Some of these drugs have been approved by the FDA as medicines, while there are others in various stages of clinical trials, while others are still in their infancy of development (Chin, *et al.*, 2006). However, irrespective of the source, a drug can take up to 15-years to reach the market from discovery through preclinical and then clinical trials at a cost of $802 million (DiMasi, 2001; Adams and Brantner, 2006).

The compounds spongouridine and spongothymidine, discovered by serendipity from the Caribbean sea sponge *Cryptotheca crypta* (Jimeno, *et al.*, 2004; Newman and Cragg, 2004a), along with the synthetic analogue, 1-beta-D-arabinofuranosylcytosine (ARA-C) demonstrated anti-proliferation activity against acute myeloid leukaemia (Wolf, *et al.*, 1985; Jimeno, *et al.*, 2004). The analogue ARA-A was developed from this ARA-C and later commercialised (Newman and Cragg, 2004b). More recently, ARA-A and ARA-C were approved as a orphan drug in the treatment of soft tissue sarcromas and in ovarian cancer (von Schwarzenberg and Vollmar, 2010). Agelasphin compounds isolated from the Okinawan sea sponge, *Agelas mauritianus*, have anti-tumour activity *in vitro* and *in vivo* (Natori, *et al.*, 1994), and the synthetic derivative KRN7000 has anti-tumour activity in phase I clinical trials (Giaccone, *et al.*, 2002).

The drug Trabectedin (Ecteinascidin-743) or Yondelis® (PharmaMar), derived from the tunicate *Ecteinascidia turbinate* in the Caribbean (Rinehart, 2000) demonstrated anticancer activity in phase I clinical trials against advanced-stage cancer, including breast and ovarian cancer (Verweij, 2005). More recently Yondelis®, has been approved as the first anticancer marine drug in European Union for the treatment of platinum-sensitive ovarian cancer (Carter and Keam, 2010; von Schwarzenberg and Vollmar, 2010). Aplidine^{R}, from the marine tunicate *Aplidium albicans* has shown activity against certain types of tumours including ovarian carcinomas *in vivo* and phase I trials (Taraboletti, *et al.*, 2004; Chin, *et al.*, 2006), and is now in the process of phase II clinical trials (Jimeno, *et al.*, 2004; Chin, *et al.*, 2006).

1.3 Marine Molluscs as a Source of Bioactive Compounds

The class Gastropoda comprises the largest group of molluscs and include a wide number of species (Commonwealth Scientific and Industrial Research, 1998). The bioactive compounds developed from molluscs have been well documented (Chin, *et al.*, 2006; Benkendorff, 2010). The only mollusc derived pharmaceutical to be successfully commercialised in the last 20-years is Ziconotide®, or Prialt, developed from the gastropod *Conus magnus* and is currently used to treat severe chronic pain (Chin, *et al.*, 2006). Seaton or Lyprinol®, derived from the green-lipped/green-shell mussel from New Zealand, *Perna canaliculus*, is a commercialised nutraceutical sold for the treatment of pain associated with chronic arthritis (Jeffs, *et al.*, 1999; Cobb and Ernst, 2006). Other molluscan products are at the research phase and not yet commercially available. These include the linear peptides Dolastatin-10 and 15 derived from the shell-less mollusc *Dollabella auricularia* (Pettit, *et al.*, 1987). In Phase I trials, Dolastatin-10 and 15 have anti-tumour activity against breast and liver cancer (Tran, *et al.*, 1997). More recently TZT-1027 a synthetic derivative of dolastatin-10 was active against advanced solid tumours in phase I clinical trials (Yamamoto, *et al.*, 2009). The bioactive compound Kahalaide F, was discovered in the marine mollusc, *Elysia rufescens*, from Hawaii (Hamann, *et al.*, 1996) and in phase I clinical trials Kahalaide F has shown anti-tumour activity in breast, hepatoma, melanoma and pancreatic carcinomas (Janmaat, *et al.*, 2005; Chin, *et al.*, 2006).

1.4 Muricidae Molluscs

The family of marine Mollusca, Muricidae comprises a wide group of predatory whelks including over 2000 species that are found in many parts of the world (Commonwealth Scientific and Industrial Research, 1998; Ramón and Amor, 2002). Australia alone has over 180 different species, the majority of which usually inhabit

shallow rocky subtropical tidal, and intertidal regions, with a few species known to inhabit more temperate intertidal regions (Tan, 2003).

Dicathais orbita (Figure 1.1) is one of the larger of the muricid species and is only found in the southern regions of Australia, New Zealand, Norfolk Island and Lord Howe Island (Figure 1.2; Phillips and Campbell, 1974; Tan, 2003). This predatory gastropod (whelk) is carnivorous and feeds on a variety of living and dead marine animals, including limpets, carrion and mussel beds (Phillips, 1969; Morton, 1999). *D. orbita*, as a species, is generally only found on hard rocky surfaces, often reef platforms close to the shore (littoral) and prefer mild temperatures (18^{o}C), as the lethal temperature has been noted at ~37-38^{o}C (Phillips, 1969). However, *D. orbita* have also been found in sublittoral areas (Tan, 2003). Phillips (1969) noted that there is often a high rate of mortality with juveniles of this species which often occurs during the summer months because of increased temperatures and predation from other marine animals including octopodes, crayfish and other species of gastropods.

Figure 1.1: Specimens of marine whelk *Dicathais orbita*.
Specimens of the marine whelk *Dicathais orbita* held in re-circulating tanks at Flinders University, South Australia (photographed by Nikki Sperou, February 2008).

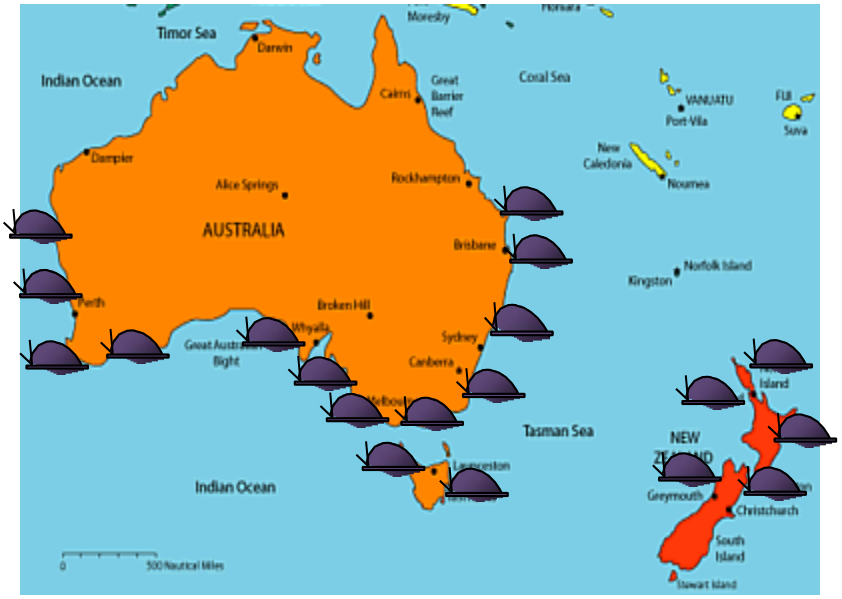


Figure 1.2: Geographical Distribution of *Dicathais orbita*
Geographical Distribution of *Dicathais orbita* in Australia and New Zealand (adapted from Tan, 2003; Map from http://www.transitionsabroad.com/images/maps/australia_oceania_australasia.gif)

Unlike some species of gastropods, *D. orbita* is not a hermaphrodite, there is a distinction between the male and female of the species, and breeding occurs usually between May and November (Phillips and Campbell, 1974). In the female, the egg travel through the oviduct and into the albumen gland where it is believed, internal fertilization takes place (Tan, 2003). Females lay large masses of white vase-shaped egg capsules which turn yellowish and then eventually purplish during the hatching period of spring and summer (Figure 1.3; Phillips and Campbell, 1974; Benkendorff, *et al*., 2000).

The growth rate of *D. orbtia* has been noted to be quite slow and maturity is often only reached after three years, with a life-span generally greater than five years, and growth to ~60-70mm in length and up to ten millimetres in shell height (Phillips and Campbell, 1974). However, recent growth trials on juveniles in aquaria have shown that the body mass of *D. orbita* can approximately double in a three month period with maximum growth rate of two millimetres per month (Woodcock and Benkendorff, 2008). It has been suggested the largest specimen recorded lived up to 19-years (Phillips and Campbell, 1974; Tan, 2003).

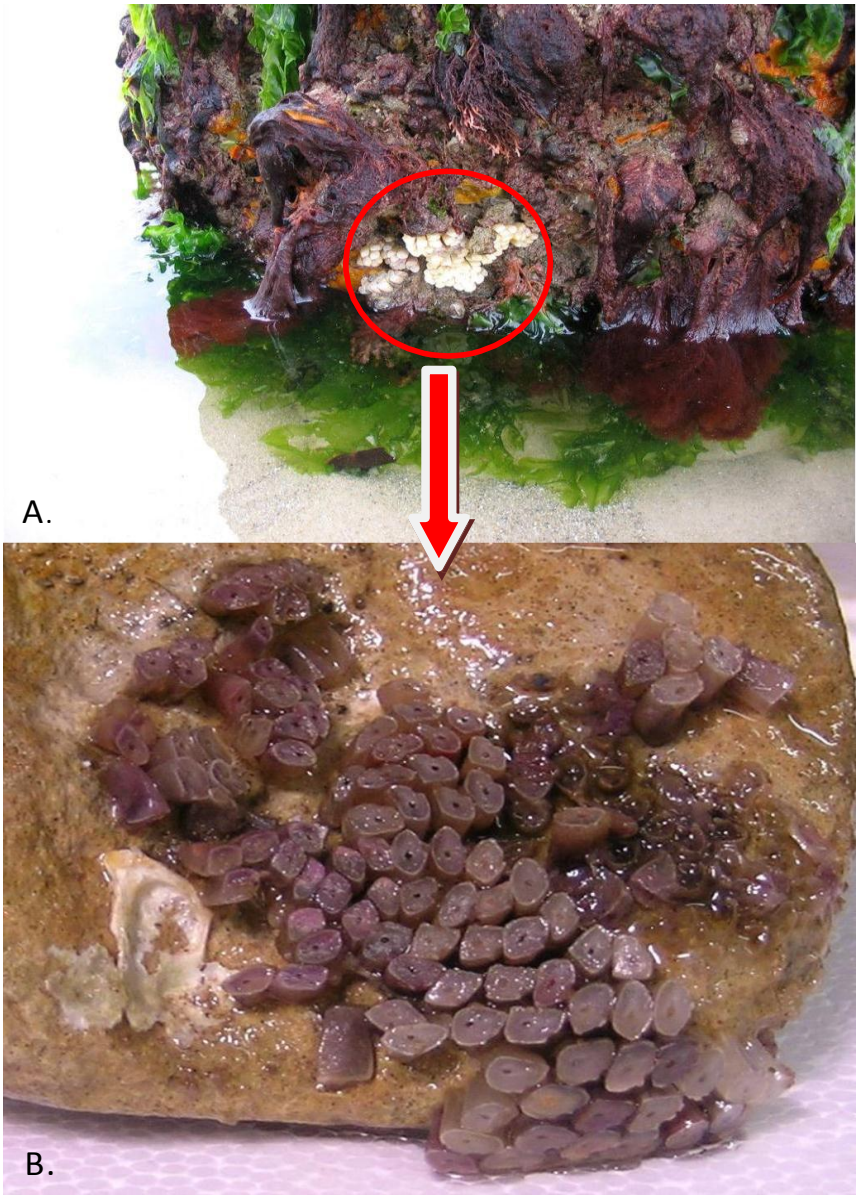


Figure 1.3: The egg masses of *Dicathais orbita*
Dicathais orbita whelks lay large masses of white vase-shaped egg capsules often on the pylons of piers (A) and rocks during the summer months in Australia (from January through to March), which turn yellowish and then eventually purple (B) (source of Tyrian purple) during the hatching period of spring and summer with oxidation and exposure to light (Photographed at Brighton Beach Pier, Adelaide, South Australia by Cassandra McIver in December 2007 and in the aquarium at Flinders University, South Australia by Nikki Sperou in February 2008).

1.5 Discovery of 6'6-dibromoindigotin (Tyrian purple)

The family of Muricidae molluscs dates back to ancient times in the Mediterranean (Jensen, 1963; Baker, 1974). The civilizations of the ancient Semetic peoples, the Phoenicians and Canaanites discovered the art of crushing the shells of the genus *Murex* to produce a vibrant purple dye for colouring cloth, and evidence of this has been found by mounds of broken purple shells near Tyre (Allan, 1934; McGovern and Michel, 1990; Cooksey, 2001). Numerous civilizations since have adopted the ancient industry of purple dying using various species of marine whelks, including the prehistoric inhabitants of Britain and Norway (*Nurella lapillus*), the ancient Greeks and Minoans (*Murex brandaris* and *Murex trunculus*), the people of the West Indies (*Purpura patula*), the Japanese (*Rapana thomasiona* and *Rapana venosa*), the Mexicans and Peruvians (*Plicopurpura pansa*) and the Romans (*T. hemastroma*) (Hutchinson, 1962; Jensen, 1963; Cooksey, 2001; Naegel and Alvarez, 2005).

The production and industry of Tyrian purple from various murex species predates knowledge of its chemistry, although circa AD 77-79 Pliny the Elder described the formation of different colours in the dyeing process, "It (Tyrian purple) is most appreciated when it is the colour of clotted blood, dark by reflected, and brilliant by transmitted light. This is why Homer used the adjective "purple" of blood" (Bailey, 1929). In the latter part of seventeenth century, William Cole (1685) went on to describe the complete colour reaction in the formation of Tyrian purple using the species of muricid, *Nucella lapillus.* The white secretion extracted from the hypobrancial glands of live species of *N. lapillus,* when applied to cloth such as silk, sequentially changed colour from yellow, light green, deep green, sea green, blue, purple red and deep purple red, in the presence of oxygen and light (Cole, 1685). In

the early part of twentieth century chemical analysis confirmed the structure of Tyrian purple to be 6,6'-dibromoindigotin (Figure 1.4; Friedländer, 1909). Friedländer (1909) used 12,000 specimens of the marine murex species, *M. brandaris,* to produce only 1.4g of the purple compound (Baker and Sutherland, 1968).

1.6 Formation of 'Tyrian Purple'

It wasn't until the late 1960's that Baker and Sutherland (1968) identified the precursor compound to 6,6'dibromoindigotin (Tyrian purple) from the Australian mollusc *Dicathais orbita* (Gmelin). Ethanol extraction of the hypobrancial gland of *D. orbita,* confirmed that sodium tyrindoxyl sulphate was the precursor compound of 6,6'-dibromoindigotin (Baker and Sutherland, 1968). Baker and Duke (1973) later identified other compounds from the hypobranchial gland of *D. orbita* in the chemical pathway to the formation of Tyrian purple. These compounds included, 6-bromo-2-methylthioindoxyl more commonly known as Tyrindoxyl (Baker and Duke, 1973a). When oxidised, tyrindoxyl was found to convert readily to 6-bromo-2-methylthioindoleninone or tyrindoleninone (Baker and Duke, 1973a). Hydrolysis of tyrindoleninone then led to the formation of 6-bromo-2,2-dimethylthioindolin-3-one, commonly known as tyrindolinone (Baker and Duke, 1973a; Baker, 1974). Tyriverdin, another precursor compound to 6,6'dibromoindigotin (Tyrian purple) was later identified by ^{1}HMR (Christophersen, *et al.*, 1978). The complete chemical pathway to the formation of 6,6'dibromoindigotin, in the hypobranchial gland of *D. orbita,* has been clearly described by Cooksey (2001; Figure 1.4).

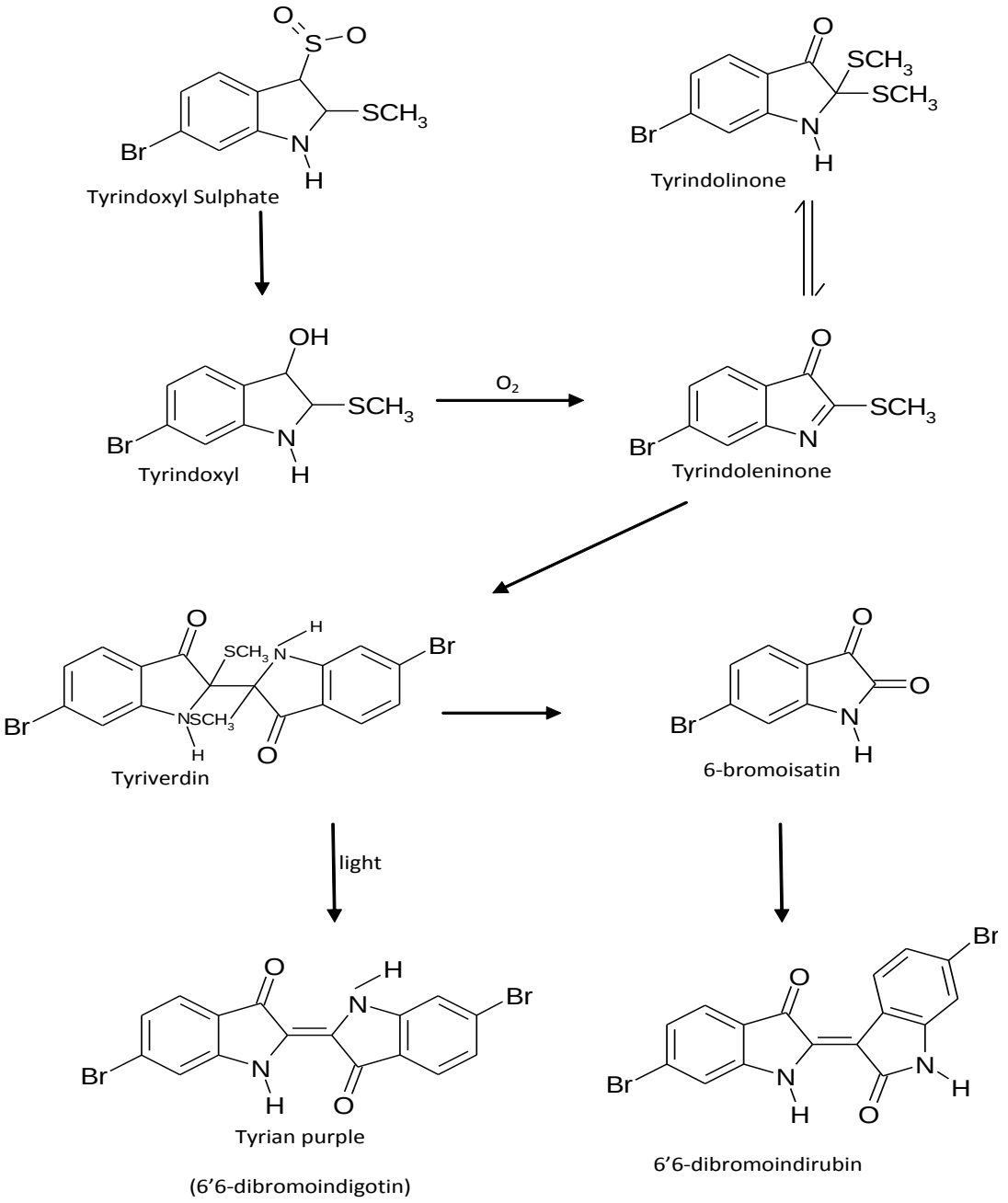


Figure 1.4: Chemical pathway of Tyrian purple
The formation of 6,6'-dibromoinigotin developed from the precursor compound tyrindoxyl sulphate found in the tissues of specimens of muricid whelks. 6,6'-dibromoindigotin, more commonly known as Tyrian purple was structurally identified in 1909 by Friedländer, from the species of murex *M. brandaris*. Friedländer produced 1.4g of the purple compound from 12,000 molluscs (Friedländer, 1909). Friedländer later also identified that the species *M. trunculus*, *P. aperta* and *P. lapillus* were able to produce 6,6'-dibromindgotin (adapted from Cooksey, 2001 and these figures were constructed using MDL ISISTM/Draw Software Version 2.5).

1.7 Bioactive Properties of Muricidae Secretions

The bioactive effects of the compounds from the hypobranchial gland of mollusca were first reported in *in vivo* assays in 1909 in both cold and warm blooded animals (Dubois, 1909). Dubois (1909) noted that, the toxic compounds first restricted movement and then finally caused paralysis of the animal *in vivo*. Research has shown that the hypobranchial glands from a number of different families of marine mollusca, contain not only brominated indoles but several other bioactive compounds some of which have been identified (Roseghini, *et al.*, 1996). Choline esters, including β[imidazolyl-4(5)acrylcholine] or more commonly known as murexine or urocanylcholine, β,β-dimethylacryloycholine more commonly known as senecioylcholine and dihydromurexine, have been identified in the several species of the marine mollusc superfamily, Muricoidea (Whittaker, 1960; Roseghini, *et al.*, 1996). Erspamer and Glässer (1957) summarised several studies on the pharmacological actions of murexine in several species, including the mouse, rabbit, cat, dog and clinical trials in man. In human clinical trials, murexine administered at 1 to 1.2mg/kg intravenously caused paralysation within 45-60 seconds lasting from 3 to 6 minutes (Erspamer and Glässer, 1957). Both murexine and senecioylcholine have been identified in the hypobranchial glands of *D. orbita*; however the choline ester dihydromurexine has not (Baker, 1976; Roseghini, *et al.*, 1996). Preliminary observations after ingestion of hypobranchial glands from *D. orbita* have produced numbing effects in the mouth, which may be attributed to choline esters (Benkendorff, *et al.*, 2011).

Another group of compounds, the indirubins have also been isolated. Meijer *et al* (2003) isolated the indirubins, 6'-bromoindirubin and 6, 6'-dibromoindirubin from the mollusc *Hexaplex trunculus* and found that they were selective kinase inhibitors

of glycogen synthase kinase-3 (GSK-3). Indirubin is also the active ingredient commonly used in traditional Chinese medicine such as the Chinese recipe Danggui Longhui Wan, has been used for the treatment of various diseases including cancer (Han, 1994; Xiao, *et al.*, 2002; Meijer, *et al.*, 2003). More recently derivatives of indirubin have been shown to inhibit proliferation of cancer cells *in vivo* (Kim, *et al.*, 2007). Clinical trials have shown that meisoindigo is effective in the treatment of chronic myelogenous leukaemia (Xiao, *et al.*, 2002). Due to poor adsorption and side-effects of indirubin, such as irritation to the gastrointestinal tract, more recently research has been focused on new derivatives of indirubins (Kim, *et al.*, 2007).

1.7.1 The Homeopathic Remedy 'Murex Purpurea'

The family of Muricidae molluscs are also the source of a homeopathic remedy 'Murex purpurea', the origin of which dates back to the nineteenth century (Dunham, 1864). 'Murex purpurea' was introduced into the homeopathy Materia Medica by Dr Petroz in the '*Revue Oritique et Retrospective de la Matiere Medicale,* Vol.III 1841'(Dunham, 1864). The active ingredient of the remedy was not stated in the original homeopathic materia medica, however Weber in Dunham (1864, pg 307) states that, 'the coloring matter which furnishes the purple is a juice and is first obtained, colorless in a distinct little sack in the majority of molluscs situated between the heart and liver. When brought into contact with the atmosphere this juice becomes successively yellow, green, blue and finally a reddish purple'. This suggests that the active ingredient that was used in the homeopathic remedy at the time was the purple compound, Tyrian purple.

In the nineteenth century, the 'Murex purpurea' remedy was used to treat what was then referred to as women's problems, including a nervous temperament, leucorrhoea

and pain of the uterus or dysmenorrhoea (Dunham, 1864). 'Murex purpurea' was later mentioned in the homeopathic, Materia Medica (Peterson, 1905). Today, 'Murex purpurea' is still sold commercially by several Homeopathy companies worldwide for treatment of a range of gynaecological disorders including cancer of the uterus, and dysmenorrhoea (painful menstruation), menorrhagia (heavy menstruation), and leucorrhoea (abnormal menstruation), cysts of the ovaries, endometriosis and polycystic ovary syndrome (Boericke, 2005; Cazalet, 2008).

Very few homeopathic remedies, including 'Murex purpurea', have actually been scientifically tested for their efficacy and cytotoxicity (Straus, 2000; Fisher, 2001; Dantas, *et al.*, 2007). Consequently, in many cases the benefits of homeopathic treatment are often attributed to the placebo effect (Fisher, 2001). In fact, preliminary testing of the anticancer properties of 'Murex purpurea' remedy has revealed no cytotoxicity against some solid tumour and leukaemia cell lines *in vitro* (Benkendorff, *et al*., 2011).

1.8 Bioactive Indoles and Other Compounds in the Egg Masses

Three of the precursor compounds to Tyrian purple, extracted from the egg masses of *D. orbita*, tyriverdin, tyrindoleninone and 6-bromoisatin have demonstrated bioactivity against both human and marine bacteria (Benkendorff, *et al.*, 2000). Semi-purified extracts from *D. orbita* have cytotoxic activity towards cancer cell lines *in vitro*, including the lymphoma cell lines, U937 and Jurkat (Westley, *et al.*, 2006; Vine, *et al.*, 2007a) and solid tumour cell lines CaCo2 from the colon as well as the breast cancer cell line, MCF-7 (Vine, *et al.*, 2007a; Benkendorff, *et al.*, 2011). Benkendorff *et al.*, (2011) demonstrated that a crude sample of *D. orbita* have shown

less activity to primary human mononuclear cells (MNC) in comparison to a range of cancer cells lines including HT29 (70% reduction at 0.5mg/ml; colon carcinoma) and MCF7 (40% reduction at 0.5mg/ml; breast cancer cell line) after 4h of treatment. Furthermore, recent animal assays have shown that tyrindoleninone and 6-bromoisatin are pro-apoptotic, in the distal colon in response to genotoxic carcinogens (Westley, *et al.*, 2010).

1.9 Separation & Identification of the Bioactive Compounds

Various solvents have been employed in the initial extraction of the precursor compounds (chromagens) of 6'6-dibromindigotin, from the hypobranchial glands and egg masses from *D. orbita*, including ether, ethanol, chloroform, methanol and/or a combination of these solvents (Baker and Sutherland, 1968; Baker and Duke, 1973a; Benkendorff, *et al.*, 2000).

Analytical identification and separation of 6'6-dibromoindigo compounds from crude samples have included thin layer chromatogranpy (TLC), liquid chromatography (LC), high performance liquid chromatography (HPLC), ultraviolet (UV), visible, and infrared spectroscopy, gas chromatography (GC), mass spectrometry (MS), electron spectroscopic chemical analysis, electron spin resonance and carbon, hydrogen and bromide nuclear magnetic resonance (NMR) (McGovern and Michel, 1990; Puchalska, *et al.*, 2004).

After the active compounds have been extracted from the hypobranchial glands, Baker and Duke (1973) identified the insoluble compounds, tyrindoleninone and tyrindolinone, on alumina preparative-layer plates, and then eluted the fractions with

ether. McGovern and Michel (1990) found that high-resolution mass spectrometry was effective for identifying indigo compounds originally sourced from marine molluscs. Benkendorff *et al.* (2001) identified the bioactive compounds, tyrindoleninone, tyriverdin and 6-bromoisatin by GC/MS. Puchalska *et al.* (2004) found that reverse-phase HPLC together with electron-spray ionization mass spectrometry is an improvement on normal HPLC for identifying isomeric indigo compounds extracted from both natural and synthetic resources, especially when the poorly soluble compounds are dissolved in dimethylsulphoxide (DMSO).

1.10 Reproduction & Hormone Synthesis

Reproduction involves numerous enzymes and steroid hormones, such as progesterone and estradiol, which are vital for normal reproductive functions in females, including the establishment and maintenance of pregnancy, and mammary gland development (Chen, *et al.*, 2005a). The female hormonal cycle is characterized by the follicular phase and the luteal phase, both of which are controlled by the hypothalamic pituitary axis, which interacts with the anterior pituitary and ovaries (Vander, *et al.*, 1998). The hypothalamus releases gonadotrophin releasing hormone (GnRH), which in turn, stimulates the anterior pituitary to release the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH) that act upon the ovaries in various ways depending on the time of the ovarian cycle in a negative feedback mechanism (Figure 1.5; Jones and Lopez, 2006).

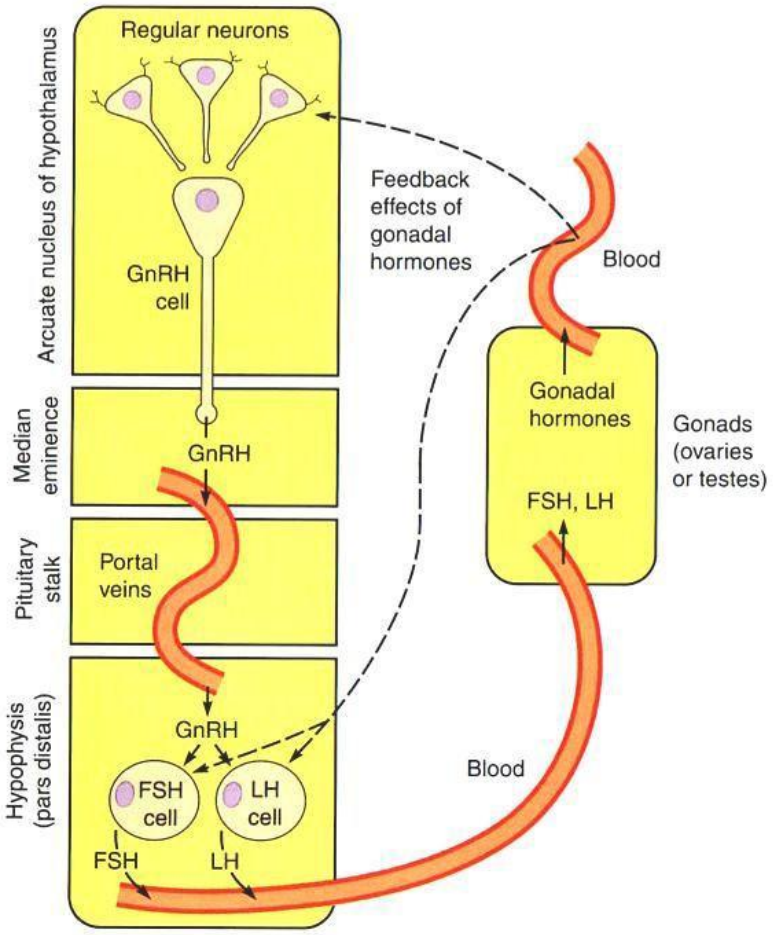


Figure 1-15 Schematic diagram of the control of the reproductive system by the brain and pituitary gland and the sites of feedback by gonadal hormones on this control. tes of 2006, pp. 26 reproduced with permission from the authors/editors)

Female humans have a twenty-eight day cycle, starting with the follicular phase from day one to approximately day fourteen, followed by the luteal phase from day fifteen to approximately day twenty-eight, ending in menstruation (Figure 1.6; Vander, *et al.*, 1998). At the beginning of the follicular phase or growing phase, serum levels of progesterone and estrogen are low, which results in a gradual increase in circulating FSH/LH. FSH rescues the ovarian primordial follicles from atresia and approximately 3-30 follicles begin to grow (Vander, *et al.*, 1998). However, only one dominant follicle, the preantral follicle, continues growth, eventually matures and

then ovulates; while all the other competing follicles undergo atresia (Vander, *et al.*, 1998). FSH acts on specific FSH receptors to stimulate the hormone producing cells of the follicle, the granulosa and theca cells (Figure 1.7), to gradually increase the secretion of the steroid hormone estrogen (Leung and Adashi, 2004). FSH and estrogen work synergistically to increase FSH receptors of the dominant follicle and stimulate the proliferation of granulosa cells (Speroff and Fritz, 2005).

Estrogen is also responsible for causing the uterine wall to thicken in preparation for implantation of the fertilized oocyte (Jones and Lopez, 2006). Just prior to ovulation there is a surge in the pituitary gonadotrophin, LH and a mild peak of the steroid hormone progesterone (Leung and Adashi, 2004). After ovulation, a structure known as the corpus luteum is formed by the luteinized and differentiated granulosa, and theca cells (Figure 1.7; Moore and Persaud 2003).

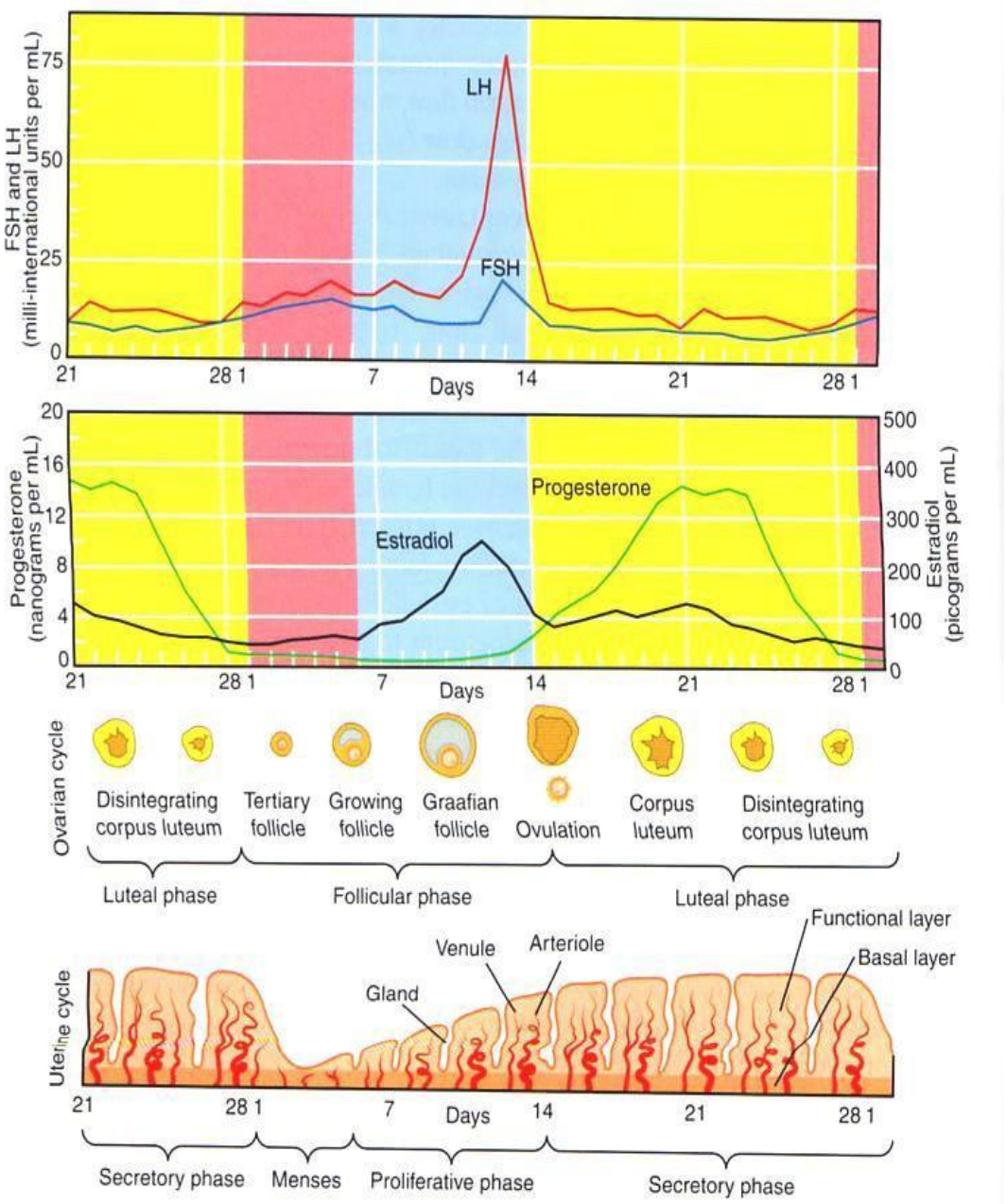


Figure 1.6: Overview of the female human menstrual cycle.
During the 28-day menstrual cycle blood levels of hormones change along with the uterine endometrium. Plasma FSH acts on receptors of the granulosa. The ratio of estradiol to progesterone is high during the follicular phase and lower during the luteal phase, and the estradiol peak precedes the LH surge (Jones, R.E. and Lopez, K.H., 2006, pp. 78 reproduced with permission from the authors/editors).

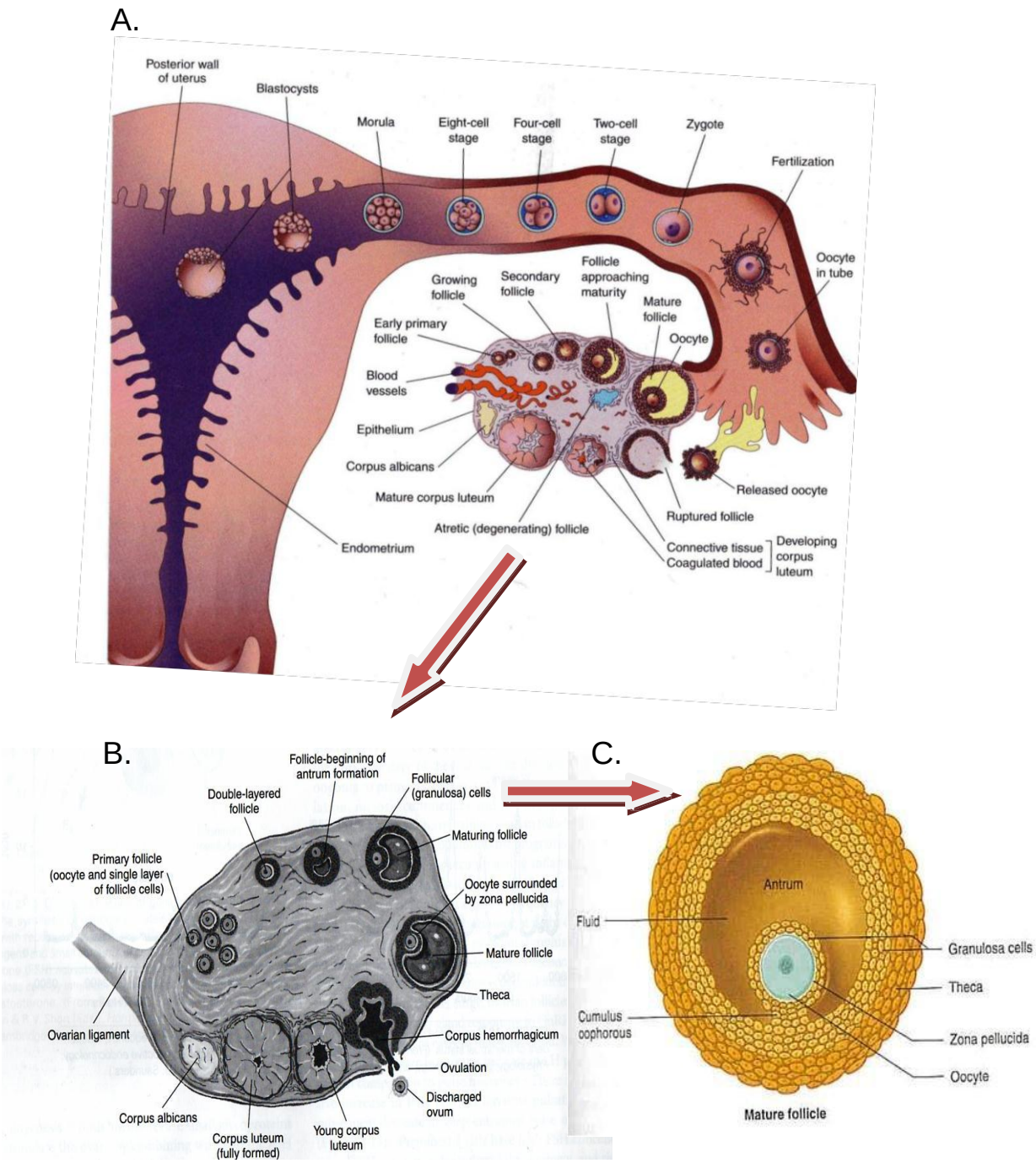


Figure 1.7: Diagrammatic overview of the ovarian cycle
Diagrammatic overview of the ovarian cycle, fertilization and human development during the first week (A), an enlarged view of the cross section of the ovary (B), and the mature follicle surrounded by granulosa and theca cells (C). As the developing follicle maturates an increase layer of hormone producing cells, the granulosa and theca cells, are rapidly growing and surround the dominate follicle. After ovulation the layer of granulosa and theca cells differientiate and lutenize, and go on to form the corpus luteum that continues to secrete progesterone and estrogen, controlled by hCG, which itself is secreted by the developing embryo (Moore, K.L. and Persaud, T.V.N., 2003 reproduced with permission).

Ovulation, or release of an oocyte (more commonly known as an egg) from the ovary, occurs at approximately day fourteen of the cycle (Vander, *et al.*, 1998). At the onset of the follicular phase the pituitary secretes the gonadotrophin FSH mediated by estradiol (E_2) and inhibin, and FSH peaks just prior to ovulation (Figure 6; Sherwood, 2004). On the other hand, LH is constant at very low concentrations during the follicular phase until approximately eighteen hours before ovulation, when there is a surge of LH followed by a slow decline during the luteal phase (Sherwood, 2004). This surge of LH induces ovulation and stimulates cells of the follicle, granulosa, and theca cells to differentiate, luteinise and transform into the corpus luteum (Sherwood, 2004).

The corpus luteum secretes the hormones estrogen, progesterone and inhibin in large quantities (Figure 1.7B; Vander, *et al.*, 1998). Progesterone and estrogen cause a decrease in the secretion of gonadotrophins by the pituitary axis, by acting on the hypothalamus to suppress the secretion of GnRH in a negative-feedback control of hormone levels (Vander, *et al.*, 1998). If the oocyte is not fertilized within two weeks, the corpus luteum degenerates, which in turn causes a decrease in estrogen and progesterone (Sherwood, 2004). At the same time the hypothalamus begins to secrete GnRH, this results in the gradual secretion of plasma FSH and LH, and hence, the follicles begin to grow at the start of the new cycle (Vander, *et al.*, 1998; Sherwood, 2004).

If the oocyte is fertilized, the corpus luteum does not degenerate and continues to secrete the hormones estrogen and progesterone under the control of human chorionic gonadotrophin (hCG) (Vander, *et al.*, 1998). By the time the fertilized

oocyte has been transported down the oviduct to the uterus, it has developed into a blastocyst (Figure 7A, Vander, *et al*., 1998). The blastocyst has an outer layer of cells, the trophoblast cells, which are responsible for the secretion hCG (Sherwood, 2004). This facilitates embryo implantation and also stimulates the secretion of progesterone and estrogen by the corpus luteum (Vander, *et al.*, 1998). The hCG stimulation of progesterone and estrogen production is essential for the maintenance of pregnancy (Vander, *et al.*, 1998; Sherwood, 2004). By approximately week five of gestation the developing placenta begins to take over the secretion of progesterone and estrogen for the continuation of pregnancy (Speroff and Fritz, 2005).

1.11 Steroidogenesis in granulosa cells

The granulosa cells not only play an extremely important role in the maturation of the oocyte, but along with the theca cells also form the corpus luteum, which secretes the hormones progesterone and estrogen (Vander, *et al.*, 1998; Leung and Adashi, 2004). There are number of factors involved in the rate of steroidogenesis or steroid hormone biosynthesis in granulosa cells, including the secretion of gonadotrophins, and numerous enzymes, receptors and genes (Azhar, *et al.*, 2003). The first step of hormone synthesis involves the activation of a G-coupled protein receptor on the surface of the cell by binding of hCG or LH, causing a conformational change and resulting in a signalling cascade (Leung and Adashi, 2004). This involves the activation of adenosine 3',5'-cyclic monophosphate (cAMP) by adenosine triphosphate (ATP; Azhar, *et al*., 2003). cAMP is then converted to protein kinase A (PKA), which itself is phosphorylated, and activates steroidogenic acute regulatory (StAR) gene transcription and translation in the nucleus of the cell (Leung and

Adashi, 2004). This leads to the uptake of cholesterol into the mitochondria followed by the synthesis of hormones (Azhar, eta!., 2003; Figures 1.8 and 1.9).

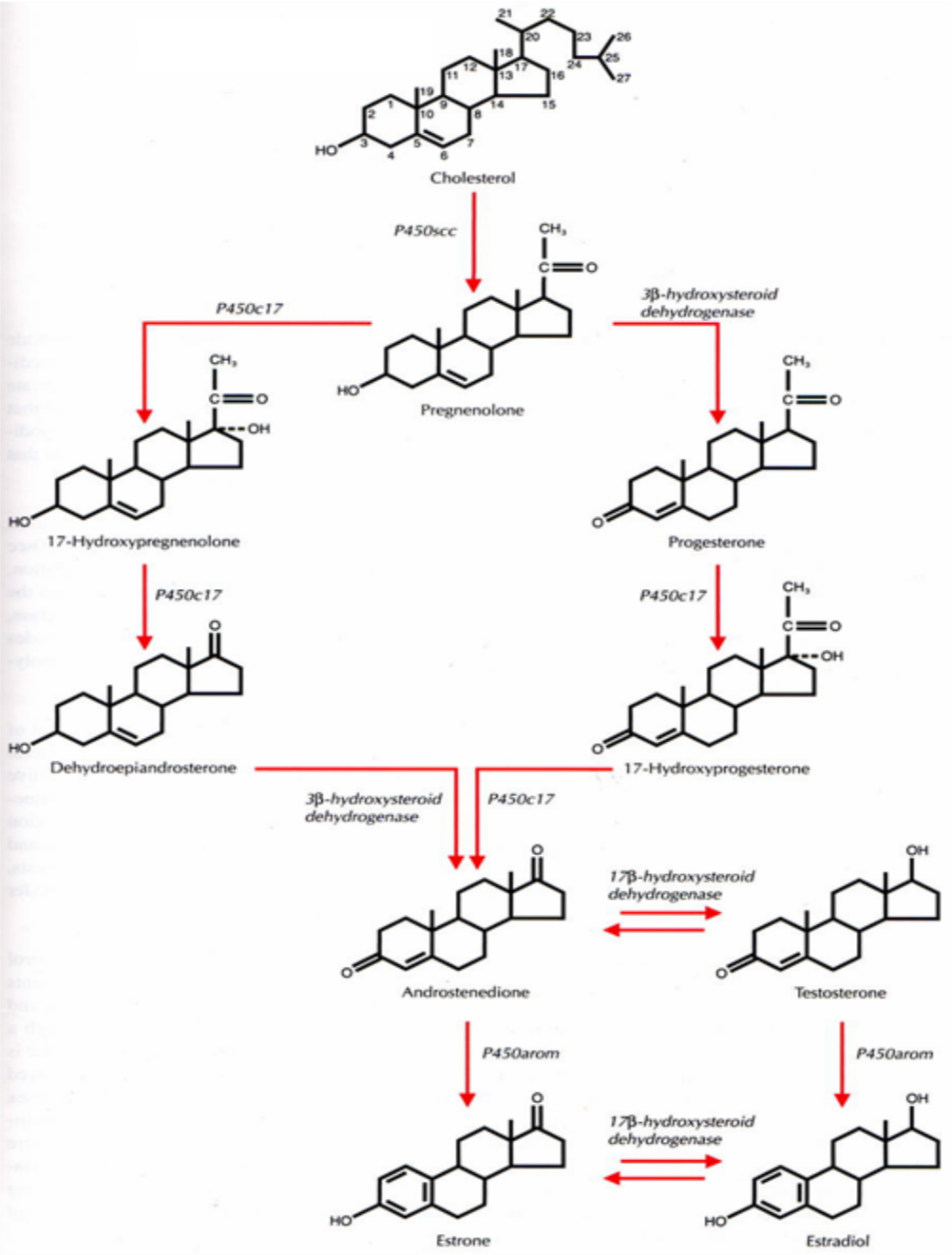


Figure 1.8: Overview of Steroidogenesis.
The biosynthesis of steroid hormones begins with the conversion of chlosterol to pregnenolone. Pregnenolone is then converted to progesterone, catalyzed by 3β-HSD. P450c17 then catalyzes the conversion of progesterone to 17-hydroxyprogesterone which is then converted to androstenedione. Androstenedione is then converted to estrone by $P450_{arom}$, and estrone to estradiol by 17β-hydroxysteroid dehydrogenase. Alternatively, pregnenolone can be directly converted to the DHEA, by 17α-hydroxylation and cleavage of the side chain. DHEA is then converted to androstenedione, which is either converted directly to estradiol or androstenedione is converted to testosterone by the 17β-HSD enzyme Testosterone is then reduced to estradiol by $P450_{arom}$ (Speroff, L. and Fritz, M.A., 2005, pp.33 reproduced with permission from the authors/editors).

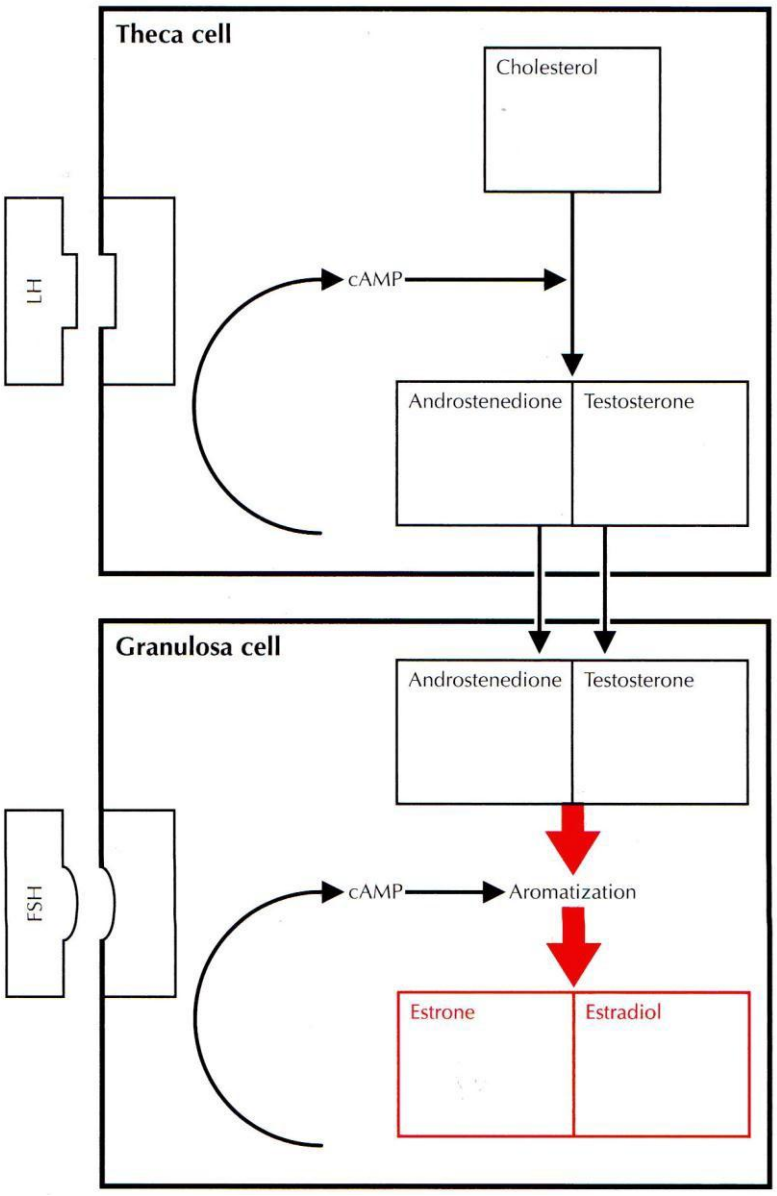


Figure 1.9: Estradiol synthesis *in vivo*
Estrogen synthesis by the two cell, two gonadotrophin system. The interaction between the granulosa and theca cells results in estrogen synthesis. In preantral and antral follicles LH receptors are only present on the theca cells and FSH receptors on the granulosa cells. The theca cells express the P450c17 enzyme, the rate limiting step in the conversion of C-20 carbons to androgens which are then converted to estrogens in the granulosa cells by the $P450_{arom}$ enzyme (Speroff, L. and Fritz, M.A., 2005, pp.193 reproduced with permission from the authors/editors).

1.12 Infertility

Infertility affects between 13-15% of couples world-wide, and while it is not life-threatening, it can have a tremendous psychological impact on couples trying to conceive (Kamel, 2010). In the United States alone it is expected that twenty-five percent of women will experience some form of infertility during their reproductive years (Meletis and Barker, 2004). A number of different factors have been attributed to the etiology of infertility, however, the majority of cases are unexplained factors (28%), followed by male factors (24%), ovarian dysfunction or anovulatory infertility (21%), tubal factors (14%) and others, such as endometriosis (14%) (Jose-Miller, *et al.*, 2007; Kamel, 2010).

1.12.1 Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) has been shown to account for up to 75% of anovulatory infertility in women (Amer, 2006). Excess ovarian androgen production, insulin resistance and hyperinsulinemia, and an imbalance in the hypothalamic pituitary-ovarian axis, are all important factors in PCOS (Amer, 2006). Research indicates that PCOS is associated with abnormal folliculogenesis causing deficient FSH, an abnormal LH surge and a deficiency in growth factors (Erickson, *et al.*, 1992; McAllister, 1995; Amer, 2006). The deficiency in FSH has been shown to inhibit the growth of the small antral follicles and the abnormal LH surge results in the small follicles acquiring LH receptors prematurely (Amer, 2006). As a result, these small antral follicles switch from proliferation to differentiation, therefore arresting the follicle in development and preventing growth of the dominant follicle (Amer, 2006). It has further been demonstrated that an excess secretion of ovarian androgens by small theca cells results in an increase in precursors for estrogen synthesis in the granulosa cells thus resulting in an increase in estrogen production

(Erickson, *et al.*, 1992; Mason, *et al.*, 1994; Amer, 2006). This high level of circulating estrogen results in a negative feedback on FSH secretion, causing both an abnormal folliculogenesis and abnormal steroidogenesis (Amer, 2006).

1.12.2 Endometriosis

The endometrium is a major determinant in successful implantation of a fertilized oocyte, but conception is unlikely to occur if the endometrium is abnormal in thickness (Raine-Fenning, *et al.*, 2004). Hence, endometriosis is also thought to be the underlining cause of infertility in some women (Wu, *et al.*, 2010). Additionally, endometriosis is associated with dysmenorrhoea (painful menstruation) and also pregnancy loss (Wu, *et al.*, 2010). In women who suffer from endometriosis, the endometrium is found outside the uterine cavity (Olive and Schwartz, 1993; Wu, *et al.*, 2010). The exact cause of endometriosis is currently not known, although there are several conflicting factors that are thought to contribute to the etiology (Wu, *et al.*, 2010). Women with endometriosis generally have a longer follicular phase, a small LH peak and reduced estrogen secretion (Harlow, *et al.*, 1996). Harlow *et al.*, (1996) demonstrated that even after hCG stimulation, the LH surge is still reduced in patients with endometriosis. The authors suggested that the dysfunction in LH surge was related directly to the follicle and not the pituitary axis (Harlow, *et al.*, 1996). In contrast, other research suggests that endometriosis is an estrogen-dependent disease, requiring estrogen for proliferation of the endometrium (van Kaam, *et al.*, 2007). More recently prostaglandin E_2 (PGE_2) has been implicated in endometriosis (Wu, *et al.*, 2010). Concentrations of PGE_2 in the peritoneal fluid of women with endometriosis have been shown to be much higher than in normal women (Wu, *et al.*, 2010).

1.12.3 Progesterone Deficiency in Infertility

Insufficient progesterone during implantation or early pregnancy can result in infertility, spontaneous abortions and/or recurrent miscarriage (Daya, 2009). Progesterone is essential not only for conception but throughout pregnancy (Speroff and Fritz, 2005). During the luteal phase, progesterone along with estradiol, prepares the uterus for implantation of the embryo (Speroff and Fritz, 2005). In the late follicular phase insufficient progesterone secretion can result in an inhibition of the LH induction of ovulation, and can also lead to failed implantation at the early stages of pregnancy (Leung and Adashi, 2004). Progesterone is also essential for suppressing maternal rejection of the foetus, by preventing uterine contraction and spontaneous abortion, and by changing the environment of the uterus to allow semen to survive longer (Leung and Adashi, 2004). Progesterone is a substrate for foetal adrenal production and along with estrogen, prepares the breast tissue for lactation (Leung and Adashi, 2004; Speroff and Fritz, 2005). Measurements of mid-luteal phase serum progesterone found that progesterone concentrations < 15nmol/L are directly related to defective follicle growth, abnormal ovarian functions and infertility (Baird, *et al.*, 2000; Banerjee, *et al.*, 2001). Further clinical studies found that serum mid-luteal progesterone threshold for infertility was actually 32nmol/L (van Zonneveld, *et al.*, 1994). In a study by van Zonneveld, *et al*., (1994), only nine out of fifty women (18%) whose progesterone levels were < 32nmol/L had normal cycles. Consequently, infertile women with low progesterone levels and known reproductive disorders are often referred to undertake assisted reproductive technologies (ART) to improve their chance of conception (Chambers, *et al.*, 2006; Wang, *et al.*, 2007; Kamel, 2010).

1.12.4 Assisted Reproductive Technology

Since the birth of the first IVF baby, Louise Brown in 1978, Assisted Reproductive Technology (ART) has changed the lives of thousands of couples with an estimated 3.5 million children born world-wide (ESHRE, 2008). Australia and New Zealand combined reported over 50,000 ART cycles in 2006, resulting in the birth of over 9,000 babies (Chambers, *et al.*, 2006; Wang, *et al.*, 2007). However, despite the growing success rate, there is still a percentage of failed cycles and early pregnancy losses caused by failed oocyte fertilization, spontaneous miscarriage, ectopic and unexplained loss, just to name a few (Wang, *et al.*, 2007). Furthermore, the associated expense of ART is often out of reach for many couples, with the average treatment cost per cycle of between $6000-9000 AUD in 2006, although approximately half of the cost is covered by Pharmaceutical Benefit Schemes in most Western countries (Chambers, *et al.*, 2006). As a result, instead of ART many couples investigate and explore complementary and alternative treatments in the hope of improving their fertility, and becoming pregnant (Stankiewicz, *et al.*, 2007; Smith, *et al.*, 2010).

1.13 Importance of Reproductive Toxicity Screening

Reproductive toxicity can occur at any point of the reproductive cycle including female or male fertility, prenatal and postnatal development, or an accumulation, which only becomes apparent in later generations (Spielmann, 2009). Risks may therefore occur not only to the individuals exposed to the toxic compound, but potentially have irreversible effects on their offspring (Spielmann, 2009). Therefore, strict reproductive toxicity screening guidelines are required (ICH, 1993).

Reproductive toxicity screening guidelines for new pharmaceuticals are currently set by The International Conference on Harmonisation (ICH, 1993) and the Organisation for Economic Co-Operation and Development (OECD, 2004), and are conducted at the *in vivo* stage of drug development as animal assays (OECD, TG 414, TG 415, TG 416, TG 421 and TG 422, 2004). These studies are vital as they address potential adverse effects of all new drug candidates on sexual functions in adult males and females, as well as developmental toxicity on the offspring (OECD, 2004). Multigeneration studies are also conducted to determine adverse effects on growth and development (OECD, 2004). Studies during pregnancy are required to ascertain the effects of the drug candidate on birth and pregnancy length, and lactation, along with effects on maturation on the foetus (OECD, 2004). Repeated dose screening further provides data on potential systemic toxicity by examining growth and biochemical effects of new drug candidates (OECD, 2004).

I*n vivo* animal studies provide essential data on the potential reproductive risks of new drug candidates, however they require large numbers of animals and therefore are time-consuming and very expensive (Seiler, *et al.*, 2004; Spielmann, 2005). These studies are required to provide data on how a drug candidate is absorbed, distributed and metabolised in the body, along with how the drug is eliminated and its overall toxicity in the body (Hodgson, 2001; Davis and Riley, 2004). However, animal studies whether single or multiple species are tested, do not always reflect the biochemical human response to a drug candidate (Nau, 1986). A prime example is the thalidomide tragedy in the late 1950's-1960's, which resulted in over 10,000 birth defects in new infants (Schumacher, *et al.*, 1968; Nau, 1986). Medical disasters such as the thalidomide tragedy, lead to the development of alternative methods for

screening new drug candidates for their potential reproductive toxicity (Seiler, *et al.*, 2004; Spielmann, 2005; Spielmann, 2009).

Studies using whole embryo culture (WEC) systems have been developed to examine folliculogenesis and oogenesis from early follicle growth to fertilization (Cortvrindt and Smitz, 2002). The concentration of the drug candidate and the metabolites can be easily monitored in both the medium and embryo samples (Spielmann, 1998; Spielmann, 2005). These assays have in fact been around for over 20-years and provide data of potential teratogenic compounds (Spielmann, 2005). WEC studies examine the mechanism of drug teratogenicity on embryos, which can supplement *in vivo* animal studies, resulting in a reduction in costs and number of animals required *in vivo* (Spielmann, 1998; Spielmann, 2005). However, WEC studies can only examine a part of reproductive toxicity and, are still conducted using animal embryos including rat, mouse and rabbit embryos, therefore these studies do not always reflect the reprotoxicity of the drug candidate in humans (Spielmann, 2005).

The embryonic stem (ES) cell test is another *in vitro* culture technique that employs blastocyst-derived pluriopotent ES cells from mice (Seiler, *et al.*, 2004; Spielmann, 2005). These assays were developed to examine the embryotoxicity effects of new drug candidates *in vitro* (Seiler, *et al.*, 2004). ES studies are considered reliable techniques for testing embryotoxicity because a number of different molecular end-points can be evaluated (Seiler, *et al.*, 2004). ES studies can evaluate intracellular processes, such as the effects of a drug candidate on gene expression, or a reporter gene construct can be added to the cells to monitor the effects of the drug candidate on different target cells (Seiler, *et al.*, 2004). Furthermore, these ES cells can be

developed into cell lines and therefore have the added bonus of long time exposure of drug candidates (Spielmann, 2005). Therefore, the ES assay can provide additional data on tetragencity of new drug candidates, thereby reducing the number of animals required for *in vivo* studies.

Both WEC and ES are useful assays because they provide supplementary data for *in vivo* studies, however they are still performed using animal tissues. Ideally reproductive toxicity studies using human tissues or cells would provide a more accurate account of the adverse effects of a drug candidate on some aspects of human reproduction (Edwards, *et al.*, 2008). These *in vitro* cell culture assays could provide beneficial information not only on the immediate cytotoxicity of a new drug candidate but how the drug compound affects different aspects of hormonal synthesis. *In vivo*, the hormones progesterone and estrogen play an essential role not only in normal reproductive functions but also during conception and throughout pregnancy (Leung and Adashi, 2004). *In vitro* cell culture techniques that could model the effects of a drug candidate on progesterone and or estrogen synthesis could potentially give some insight on how the drug candidate works *in vivo*. Human cell culture *in vitro* systems will never be able to predict adverse effects of drug candidates on the entire reproductive cycle, however these assays like WEC and ES studies, could provide additional useful data on specific areas of reproductive toxicity and therefore could aid current *in vivo* animal studies.

1.14 Apoptosis or Programmed Cell Death

Apoptosis or programmed cell death is a highly regulated efficient form of cell death that results in the ordered genetic elimination of unwanted or excess cells

(Hengartner, 2000; Jin and El-Deiry, 2005). It is important in a range of normal cellular processes, including fetal development, tissue homeostasis and immune responses etc (Elmore, 2007). In fact, dysregulation of apoptosis has been shown to play a major role in numerous diseases (Fischer and Schulze-Osthoff, 2005). For example, mutations that result in insufficient cell death are known to contribute to the etiology of cancer (Fischer and Schulze-Osthoff, 2005; Jin and El-Deiry, 2005).

In comparison, necrosis is a process of cell death usually brought about by acute injury to the cell (Jin and El-Deiry, 2005). The injury leads to the loss of membrane integrity and swelling of the cell which results in the uncontrolled release of the cellular contents into the surrounding tissue (Jin and El-Deiry, 2005). However, recent evidence now suggests that, like apoptosis, necrosis is highly regulated involving ATP, death receptors and caspase inhibitors (Kroemer, *et al.*, 2009).

The mechanism of programmed cell death or apoptosis is extremely complex, it can be activated by DNA damage resulting in intracellular signalling (Fischer and Schulze-Osthoff, 2005). In addition, apoptosis can be activated by extracellular signals, including environmental stress, down regulation of growth factors and exposure to cytokines (Jin and El-Deiry, 2005; Elmore, 2007). Either way, cells undergoing apoptosis exhibit characteristic morphological changes induced by a family of cysteine proteases called caspases, which are highly conserved across species (Hengartner, 2000; Elmore, 2007). These morphological changes in apoptotic cells occur in a series of events and include nuclear and chromatin condensation, loss of microvilli and gap junctions, DNA fragmentation, cell shrinkage and eventually surface blebbing or microspikes (protusions) leading to the formation of apoptotic

bodies, which can be engulfed by surrounding cells or immune cells (Collins, *et al.*, 1997; Young, *et al.*, 1997; Elmore, 2007).

The activation of programmed cell death or apoptosis begins with an initial physiological or pathological stimulus to the cell, signalling either cell surface receptors or receptors inside the cell itself (Morita and Tilly, 1999). A number of death promoting proteins are involved, including the Fas ligand (FasL), CD95 or APO-1, tumor necrosis factor-related apoptosis (TRAIL), the tumor necrosis factor (TNF) superfamily and the Bcl-2 family of proteins (Morita and Tilly, 1999; Elmore, 2007). This stimulus results in intracellular signalling events involving, the tumor suppressor genes p53 and WT-1, c-Myc gene, Akt protein family and the protein kinase, SAPK (Maheswarran 1993) (Tilly, 1996; Amsterdam and Selvaraj, 1997; Morita and Tilly, 1999). This in turn triggers a number of regulator proteins that belong to the family of Bcl-2 proteins; proteins that are both anti-apoptotic and pro-apoptotic (Tilly, 1996). The pro-apoptotic proteins interact (Glamoclija, *et al.*, 2005), resulting in an auto or transcatalytic change in caspases such as procaspase-9 (Elmore, 2007). Caspase-9 then initiate a sequential caspase cascade, by the activation of death effector caspases, including caspase-3, -6 and -7 (Glamoclija, *et al.*, 2005). There are three main pathways known to be involved in this apoptotic cascade, two of which are linked and can interact, the intrinsic and extrinsic pathways, and a third pathway the granzyme pathway (Figure 10; Elmore, 20007). All three pathways involve specific genes and a cascade of molecules and enzymes (Garcia-Calvo, *et al.*, 1999; Elmore, 2007).

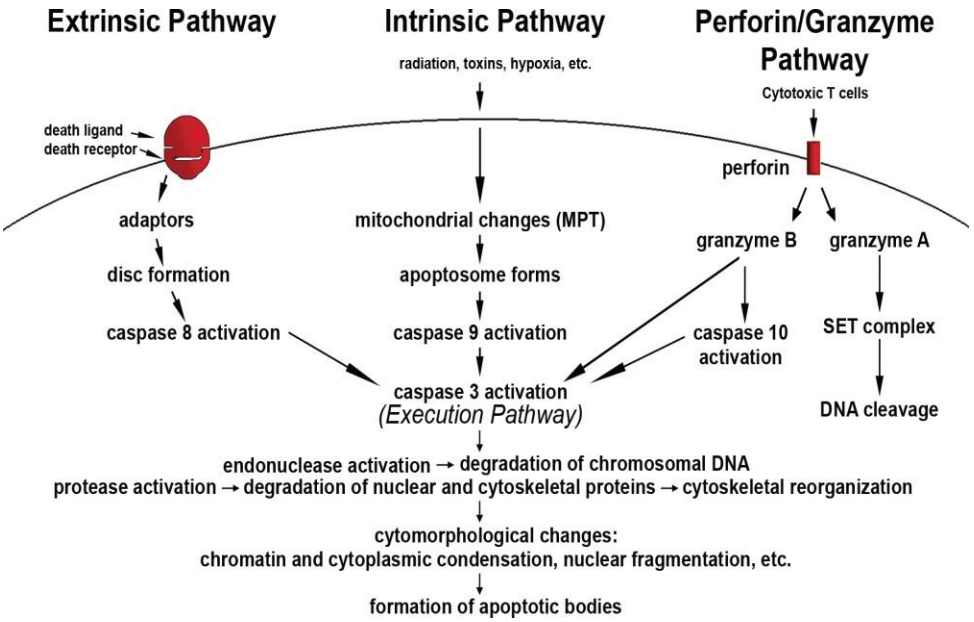


Figure 1.10: Apoptosis pathways.
The different pathways of apoptosis showing the two main pathways intrinsic and extrinsic, along with a third pathway the granzyme pathway. Each pathway activates its own caspases (8, 9 10) which eventually activate the executioner caspase 3, sending the cell into irreversible cell death (Elmore, 2007).

The intrinsic or mitochondria pathway is activated via a non-cell-surface receptor stimuli, which initiates intracellular signals (Figure 1.11; Jin and El-Deiry, 2005; Elmore, 2007). A diverse range of apoptotic stimuli at the mitochondria results in the release of cytochrome C into the cytoplasm (Jin and El-Deiry, 2005). Cytochrome C binds to the apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, this results in the formation of a DISC-like complex, known as apoptosome (Jin and El-Deiry, 2005). The formation of the apoptosome leads to the activation of caspase-9, which in turn leads to the sequential activation of effector caspases including caspase-3 (Jin and El-Deiry, 2005; Elmore, 2007).

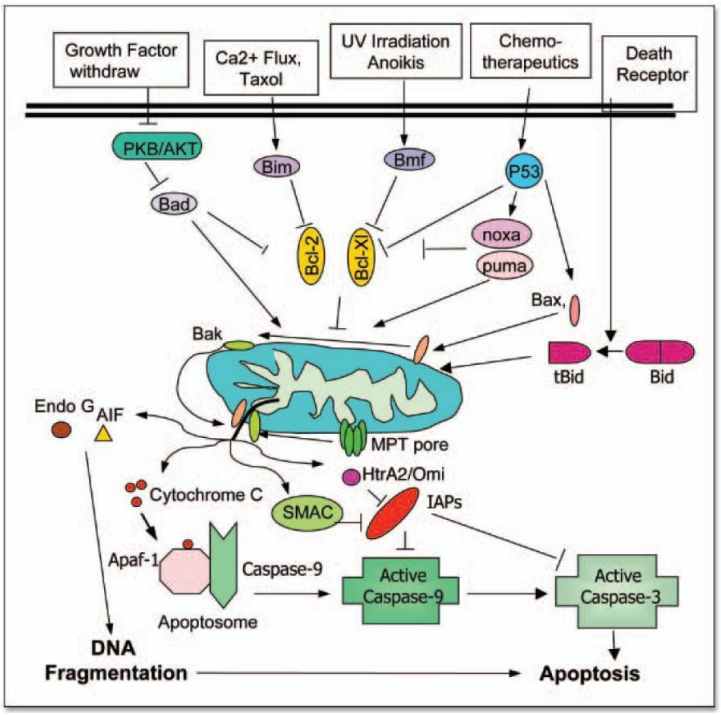


Figure 1.11: The intrinsic apoptotic pathway.
The intrinsic pathway mediated by the mitochondria (Jin and El-Deiry, 2005).

On the other hand the extrinsic pathway (Figure 1.12) is mediated by cell surface trans-membrane (death) receptor binding, involving the tumor necrosis factor (TNF) receptor gene family, FasL-Fas receptors or TRAIL-DR4-DR5 receptors (Jin and El-Deiry, 2005; Elmore, 2007). Death receptor binding results in the activation of adaptor proteins such as FAD or TRADD and recruits caspase-8, forming a death inducing signalling complex (DISC) (Jin and El-Deiry, 2005). Auto-activation of caspase-8 at DISC, activates downstream effector caspases, including caspase-3, -6 and -7, resulting in cell death (Jin and El-Deiry, 2005).

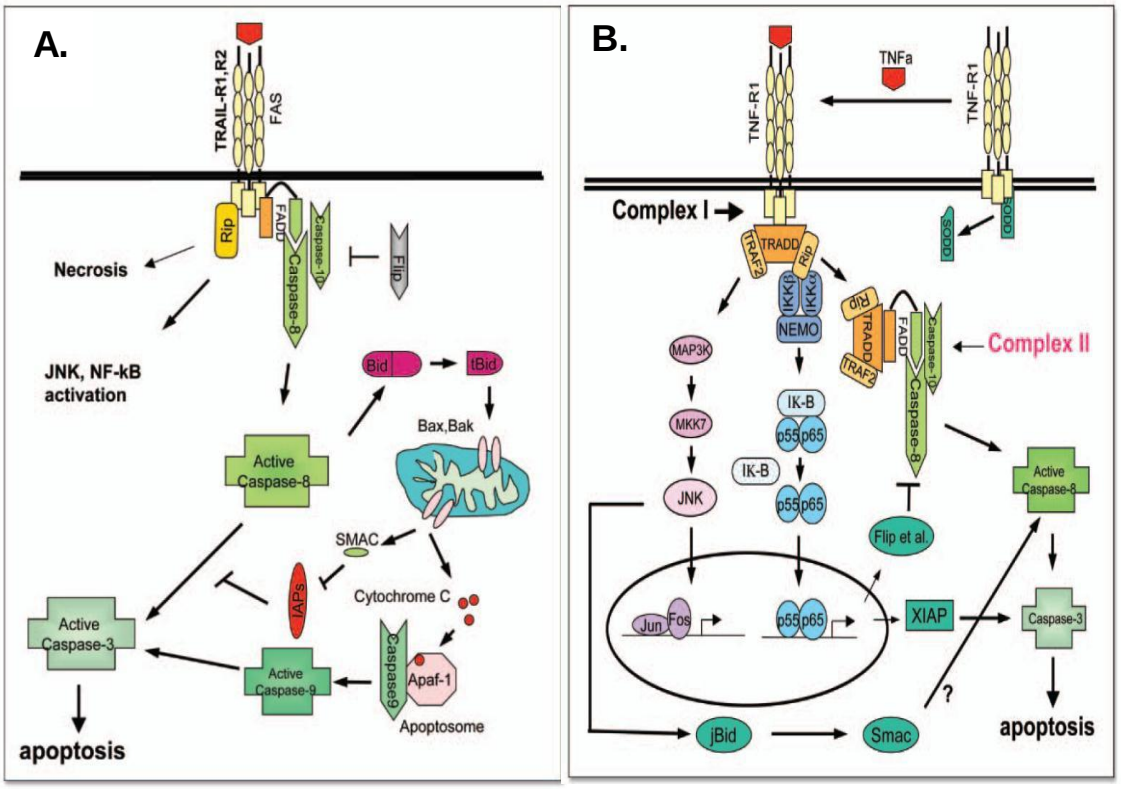


Figure 1.12: The extrinsic apoptotic pathway.
The extrinsic apoptotic pathway. (A) The extrinsic pathway can be activated by different death receptors including TRAIL-DR4-DR5 or FasL-Fas or (B) TNF-TNFR1 (Jin and El-Deiry, 2005).

The third pathway, the granzyme pathway, induces apoptosis by T-cell mediated cytotoxicity; via granzyme A or B pathways (Figure 1.10; Elmore, 2007). A central element of all three pathways is the activation of a series of initiator or procaspases (caspase 8, 9 10), and effector caspases including the key effector caspases-3 (Nguyen and Wells, 2003; Elmore, 2007). Caspase-3 (known as an executioner caspase) is considered to be one of the most important caspases in apoptosis because once caspase-3 is activated the cell is irreversibly committed to cell death (Figure 1.10; Nguyen and Wells, 2003; Elmore, 2007). The execution pathway results in DNA degradation by endonucleases, chromatin condensation and cell shrinkage and finally the formation of apoptotic bodies (Elmore, 2007; Wieder, 2008).

1.15 Reproductive Cancer Cell lines

1.15.1 The OVCAR-3 Human Ovarian Carcinoma cell line

The human ovarian carcinoma cell line, OVCAR-3 was developed from adenocarcinoma tissue of the ovary (Hamilton, *et al.*, 1983). Cultured OVCAR-3 cells exhibit functional androgen and estrogen receptors (Hamilton, *et al.*, 1983). OVCAR-3 cells are useful in pharmacological studies of drug resistance as these cells have been shown to be resistant to antineoplastic drugs, including adriamycin, melphalan and cisplatin (Hamilton, *et al.*, 1983).

1.15.2 Granulosa Cell lines

A number of human ovarian granulosa cell lines have been established to study the mechanisms and regulations of steroidogenesis (Havelock, *et al.*, 2004). The first human granulosa cell line developed was the HTOG cell line, which was isolated from an ovarian tumour of a 75-year old female (Ishiwata, *et al.*, 1984). The immortalised COV434 human granulosa cell line was developed from a granulosa cell carcinoma taken from a 27-year old female and was the first human cell line developed that responded to FSH stimulation due to a functional FSH receptor (Van Den Berg-Bakker, *et al.*, 1993; Zang, *et al.*, 2000). Rainey *et al*. (1994) developed the HGL5 human granulosa adherent cell line using open reading frames (E6 & E7) from the human papillomavirus strain 16 (HPV 16). The transfection was performed by inserting HPV16 E6/E7 DNA into the retroviral vector pLXSN (Rainey, *et al.*, 1994).

The KGN human granulosa cell line was established from a granulosa cell tumour taken from a 65-year old female (Nishi, *et al.*, 2001). KGN cells are an adherent cell line and were found to grow in monolayers (Nishi, *et al.*, 2001). Stability of the cell line was maintained after 5-years with over 100 passages, and the doubling rate was

estimated at 46.4 hours (Nishi, *et al.*, 2001). Nishi *et al.* (2001) identified the KGN cells as steroidogenic cells by immunohistochemical staining against the human cytochrome P450arom (aromatase enzyme that catalyses the conversion of androgens to estrogens). Aromatase activity was found to be similar to normal human primary-derived granulosa cells and the activity increased when treated with FSH and human menopause gonadotrophin (hMD) hence the authors concluded that the KGN must exhibit a functional FSH receptor (Nishi, *et al.*, 2001). Furthermore, Nishi *et al.* (2001) demonstrated that KGN cells secrete the hormones pregnenolone and progesterone in culture and concentrations of the hormones were increased when stimulated dose-dependently with dibutyryl cAMP. Nishi, *et al.*, (2001), have demonstrated that like primary-granulosa cells, the KGN cancer cell line can undergo apoptosis via the Fas-Fas ligand pathway.

1.15.3 The Human Choriocarcinoma JAr Cell Line

The human JAr choriocarcinoma cell line was initially developed in 1971 from a trophoblastic tumor from a human placenta of a twenty-five year old female (Pattillo, *et al.*, 1971). This cell line is known to produce the steroidal hormones estrogen, progesterone, gonadotrophic hormones (hCG) and lactogenic hormones (HPL) in cell culture (Pattillo, *et al.*, 1971; Nilkaeo, 2003). It has been noted that the JAr cell line grows rapidly *in vitro* with a doubling rate of approximately 15h (Adler, *et al.*, 1995). Plessinger and Miller (1999) demonstrated that JAr cells proliferate rapidly and exhibit invasive characteristics, changes that are known to occur in the early placenta. The JAr cell line has been used to investigate regulation pathways including transportation of serotonin activated by cAMP regulation (Cool, *et al.*, 1991) and in apoptosis studies (Zhang, *et al.*, 2001). Hallamann *et al*. (2004) partially characterised apoptosis in JAr cells using menadione 2 methyl-1,4-napthoquinone

(MEN) and hydrogen peroxide as inducers of oxidative stress and apoptosis. Chen et *al.* (2005) investigated mechanisms of tumour invasion of the placenta with JAr cells.

1.16 Aim, Objectives and Hypotheses

The overall aim of this research was to investigate the anticancer effects and steroidogeneic activity of bioactive compounds derived from the marine whelk *Dicathais orbita* along with similar synthetic compounds on primary-derived female human reproductive cells and compared with female human reproductive cancer cells.

1.16.1 Objectives

1. Compare three colourmetric assays, MTT, MTS and crystal violet for use in determining cell viability in reproductive cells.
2. Optimise the culture conditions for the reproductive cancer cell line, JAr as follows:
    - Determine the minimum time required for JAr cells to firmly attach to treatment cell culture plates.
    - Determine the optimum concentration of MTT substrate for the JAr cell line.
    - Determine the optimum human choronic gonadotrophin (hCG) concentration required to stimulate progesterone synthesis.
    - Comparison the ELISA and RIA assay for measurement of progesterone synthesis.
    - Establish an effective positive control for the JAr cell line that can then be used when testing novel compounds
3. Examine the effects of synthetic brominated-indole compounds on primary-

derived human granulosa cells and compare against the JAr reproductive cancer cell line.

4. Isolate, purify and identify the bioactive compounds from the hypobranchial glands and egg masses of the marine mollusc *D. orbita*.
5. Investigate the cytotoxic effects of the semi-purified fractions from *D. orbita* containing bioactive compound on primary-derived human granulosa cells and compare with the female reproductive cancer cell lines, KGN (equivalent cancer granulosa cell line), JAr and OVCAR-3.
6. To examine the effects of the semi-purified fractions on basal progesterone and estradiol synthesis and human choronic gonadotrophin (hCG) and dibutyryl adenosine 3‘,5‘-cyclic monophosphate (db-cAMP) stimulated progesterone and estradiol synthesis by the reproductive cells
7. To determine if the semi-purified *D. orbita* fractions induce apoptosis in the primary-derived human granulosa cells and the reproductive cancer cell lines by measurement of caspase-3/7 activity, apoptotic DNA fragmentation using the TUNEL assay and DAPI staining or necrosis by loss of membrane integrity and LDH release.

1.16.2 Hypotheses

1. The synthetic brominated indole compounds will inhibit proliferation of reproductive cancer cells but have minimal effect on primary-derived human granulosa cells.
2. Bioactive compounds from *D. orbita* will kill, inhibit or arrest growth of human reproductive human cancer cell lines without affecting normal healthy reproductive cells.
3. The compounds from *D. orbita* will induce apoptosis in the human

reproductive cancer cells at lower concentrations than in the pnmary-derived human reproductive cells.

4. The muricid compounds will mcrease progesterone and estrogen production in primary-derived human reproductive cell lines.

Chapter 2 Materials & Methods

2.1 Reagents

All chemical reagents used throughout this research were HPLC grade and purchased from the company Sigma-Aldrich unless otherwise stated (Appendix I). Reagents are grouped under the experimental categories for which they are generally used and experimental categories are listed in alphabetical order.

2.1.1 Apoptosis and Necrosis Reagents

2.1.1.1 Caspase-Glo 3/7 Reagent

The Caspase-Glo® 3/7 Assay kit (Promega) was stored at -20^{o}C protected from light prior to use. For assays, the pre-equilibrated buffer (10mL) was added to the lyophilized substrate (25^{o}C) and mixed by gentle inversion to dissolve the substrate. Any remaining substrate not used on the day of the experiment was stored at 4^{o}C protected from light for 3-days (all experiments were performed within this time period).

2.1.1.2 CytoTox-ONE Reagent

The CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega) was stored at -20^{o}C until required. For assays, the substrate mix (supplied by manufacturer) containing resazurin, was equilibrated to 22^{o}C and then dissolved in 11mL of assay buffer (supplied by manufacturer), pre-equilibrated to 22^{o}C; the solution was mixed gently by inversion to dissolve the substrate. Unused substrate was stored at -20^{o}C protected from light and used within 1-week of preparation.

2.1.1.3 4',6-Diamidino-2-phenylindole dihydrochloride for Fluorescence (DAPI)

2.1.1.3.1 Cell Fixative (CH_3CH_2OH, $CHCl_3$, CH_3CO_2H)

A solution of ethanol (Merck), chloroform (BDH), and acetic acid (6:3:1, v/v/v) was prepared in a final volume of 100mL and mixed with stirring. The solution was stored at room temperature in a glass Pyrex bottle until required

2.1.1.3.2 DAPI stain (4′,6-Diamidino-2-phenylindole dihydrochloride)

A 1mg/mL stock solution of DAPI was prepared in sterile water and mixed by vortexing. Aliquots of 0.02mL were stored at -20^{o}C. For assays, stock DAPI stain was diluted in sterile PBS (section 2.1.2.5) to a working concentration of 1µg/mL.

2.1.1.4 DNase I Buffer

2.1.1.4.1 Tris-HCl (1M; pH 7.9)

A 1M Tris-HCl solution in a final volume of 50mL was first prepared by dissolving 6.057g of Tris-HCl in 40mL of sterile water for injections (AstraZeneca). The pH was adjusted to 7.9 and then the volume was increased to 50mL with sterile water for injections (AstraZeneca). The solution was autoclaved and stored in a glass Pyrex bottle at room temperature.

2.1.1.4.2 DNase I Buffer (50mL)

Tris-HCl (2mL; 40mM) was added to a glass beaker along with NaCl (29.215mg; 10mM), $MgCl_2$ (60.03mg; 6mM) and $CaCl_2$ (55.49mg; 10mM) with 40mL of sterile water for injections (AstraZeneca) and mixed with stirring. The solution was then increased to 50mL with sterile water for injections (AstraZeneca) and stored at 4^{o}C until required.

2.1.1.5 Glycerol Buffered Mounting Medium (pH 8.6) for Mounting Slides

2.1.1.5.1 Sodium Carbonate (0.5M Na_2CO_3) Solution A

A 100mL solution of Na_2CO_3 was prepared by dissolving 5.3g of Na_2CO_3 in 80mL of MilliQ water. The solution was mixed with stirring and the pH adjusted to 11.5. The solution was then increased to 100mL with MilliQ water and stored at room temperature in a glass Pyrex bottle.

2.1.1.5.2 Sodium Bicarbonate (0.5M Na_2HCO_3) Solution B

Solution B (200mL) was prepared by adding 8.4g of Na_2HCO_3 to 180mL of MilliQ water and mixed with stirring. The pH was then adjusted to 8.16 and volume

increased to 200mL. Solution B was stored at room temperature in a glass Pyrex bottle.

2.1.1.5.3 Glycerol Buffered Mounting Media (90ml)

50mL of Solution B (Na_2HCO_3; section 2.1.2.5.2) was added to a glass beaker and the pH was adjusted to 8.6 with solution A (Na_2CO_3; section 2.1.2.5.1; ~2mL). Then 60mL of glycerol (UNIVAR) was added to 30mL of buffered Na_2HCO_3 solution (2:1; v/v) and mixed with stirring. Glycerol buffered mounting media was stored at room temperature in a glass Pyrex bottle.

2.1.1.6 TUNEL Reagents

2.1.1.6.1 rTdT Incubation Buffer

The rTdT incubation buffer was prepared by adding equilibrium buffer, nucleotide mix and rTdT enzyme (all supplied by manufacturer; and thawed on ice just prior to use) at a working ratio of 90:10:2 respectively in a final volume of 50µL/slide. For negative control slides the incubation buffer was prepared with nucleotide mix and sterile water minus the rTdT enzyme.

2.1.1.6.2 Paraformaldehyde 4% Solution for Fixing Cells (100ml)

Paraformaldehyde (4g: BDH) was added to 80mL of sterile PBS (section 2.1.2.5), and mixed with stirring. The volume was then increased to 100mL and the solution was heated to 60^{o}C to produce a clear solution. The paraformaldehyde solution was stored in glass at 4^{o}C for no longer than 2-weeks.

2.1.1.6.3 Propidium Iodide (PI) for Staining the Nucleus of Cells

A stock solution of PI (1mg/mL) was prepared by dissolving PI (1mg) in MilliQ water (1mL). The solution was mixed and stored in 0.01mL aliquots at 4^{o}C. For cell assays, the stock PI solution was prepared fresh in 1x sterile PBS (section 2.1.3.5) to give a working concentration of 1µg/mL.

2.1.1.6.4 2x Sodium Chloride and Sodium Citrate (SSC) Solution

20x of sodium chloride and sodium citrate solution (SSC; supplied by manufacturer) was diluted 1:10 with MilliQ water in a final volume of 100mL by adding 10mL of stock SSC to 90mL of MilliQ water to give a working concentration of 2x. The solution was mixed by inversion and stored at room temperature until required.

2.1.1.6.5 Triton X-100 Solution (0.2%)

0.2mL of Triton X-100 was added to 90mL of sterile 1x PBS (section 2.1.2.5) and mixed with stirring. The solution was then increased to 100mL with PBS (2.1.2.5) and stored in a glass Pyrex bottle at room temperature until required.

2.1.2 Medium, Maintenance and Characterisation Reagents

2.1.2.1 DMEM Media

2.1.2.1.1 DMEM Wash Medium

Dulbeccos Minimum Essential Medium/HAMS F12 powder (12g/L; Invitrogen Corporation), sodium bicarbonate (14.28ml/L), penicillin (0.6g/L) and streptomycin (0.05g/L) were added to 4L of sterile MilliQ water (Baxter). The pH was adjusted to 7.4 and then sterile MilliQ water (Baxter) was added to increase volume to 4.5L. The solution was filtered through a 0.22µm Minisart Millipore filter with suction under aseptic conditions and stored in 500mL aliquots at -20^{o}C until required in sterile glass 500ml Pyrex bottles. Stock medium was stored for no longer than 12-months.

2.1.2.1.2 Insulin, Transferrin & Selenium (ITS)

2.1.2.1.2.1 Selenium Sodium Selenite (1mg/mL) Stock Solution

A 1mg/mL stock solution of selenium sodium selenite was prepared in sterile 1x PBS (section 2.1.2.5) in a final volume of 3mL and stored in 0.02ml aliquots at -20^{o}C until required.

2.1.2.1.2.2 ITS 40mL Solution

A 40mL ITS solution was prepared at 100x the final concentration to give concentrations of Insulin solution human (0.5mg/mL), Transferrnin Apo-human (0.5mg/mL) and Selenium sodium selenite (0.5µg/mL). Insulin solution human

(2mL; 10mg/mL), Transferrin Apo-human (20mg) and selenium (0.02mL of stock solution; section 2.1.2.1.2.1) were added to DMEM base media (37.98mL) and mixed with stirring. The ITS solution was then filtered through a sterile 0.22µm filter (Sartorius) and syringe and stored at -20^{o}C in 1.1mL aliquots. For complete media, 1mL of stock ITS solution was added per 100mL of base media to give working concentrations of 5µg/mL of insulin and transferin apo-human and 5ng/mL selenium sodium selenite.

2.1.2.1.3 DMEM Complete Medium

DMEM complete medium was prepared by adding 10% heat inactivated fetal bovine serum (section 2.1.2.3) and ITS solution (1mL/100mL; section 2.1.2.1.2.2) to DMEM wash media (section 2.1.2.1.1). The medium was adjusted to pH 7.4 and then filtered through a 250mL sterile filter under suction and aseptic conditions. The medium was then stored in a 500mL sterile glass Pyrex bottle at 4^{o}C; medium was equilibrated to 37^{o}C as required. Complete medium was used within one week of preparation.

2.1.2.2 Freezing Medium for Cryopreservation and Long Term Storage of Cells

20% heat inactivated fetal bovine serum (FBS; section 2.1.2.3) and 5% sterile dimethyl sulfoxide (DMSO), were added to 7.5mLs of sterile complete medium (section 2.1.2.1.3 or 2.1.2.6.4). The freezing media was stored at 4^{o}C for no longer than a week. When used for freezing down cells, the freezing medium was put on ice 30 minutes prior to use.

2.1.2.3 Heat Inactivation of Fetal Bovine Serum (FBS)

Sterile fetal bovine serum (FBS; Invitrogen Corporation; 500ml) was heated to 56^{o}C for 30 minutes in a water-bath (American Type Culture Collection; ATTC). The serum was stored at -20^{o}C in 10mL aliquots and when required the heated inactivated

FBS was equilibrated to 37^{o}C and used at a final concentration of either 10 or 20% depending on cell line.

2.1.2.4 3-β-Hydroxysteroid Dehydrogenase Stain (3- βHSD)

2.1.2.4.1 Trans-Dehydroandrosterone (DHA)

A stock solution of trans-dehydroandrosterone (DHA) at 100x working concentration of 0.5mg/mL was prepared in chloroform (BDH) by adding 50mg of DHA to 1mL of chloroform in a sterile glass McConkey bottle. For assays, the stock solution was diluted in sterile PBS (section 2.1.2.5) to a working concentration of 0.5mg/mL.

2.1.2.4.2 Formalin Solution (10%)

10mL of formaldehyde was measured out in a volumetric flask and added to 90mL of sterile PBS (section 2.1.3.5). The solution was stored in a glass Pyrex bottle at room temperature.

2.1.2.4.3 3 β-Nictoinamide Adenine Dinucleotide (NAD)

A stock solution of β-nictoinamide adenine dinucleotide (NAD) was prepared at 10x the working concentration of 2mg/mL by adding 200mg of NAD to 10mL of sterile MilliQ water (Baxter). Stock NAD solution was stored at -80^{o}C in 0.5mL aliquots until required. For assays, NAD stock solution was diluted in sterile 1x PBS (section 2.1.2.5) solution to a working concentration of 2mg/mL.

2.1.2.4.4 Nitroblue Tetrazolium (NBT)

A stock solution of Nitroblue tetrazolium (NBT; BIORAD) was prepared at 100x the working concentration of 0.5mg/mL by adding 50mg of NBT to 1mL a 70% solution of dimethyl formamide. Stock NBT solution was stored at -20^{o}C. For assays, stock NBT was dissolved in sterile 1x PBS (section 2.1.2.5) to a working concentration of 0.5mg/mL.

2.1.2.4.5 3-βHSD Stain

DHA (0.5mg/mL; section 2.1.2.4.1), NAD (2mg/mL; section 2.1.2.4.3), and NBT (0.5mg/mL; section 2.1.2.4.4) were added to sterile 1x PBS (section 2.1.2.5) in a

sterile McConkey bottle under aseptic conditions. The solution was mixed by gentle inversion of the bottle. 3βHSD was used immediately on cells after preparation.

2.1.2.5 Phosphate Buffer Saline (PBS)

NaCl (AJAX Chemicals; 98g), KCl (0.2g), $Na_2H_2PO_4$ (AJAX Chemicals; 1.44g) and KH_2PO_4 (AJAX chemicals; 0.24g) were added to 950mL of MilliQ water. The pH was adjusted to 7.4 and then the volume increased to 1L with MilliQ water. PBS was filter sterilized through a 250mL filter in 4 aliquots and stored at room temperature in a sterile 1L glass Pyrex bottle.

2.1.2.6 RPMI 1640 Medium

2.1.2.6.1 Base Medium

RPMI 1640 medium (10.44g/L; without glucose and L-glutamine), sodium bicarbonate (1.5g/L), HEPES (10mM), 1mM sodium pyruvate (0.11g/L) and penicillin/streptomycin (5000μg/mL) were added to 8.5L of sterile MilliQ water (Baxter) and mixed with stirring. The base medium solution was adjusted to pH 7.4 and the volume increased to 10L. The medium was filtered using a 0.22μm Minisart Millipore filter using a peristatic pump under aseptic conditions and stored at -20^{o}C in 500mL sterile glass Pyrex bottles.

2.1.2.6.2 Glucose (100x Working Concentration of 4.5g/L)

Glucose (22.5g) was dissolved in 40mL of sterile water with heat and stirring, the volume was then increased to 50mL. The glucose solution was filter sterilized into 1.1mL aliquots using a 10mL syringe and sterile 0.22μm filter (Sartorius) and stored at -20^{o}C until required, when it was diluted 1:100 to give a final working concentration of 4.5g/L.

2.1.2.6.3 L-glutamine (100x Working Concentration 2mM/L)

L-glutamine (1.46g) was added to 50mL sterile water and filter sterilized into 1.1mL aliquots using a sterile 10mL syringe and sterile 0.22μm filter (Sartorius). Aliquots

were stored at -20^{o}C until required, when it was diluted 1:100 to give a final working concentration of 2mM/L.

2.1.2.6.4 RPMI Complete Medium

Glucose (4.5g/L; section 2.1.2.6.2), L-glutamine (2mM; section 2.1.2.6.3), 10 or 20% heat inactivated foetal bovine serum (section 2.1.2.3) for JAr and OVCAR-3 cell line respectively and insulin solution from bovine pancrease (0.01mg/mL) for OVCAR-3 cell line only were added to RPMI base medium (section 2.1.2.6.1). Complete medium was filtered under aseptic conditions through a 0.22µm sterile filter and stored in 200-400mL aliquots in sterile 500mL glass Pyrex bottles at 4^{o}C. The medium was equilibrated to 37^{o}C as required. Complete medium was used within one week of preparation.

2.1.2.7 [3H]-Methyl Tritiated Thymidine

A stock solution of 1mCi/ml ^{3}H-methyl tritiated thymidine was diluted in assay medium (section 2.1.2.1.3) at a working concentration of 1µCi/mL and added to the wells of Nunc Lab-Tek II CC2 Chamber Slides. The slides were then incubated at 37^{o}C + 5% CO_2 for 6h. At the end of incubation period the supernatant was removed from the wells and discarded. Cells were fixed to wells with 10% formalin (section 2.1.2.4.2) and then micro autoradiography of slides was performed (sections 2.2.1.1.2 and 2.2.1.1.3).

2.1.3 Cell Viability Assay Reagents

2.1.3.1 Crystal Violet Stain

2.1.3.1.1 Crystal Violet Solution (0.5%)

Crystal violet (0.5g; Aldrich) was dissolved in a 50% methanol solution (Merck; 100mL) and stored at room temperature in a glass Pyrex bottle in a fume hood until required. For experiments 50µL per well was added to cells in a 96-well flat-bottom plate.

2.1.3.1.2 Acetic Acid Solution (33%)

33mL of acetic acid solution (APS Chemicals) was added to 67mL of reverse osmosis water and stored in a 100mL sterile glass Pyrex bottle at room temperature.

2.1.3.2 Thiazolyl Blue Tetrazolium (MTT)

MTT cell-cultured tested (250mg) was dissolved in 50mL of sterile 1x PBS (section 2.1.2.5) to give a final concentration of 5mg/mL. This MTT stock solution was filtered using a sterile 0.22µm filter (Sartorius) and under aseptic conditions, distributed into sterile 1.5mL eppendorf tubes (1.1mL aliquots) and stored at -20^{o}C until required.

2.1.3.3 20% Sodium Dodecyl Sulfate (SDS) in 0.02M Hydrogen Chloride (HCl)

2.1.3.3.1 0.02M Hydrogen Chloride (HCl)

HCl (12M) was diluted in 1L of MilliQ water to a final concentration of 0.02M by adding 1.66mL of 12M HCl to 998.33mL of water. The solution was stored at room temperature in a glass Pyrex bottle.

2.1.3.3.2 20% Sodium Dodecyl Sulphate (SDS)

200mL of 20% SDS was prepared by adding SDS (40g) to 0.02M HCl (100mL) with stirring and heat to dissolve powder. HCl 0.02M was added to give final volume of 200mL.

2.1.3.4 Trypan Blue Reagent

2.1.3.4.1 0.9% Sodium Chloride Solution (NaCl)

A 100mL 0.9% sodium chloride solution was prepared by dissolving 0.9g of NaCl in ~ 90mL of MilliQ water with stirring. The volume was then increased to 100mL with MilliQ water, and the solution stored in a glass Pyrex bottle at room temperature.

2.1.3.4.2 Trypan Blue Solution (0.2%)

Trypan blue (0.2g) was dissolved in 0.9% NaCl saline solution (100mL) and then filter sterilized into 5mL aliquots and stored at 4^{o}C until required.

2.1.4 Hormone Measurement Reagents

2.1.4.1 Enzyme Immunoassay (ELISA) Reagents

2.1.4.1.1 ABTS (40mM)

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; 0.11g) was dissolved in 5mL of reverse osmosis water and stored in a dark bottle at 4^{o}C until required. ABTS solution was used within one month of preparation.

2.1.4.1.2 3-O-Carboxymethyloxime-Horseradish Peroxidase (HRP)

The 3-CMO horseradish peroxidise progesterone (HPR) stock conjugate (Munro and Stabenfeldt, 1983; Brown, *et al*., 2005) was stored at -80^{o}C until required. For experiments the HRP conjugate was diluted 1:200 in EIA buffer (section 2.1.4.1.4) by adding 25µL of conjugate to 4.97mL of EIA buffer and stored at 4^{o}C. The stock conjugate buffer was used within one month of preparation. For ELISA assays, the conjugate was further diluted 1:200 to give a working concentration of 1:40,000 in EIA buffer and used immediately (section 2.1.4.1.4).

2.1.4.1.3 Coating Buffer

Sodium carbonate anhydrous (Na_2CO_3; 0.795g; May & Baker) and sodium hydrogen carbonate ($NaHCO_3$; 1.465g; BDH) were added to 500mL of reverse osmosis water with stirring. The pH was adjusted to 9.6. The coating buffer was stored in a 500mL sterile glass Pyrex bottle at 4^{o}C until required. Solution was used within one month of preparation.

2.1.4.1.4 Enzyme Immunoassay (EIA) Buffer

Sodium chloride (NaCl: 4.35g; Ajax Chemicals), stock solution A (97.5mL; section 2.1.4.1.7), stock solution B (152.5mL; section 2.1.4.1.8) and bovine serum albumin (BSA; 0.5g) were added to 250mL of reverse osmosis water with stirring. The pH of the enzyme immunoassay buffer (EIA) buffer was adjusted to 7 and the solution was stored at 4^{o}C until required in a glass 500mL sterile Pyrex bottle. The buffer was used within one month of preparation.

2.1.4.1.5 CL425 Primary Antibody

The monoclonal CL425 antibody, raised in rabbit against progesterone 11α-hemisuccinyl-bovine serum albumin immunogen, was stored at -80^{o}C until required (Munro and Stabenfeldt, 1983; Brown, *et al*., 2005). Aliquots of the CL425 antibody were diluted 1:50 by adding 100µL to 4.9mL of coating buffer (section 2.1.4.1.3). 400µL aliquots were stored at -20^{o}C until required. For ELISA assays, the antibody was diluted at further 1:200 in coating buffer (section 2.1.4.1.3) to a working concentration of 1:10,000 and used immediately.

2.1.4.1.6 Progesterone Standards (0-4ng/ml)

Progesterone powder (10mg) was added to 1mL of 100% ethanol (Merck) in a sterile tube and mixed by vortex to give a stock solution of 10mg/mL. Then 0.01mL of stock progesterone solution was added to 9.99mL of cell culture media (section 2.1.2.6.4) to give a solution of 10µg/mL. A final working solution of 4ng/ml was prepared by adding 0.004mL of 10µg/mL progesterone solution to 9.996mL of cell culture media (section 2.1.2.6.4) Ten ELISA standards from 0-4ng/mL were then prepared in cell culture media (section 2.1.2.6.4) by 1:2 serial dilution. New standards were prepared for each experiment.

2.1.4.1.7 Stock Solution A (0.2M)

Sodium dihydrogen orthophosphate (NaH_2PO_4; 13.9g; BDH) was added to 500mL of reverse osmosis water with stirring. The solution was stored at 4^{o}C in a 500mL sterile glass Pyrex bottle until required. The solution A was used within one month of preparation.

2.1.4.1.8 Stock Solution B (0.2M)

Di-sodium hydrogen orthophosphate (Na_2HPO_4; 14.2g; BDH) was added to 500mL of reverse osmosis water with stirring. The solution was stored at 4^{o}C in a 500mL

sterile glass Pyrex bottle until required. The solution B was used within one month of preparation.

2.1.4.1.9 Substrate Buffer

Citric acid anhydrous (4.805g; May & Baker) was added to 500mL of reverse osmosis water with stirring. The pH was adjusted to 4 and the substrate buffer was stored at 4^{o}C in a 500mL glass sterile Pyrex bottle until required. The buffer was used within one month of preparation.

2.1.4.1.10 Wash Solution Concentrate

Sodium chloride (NaCl; 43.83g; Ajax Chemicals) and Tween-20 (2.5mLs) were added to 500mL of reverse osmosis water with stirring. The solution was stored in a 500mL sterile glass Pyrex bottle at 4^{o}C until required. For experiments, the wash solution was diluted 10-fold in MilliQ water. The wash solution was used within one month of preparation.

2.1.4.2 Radioimmunoassay Reagents

2.1.4.2.1 Anti-progesterone Primary Antibody Raised in Rabbit

An anti-progesterone primary antibody raised in rabbit (no defined concentration-titer, 1:60; MP Biomedical) was stored at -20^{o}C in 0.1mL aliquots. The primary antibody was diluted in Nafam buffer (section 2.1.4.2.7) to a concentration of 1:100 in a final volume of 0.1mL and stored at 4^{o}C. For radioimmunoassay the antibody was diluted a further 1:100 in (section 2.1.4.2.7) to give a working concentration of 1:10,000 and used immediately.

2.1.4.2.2 Donkey Anti-Sheep/Goat IgG (DAG)

Donkey anti-sheep/goat antibody at a concentration of 39mg/mL (DAG; Chemicon) was stored at 4^{o}C until required. For the radioimmunoassay, the DAG was diluted in Nafam buffer (2.1.4.2.7) to a working concentration of 1:30 and used immediately.

2.1.4.2.3 Estradiol Standards (0-2,000pg/mL)

A stock solution of estradiol (2,000pg/mL) was prepared in a final volume of 5mL of complete medium by adding 2.5µL of a stock solution of estradiol (Diagnostics Systems Laboratories; 4µg/mL) to 4.99mL of complete medium (sections 2.1.2.1.3 and 2.1.2.6.4). Standards of 750pg/mL -0pg/mL were then prepared as per Table 2.1. New standards were prepared for each experiment.

Table 2.1: Estradiol standards 0-2,000pg/mL for the estradiol RIA

Final Concentration	From Concentration	Volume	Volume of media	Final Volume
2,000pg/mL	4,000,000pg/mL	2.5µL	4.997mL	5mL
750pg/mL	2,000pg/mL	1.875mL	3.125mL	5mL
250pg/mL	750pg/mL	1.667mL	3.333mL	5mL
50pg/mL	250pg/mL	1mL	4mL	5mL
20pg/mL	50pg/mL	2mL	3mL	5mL
0pg/mL	-	-	5mL	5mL

2.1.4.2.4 Estradiol [I^{125}] Tracer

Estradiol^{125}I radioactive tracer (no defined concentration-titer. 1:60; MP Biomedicals) was stored at 4^{o}C until required. For experiments a 10µL sample was counted on a Packard RIASTAR Gamma counter to determine activity and then tracer was diluted in Nafam buffer (section 2.1.4.2.7) to 10,000 counts per minute as required and used immediately.

2.1.4.2.5 Goat polyclonal to 17β estradiol

Goat polyclonal antibody to 17β estradiol (Sapphire Bioscience; concentration not determined because the serum contains a mixture of proteins) was stored at -20^{o}C in 0.01mL aliquots until required. The primary antibody was diluted in Nafam buffer (section 2.1.4.2.7) to a concentration of 1:1,000 in final volume of 1mL and stored at 4^{o}C. For radioimmunoassay's the antibody was diluted in Nafam Buffer (section

2.1.4.2.7) a further 1:100 to give a working concentration of 1:100,000 and used immediately.

2.1.4.2.6 Lyphochek Immunoassay Plus Controls

Lyphochek Immunoassay Plus Control Levels 1, 2 and 3 (BIO RAD; Progesterone range Level 1 0.38-1.50ng/mL, Level 2 4.25-8.83ng/mL and Level 3 10.9-22.5ng/mL and Estradiol range Level 1 22.5-144pg/mL, Level 2 66.2-312pg/mL and Level 3 191-641pg/mL) were each reconstituted by injecting 5mL of sterile MilliQ water (Baxter) into each vial with a sterile syringe under aseptic conditions and mixed by gentle inversion. After 15 minutes at room temperature the controls were stored at -20^{o}C in 1mL aliquots until required. For assays the controls were added neat to treatment tubes.

2.1.4.2.7 Nafam Buffer

$NaH_2.PO_4.2H_20$ (BDH chemicals; 1.186g), Na_2HPO_2 (BDH chemicals; 4.581g), Merthiolate (0.24g), NaCl (AJAX chemicals; 6g) and ethylenediamine tetra acetic acid (EDTA; 3.724g) were added to 800mL of reverse osmosis water. The pH was adjusted to pH 7.3 and then bovine serum albumin (BSA; 2.5g) was added with stirring. The volume was increased to 1L and the buffer was stored in a 1L glass Pyrex bottle at room temperature until required. Buffer was used within one month of preparation.

2.1.4.2.8 Normal Rabbit Serum (NRS)

Untreated rabbit blood (supplied by the Animal House at Flinders Medical Centre) was incubated at 37^{o}C in a water bath for 30 minutes and then stored at 4^{o}C for 18h. The blood was then centrifuged at 2002xg, 4^{o}C for 20 minutes. The serum was collected and stored at -20^{o}C in 5mL aliquots. For all radioimmunoassay experiments NRS was diluted in Nafam buffer (section 2.1.4.2.7) to a working concentration of 1:100 and used immediately.

2.1.4.2.9 Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) (82g) was added to reverse osmosis (RO) water (300mL) with stirring to give a final concentration of 14% PEG. Reverse osmosis water was then added to give final volume of 500mL. PEG was used within one month of preparation.

2.1.4.2.10 Progesterone Standards (0-2,000ng/mL)

10mg of progesterone powder was added to 1mL of 100% ethanol (Merck) in a sterile tube and mixed by vortex to give a concentration of 10mg/mL. From the 10mg/mL stock solution, a 0.1mg/mL solution was prepared in complete assay medium (section 2.1.2.1.3 and 2.1.2.6.4) by adding 50µL of stock to 4.95mL of complete assay medium (section 2.1.3.1.3 and 2.1.2.6.4). A final solution of 2,000ng/mL was prepared by adding 100µL of 0.1mg/mL progesterone solution to 4.9mL of complete medium (section 2.1.2.1.3 and 2.1.2.6.4). Standards of 1,000-0ng/mL were then prepared in medium as per Table 2.2. New standards were prepared for each experiment.

Table 2.2: Progesterone standards 0-2,000ng/mL for progesterone RIA

Final Concentration	From Concentration	Volume	Volume of media	Final Volume
2,000ng/mL	100µg/mL	0.1mL	4.9mL	5mL
1,000ng/mL	2,000ng/mL	2.5mL	2.5mL	5mL
500ng/mL	1,000ng/mL	2.5mL	2.5mL	5mL
100ng/mL	500ng/mL	1mL	4mL	5mL
50ng/mL	100ng/mL	2.5mL	2.5mL	5mL
10ng/mL	50ng/mL	1mL	4mL	5mL
5ng/mL	10ng/mL	2.5mL	2.5mL	5mL
2ng/mL	5ng/mL	2mL	3mL	5mL
1ng/mL	2ng/mL	2.5mL	2.5mL	5mL
0ng/mL			5mL	5mL

2.1.4.2.11 Progesterone [I^{125}] Tracer

Progesterone ^{125}I radioactive tracer (MP Biomedicals) was stored at 4^{o}C until required. For experiments a 10µL sample was counted on a Packard RIASTAR

Gamma counter and tracer was diluted in Nafam buffer (section 2.1.4.2.7) to 10,000 counts per minute as required and used immediately.

2.1.4.2.12 Sheep Anti-Rabbit Immunoglobulin (SAR)

Sheep anti-rabbit secondary antibody at a concentration of 36mg/mL (SAR; Chemicon) was stored at 4^{o}C until required. For radioimmunoassay, SAR was diluted in Nafam buffer (2.1.4.2.7) to a working concentration of 1:30 and used immediately.

2.1.5 Hormones and Hormone Stimulating Reagents

2.1.5.1 N6,2'-O-Dibutyryladenosine 3',5'-cyclic Monophosphate Sodium (cAMP)

A stock solution of 20mM N^{6}, 2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium (db-cAMP) was prepared by adding 2.5mL of sterile MilliQ water (Baxter) to 0.025g of db-cAMP powder. The solution was mixed by gentle inversion and stored at -20^{o}C until required. For cell culture assays on the KGN cell line, the stock solution was diluted in DMEM complete medium (section 2.1.2.1.3) to a working concentration of 1mM and filtered through a sterile 0.22µm filter (Sartorius) and syringe before adding to cells in culture plates.

2.1.5.2 Human Chorionic Gonadotrophin (hCG)

Sterile MilliQ water (10mL; Baxter) was injected into a stock vial of human chorionic gonadotrophin (hCG) using a sterile syringe and needle to give a working concentration of 500IU/mL. The stock hCG solution was stored in 0.2mL aliquots in sterile 0.5mL eppendorf tubes at -20^{o}C until required. For experiments, the stock hCG (0.1mL) was diluted in complete assay medium (4.9mL) to give a working concentration of 0-10,000mIU/mL where appropriate and used immediately. For assays, solvent controls were prepared by adding sterile MilliQ water (0.1mL; Baxter) to complete medium (4.9mL) at the same ratio as the hCG.

2.1.6 Positive Control for Experiments Using JAr Cells

2.1.6.1 Hydrogen Peroxide (H_2O_2)

A stock solution of hydrogen peroxide (H_2O_2; 0.3g/mL) stored at 4^{o}C in the dark was diluted in RPMI complete medium (section 2.1.2.6.4) to give a final working concentration of 10,000µg/mL. For experiments, a serial dilution of 1:10 was performed to give working concentrations of 10,000, 1,000, 100, 10, 1, 0.1µg/mL H_2O_2 in RPMI complete medium (section 2.1.2.6.4) where appropriate and used immediately.

2.1.7 Synthetic Analogues of Muricidae Extracts for JAr and Primary Granulosa Cell Experiments (Chapter 3 and 4)

2.1.7.1 Brominated Indole and Isatin Compounds

5-bromoisatin (Aldrich), indirubin (Apin Chemicals), and 6'6-dibromindirubin (synthesized by Prof. A. L. Skaltsounis) were all prepared fresh on the day of each experiment by dissolving 1mg of each powder in 0.1mL of dimethylsulfoxide (DMSO; 100%) to give a concentration of 10mg/mL; the solution was wrapped in foil to protect from light and degradation. For experiments, 5-bromoisatin, indirubin and 6'6-dibromoindirubin solutions were prepared in appropriate complete assay medium (sections 2.1.2.1.3 and 2.1.2.6.4) to give a range of working concentrations from 0.01-100µg/mL and a final concentration of 1% DMSO in each. The solutions were all filtered through a sterile 0.22µm (Sartorius) filter prior to use on cells. Chemical analysis by liquid chromatography coupled to a mass spectrometer (LC/MS; section 2.2.4.4.2) was performed on 5-bromoisatin. 6'6-dibromoindirubin and indirubin as previously described by Westley and Benkendorff (2008).

2.2 Protocols

2.2.1 Cell Maintenance and Identification

2.2.1.1 Granulosa Cell, Isolation, Identification, and Preparation

Granulosa cells were isolated from the follicular aspirates of women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at Flinders Reproductive Medicine, Flinders Medical Centre, Bedford Park, Adelaide, South Australia, (with informed consent) and ethical approval was given by the Flinders Clinical Research Ethics Committee (approval number 260/067). Participants were treated with gonadotrophin releasing hormone (GnRH) agonist, recombinant human follicle stimulating hormone (FSH) and human choronic gonadotrophin (hCG) as described in Table 1 Appendix II.

Anonymous case study notes for each patient were recorded including the date of birth of participant, the number of treatment cycles, the maximum estradiol (E_2), the number of follicles greater than 15, the follicle stimulating hormone (FSH) starting dose, the FSH total dose, the number of eggs aspirated the number of mature oocytes, the procedure they were undergoing (IVF or ICSI), the number of granulosa cells isolated and the fertility factor (Appendix II). The cases were divided into two groups based on the fertility factor of the women as characterised by Flinders Reproductive Medicine and classified as either having normal or abnormal reproductive physiology according to the following criteria and published literature (Jose-Miller*, et al.*, 2007):

1. The normal or fertile group were defined as either, a male fertility factor, which included sperm defects, decreased motility and erectile dysfunctions and/or women who had previously had ectopic pregnancies or a tubal blockage.

2. The abnormal or infertile group were defined as participants who suffered from polycystic ovarian syndrome (PCOS), single ovarian cyst, endometriosis, abnormal morphology or unexplained infertility (idiopathic).

Granulosa cells cannot be considered fertile or infertile, however for the benefit of this research the cells were subdivided subdivided into two groups and classified either, fertile or infertile, based on the original infertility factor of the woman donor.

Granulosa cells were isolated from the follicular aspirates by centrifugation at 107xg (800rpm) for 10 minutes and the pellet was re-suspended in DMEM base media (section 2.1.2.1.1); the rinse was then repeated. A lymphoprep column (Ficoll-Paque ^{TM}Plus) was then used to separate the granulosa cells from the red blood cells by adding 2mL of cell suspension to a 3mL of lymphoprep column and centrifuging at 375g (1500rpm) for 10 minutes. Isolated cells were rinsed twice with DMEM base media (section 2.1.2.1.1) and the pellet was then re-suspended in DMEM/F12 complete media (section 2.1.2.1.3). The cell number per mL was determined using the Trypan blue exclusion assay (section 2.2.2.3) on a haemocytometer (Freshney, 2005). Isolated primary-derived human granulosa cells were then identified by staining of the 3-βHSD enzyme on the mitochondria (section 2.2.1.1.1; Appendix II). For all experiments granulosa cells were first plated into either 96 well plates or chamber slides and incubated in a humidified atmosphere of 5% CO_2 at 37^{o}C for 24h to allow attachment and luteinisation before any treatments were performed. Images of cells were examined and photographed at 200x magnification using an Olympus BX50 Brightfield Microscope.

2.2.1.1.1 3-β-Hydroxysteroid dehydrogenase (3-βHSD) Staining

To identify the primary-derived human granulosa cells isolated from follicular aspirates of women undergoing IVF and ICSI treatments (section 2.2.1.1; Appendix II) staining of the 3-βHSD enzyme essential for the conversion of pregneolone to progesterone, was conducted. Cells were plated into the wells of Nunc Lab-Tek II - CC2 Chamber Slides at 30,000 cells per chamber in 0.3mL of complete assay medium (section 2.1.3.1.3) and incubated for 24h at 37^{o}C + 5% CO_2 to allow cells to adhere to the wells. At the end of the incubation period the media were removed from the wells and discarded. The slides were rinsed with sterile 1x PBS (section 2.1.2.5) and 3-βHSD (section 2.1.2.4.5) was added. The chamber-slides were then incubated for 1h at 37^{o}C + 5% $CO_{2.}$ The stain was then rinsed off with sterile 1x PBS (section 2.1.2.5) and the cells fixed to the slides with 10% formalin (section 2.1.2.4.2) for 1minute. The slides were the rinsed with deionised water and the nucleus stained with 1% neutral red for 2 minutes. The slides were then rinsed with deionised water, air dried and then dehydrated using 70, 90 and 100% ethanol washes. The slides were then dipped in Histoclear (National Diagnostics) and mounted with Clarion mounting medium (Appendix II).

2.2.1.1.2 Dual staining cells with 3-βHSD & 3H Tritiated Thymidine

To determine if female primary-derived human granulosa cells proliferate in cell culture a dual staining technique was employed using ^{3}H-methyl tritiated thymidine and 3-βHSD stain. Cells were plated into Nunc Lab-Tek II-CC2 Chamber Slides at 30,000 cells per chamber in 0.3mL of complete assay medium and incubated at 37^{o}C + 5% CO_2 to allow cell adherence. 6h prior to end of each incubation period, the media were removed and fresh media containing 1μCi/mL ^{3}H tritiated thymidine (GE Healthcare) added and slides were re-incubated. At end of incubation, medium

were removed and the cells were stained with 3-βHSD (section 2.1.2.4.5) and fixed to the slides with 10% formalin (section 2.1.2.4.2).

2.2.1.1.3 Micro Autoradiography of Slides

Slides were coated with auto-radiographic emulsion (KODAK) for 5 seconds and then left to dry for 10 minutes in a vertical position. Slides were then sealed in a container under anhydrous conditions in a cupboard in the dark to allow exposure. Slides were then developed for 2 minutes with KODAK developer (100g per 633mL of MilliQ water at 52^{o}C). The slides were then taken through a stop solution for 10 seconds (5mL glacial acetic acid + 500mL MilliQ water). After which the slides were placed in KODAK fixer for 5 minutes (100g in 543mL MilliQ water). The slides were then washed under a stream of water for 5 minutes. The nucleus of the cells was then stained with 1% neutral red, slides were dehydrated with 70, 90 and 100% ethanol; they were then treated with Histoclear and mounted with Clarion mounting media. Cells were examined and photographed at 200x magnification using an Olympus BX50 Brightfield Microscope (Appendix II).

2.2.1.2 JAr, OVCAR-3 and KGN Cell Line Maintenance

The JAr cell line derived from a trophoblastic tumour of the placenta (Pattillo, et al., 1971) and the OVCAR-3 cell line derived from an adenocarcinoma of the ovary (Hamilton, et al., 1983) were obtained from the American Type Culture Collection (ATCC; The Global Bioresource Centre) and maintained in RPMI 1640 complete media (section 2.1.2.6.4). The KGC cell line derived from a granulosa cell carcinoma (Nishi, et al., 2001) was kindly supplied by Teresa Hickey, Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of South Australia, Adelaide, South Australia and maintained in DMEM/F12 complete media (section 2.1.2.1.3).

Cells were maintained in sterile 75cm^2 tissue culture flasks, media was replaced every 1-2 days and cells were sub-cultured twice a week. Briefly, the supernatant was aspirated off and flasks were rinsed with sterile 1x PBS (section 2.1.2.5) solution, after which 2mL of 0.25% (w/v) Trypsin/0.03% (w/v) EDTA was added to the flasks which were then incubated at 37^{o}C to allow cells to detach (~5-10minutes). After cells had detached (seen as milky emulsion) 2mL of complete media was added to the flasks and cells were re-suspended gently. Cells were then sub-cultured at 1:10 or 1:4 for JAr or OVCAR-3 and KGN cell lines respectively, in 75cm^2 sterile culture flasks with complete media. Excess cells were cryopreserved with freezing media (section 2.1.2.2) in liquid nitrogen. Cell culture flasks containing cells at 80% confluence in the exponential growth phase were used for all experiments.

2.2.2 Cell Viability Assays

2.2.2.1 The Crystal Violet Cell Viability Assay

To identify viable cells in culture at the end of the experiments, the crystal violet assay was conducted which simultaneously stains and fixes the nuclei of live cells only. At the end of the assay treatment, spent medium was removed from the treatment wells and the cells were rinsed with sterile 1x PBS (section 2.1.2.5). Following this 50µL/well of the crystal violet stain (section 2.1.3.1.1) was added to all treatment wells and the plate was incubated at room temperature for 10 minutes. The plate was then rinsed extensively under a gentle stream of deionised water and air dried by inversion on paper towel. After which 50µL/well of a 33% acetic acid solution (section 2.1.3.1.2) was added to all wells to solubilise the bound stain and the plate was incubated for a further 10 minutes at room temperature. Absorbance was then measured at 570nm, with reference absorbance 630nm, using an automatic spectrophotometer with KC Junior software.

2.2.2.2 The MTT Cell Viability Assay

The MTT assay was performed to identify viable cells at the end of an experiment by measuring the metabolic function of the mitochondria. At the end of the assay period, the supernatant was removed from all the wells and a solution of 0.5mg/mL MTT (section 2.1.3.2; unless otherwise specified in the experiment) was added to wells in a final volume of 0.1mL. The plates were then incubated for 1h at 37^{o}C and 5% CO_2. At the end of the incubation period, 80µL/well 20% SDS in 0.02M HCl (section 2.1.3.3) was added and plate was incubated for a further 1h at room temperature in the dark. The absorbance was the measured at 570nm, with reference absorbance 630nm, using an automatic spectrophotometer with KC Junior software.

2.2.2.3 The Trypan Blue Exclusion Assay

Cell viability was assessed by Trypan blue exclusion assay prior to all experiments (Freshney, 2005). The Trypan blue dye (10µL; section 2.1.3.4.2) was added to a sample of re-suspended cells (10µL) and loaded onto a haemocytometer slide and counted at 10x magnification. Dead cells were permeable to the dye and so were stained blue; therefore dead cells were easily distinguished from viable cells (Freshney, 2005). The haemocytometer slide contains nine squares, the cells in four large squares were counted and the average of the number of cells in these squares was determined for each sample. The mean of the three samples was then determined and multiplied by the dilution factor of the Trypan blue (i.e.x2) and then by 1,000 to determine the number of cells per mL (i.e. each square = 0.1mm^3 and there are 1000mm^3 in 1mL therefore 0.1m^3/1000mm^3 = 1x10^{-4}). The concentration of each cell suspension was then adjusted for each experiment performed.

2.2.3 Detection of Cell Death Assays

2.2.3.1 Combined Caspase 3/7 and Membrane Integrity Assay

To simultaneously detect caspase 3/7 enzymes in an assay and determine membrane integrity by LDH release, a multiplex assay was conducted. The lactate dehydrogenase (LDH) fluorometric enzymatic assay detects membrane damage by measurement of LDH from damaged cells without damaging live cells, which is a direct measurement of necrosis (Niles, *et al.*, 2007). Combining the substrates lactate, NAD+ and resazurin, in the presence of diaphorase, results in the conversion of resazurin to the fluorescent product resorufin which is proportional to LDH release (Niles, *et al.*, 2007). The caspase 3/7 assay, based on caspase 3/7 and luciferase enzyme activity involves adding a luminogenic substrate (containing the tetrapeptide DEVD sequence) along with a cell lysis reagent (supplied by the manufacturer) to cell culture (Niles, *et al.*, 2007). Any caspase 3 or 7 in solution cleave the luminogenic substrate, this in turn releases a recombinant luciferase; which is proportional to the amount of caspase 3/7 activity (Niles, *et al.*, 2007). The caspase 3/7 assay is a measurement of apoptosis because once caspase 3 and 7 have been activated the cell is irreversibly committed to cell death by apoptosis (Elmore, 2007).

Human female primary-derived granulosa cells and the corresponding cancer cell line KGN (10,000 cells per well) in DMEM/F12 complete cell culture medium (section 2.1.2.1.3) were plated into sterile white (opaque) 96-well plates (Interpath) for 24h to allow cell adherence. Six wells containing cell culture medium only or no-cell control was added to plates to account from any signal generated from cell culture medium alone in the LDH and caspase assay. The spent medium was then removed and the cells were then treated with two semi-purified *D. orbita* fractions

(0.005-0.5mg/ml; section 2.2.4.2) in 0.1mL of DMEM/F12 cell culture medium (section 2.1.2.1.3) for 4 or 24h at 37^{o}C + 5% CO_2. The plates were then equilibrated to 22^{o}C for 30 minutes. After which 0.05mL of the LDH substrate (section 2.1.1.2) was added to all treatment wells and the plates were incubated for 10 minutes with shaking at 22^{o}C. The fluorescence in the plate was then immediately read using a fluorescence plate reader using Multimode detection software at excitation 535nm and emission 590nm. 0.1mL of supernatant was then removed from the treatment plates leaving a volume of 0.05mL per treatment well. Then 0.05mL of reconstituted Caspase 3/7 substrate (section 2.1.1.1) was added to all treatment wells giving a total volume per well of 0.1mL, and the plates was incubated at 25^{o}C with shaking in the dark for 1h. The luminescence in treatment wells was measured on plates at full light using an automatic spectrophotometer.

2.2.3.2 DAPI Staining of Cells to Detect Apoptotic Nuclei

Cells were plated into Nunc Lab-Tek II-CC2 Chamber Slides at 30,000 cells per chamber in 0.3mL of DMEM/F12 complete assay medium (section 2.1.2.1.3). After a 24h adherence period, the medium was removed and the cells were treated with the *D. orbita* fractions containing the compounds tyrindoleninone and tyrindolinone or 6-bromisatin (section 2.2.4.2) for 4 and 24h in a final volume of 0.3mL per chamber-well. The supernatant was then removed and the wells washed with sterile 1x PBS (section 2.1.2.5). The cells were then fixed by adding 0.3mL/well of ethanol/chloroform/acetic acid solution (section 2.1.1.3.1) to chamber-wells and incubating chamber slides at -20^{o}C overnight. The chamber wells were then removed and the slides were rinsed twice in 1x PBS (section 2.1.2.5) for 5 minutes each. The slides were incubated with DAPI stain (section 2.1.1.3.2) prepared in sterile 1x PBS (section 2.1.2.5) at a working concentration of 1µg/mL for 30 minutes in the dark at 25^{o}C. The slides were rinsed with 1x PBS (section 2.1.2.5) for 5 minutes, the excess

liquid was then removed and the slides were then mounted with buffered glycerol mounting media (section 2.1.1.5.3), and the edges of slide sealed with clear nail varnish. Cells were examined immediately and photographed at 10, 20 and 40x magnification using a Fluorescence Olympus AX70 Microscope with filter Chroma 31000 at excitation 340-380nm, Dichroic 400 and emission 435-485nm in the Flinders Microscopy and Image Analysis Facility in the Flinders Medical Centre, Bedford Park, South Australia.

2.2.3.3 Membrane Integrity Assay

The CytoTox-ONE Homeogeneous Membrane Integrity Assay (Promega) was conducted on the JAr and OVCAR-3 cell line to determine the effect of the semi-purified *D. orbita* fractions by measurement of non-viable cells. JAr and OVCAR-3 cells (10,000 cells per well) were plated into opaque (white) sterile flat bottom 96-well plates (Interpath) in 0.1mL of RPMI complete medium (section 2.1.2.6.4) and incubated for 2 or 24h to allow cell adherence. The medium was then removed and the cells were treated with *D. orbita* fractions (0.005-0.5mg/mL; section 2.2.4.2) in 0.1mL of RPMI cell culture medium (section 2.1.2.6.4) for 4 or 24h. After which the plates were equilibrated to 22^{o}C for 30 minutes. The LDH substrate (section 2.1.1.2; 0.1mL) was then added to all treatment wells and the plate was incubated for 10 minutes at 22^{o}C in the dark with shaking. Following which, 0.05mL of stop solution (supplied by manufacturer) was added to all wells. The plate was mixed by shaking and fluorescence read on an automatic plate reader using Multimode detection software at excitation 535nm and emission 590nm.

2.2.3.4 TUNEL assay to detect Apoptosis

To detect and quantify apoptotic cells after treatment of female human primary-derived granulosa cells and the corresponding cancer cell line, KGN with semi-purified *D. orbita* fractions, the DeadEnd^{TM} Fluorometric TUNEL System (Promega)

was performed. Cells were plated into Nunc Lab-Tek II - CC2 Chamber Slides at 30,000 cells per chamber in complete DMEM/F12 cell culture medium (section 2.1.2.1.3). After an initial 24h adherence period, the DMEM/F12 complete medium (section 2.1.2.1.3) was removed and cells were treated with the semi-purified *D. orbita* compounds tyrindoleninone or 6-bromisatin (section 2.2.4.2) prepared in DMEM/F12 complete medium (section 2.1.2.1.3) for 4 and 24h in a final volume of 0.3mL per chamber. The medium was then removed and wells washed with sterile 1x PBS (section 2.1.2.5). The cells were then fixed by adding 0.3mL/well of 4% paraformaldehyde (section 2.1.1.6.2) in sterile 1x PBS (section 2.1.2.5) for 25 minutes at 4^{o}C. Wells were then rinsed twice with sterile 1x PBS (section 2.1.2.5) for 5 minutes each, and chamber walls were removed. The slides were then treated with 0.2% Triton X-100 solution in 1x PBS (section 2.1.1.6.5) for 5 minutes to permeablize the cells. The slides were then rinsed twice by immersion in 1x PBS (section 2.1.2.5) for 5 minutes each. The positive control slide was prepared by adding 100µL of DNase I buffer (section 2.1.1.4.2) to the slide, and the slide was incubated at room temperature for 5 minutes. Excess solution was removed by blotting the sides of the slide with a tissue and then 100µL of 1µg/mL DNase I in DNase I buffer (section 2.1.1.4.2) was added and the positive control slide was incubated for 10 minutes at room temperature. The positive control slide was then rinsed thoroughly with deionised water. Treatment slides and the negative control slide were treated with 100µL of equilibrium buffer (supplied by manufacturer) for 5 minutes at room temperature. The rTdT incubation buffer (section 2.1.1.6.1.) was prepared and stored on ice just prior to use. Then 50µL of rTdT incubation buffer was added per slide to all treatment slides and positive control slide. The negative control slide had incubation buffer without rTdT enzyme. A plastic cover-slip was

placed over each slide to aid distribution of the rTdT buffer and to prevent any evaporation of buffer and the slides were incubated in a humidified chamber at 37^{o}C for 1h, protected from light. Both positive and negative slides were treated separately for all consecutive steps following and all slides were protected from light. The plastic cover slips were removed and slides were rinsed in 2x SSC solution (section 2.1.1.6.4) for 15 minutes at room temperature (25^{o}C). The slides were then rinsed by immersion in 1x PBS (section 2.1.2.5) three times for 5 minutes each. The slides were then counter-stained with 1µg/mL propidium iodide solution (section 2.1.1.6.3) for 15 minutes at room temperature in the dark. The slides were then rinsed by immersion in 1x PBS (section 2.1.2.5) three times for 5 minutes each. Any excess liquid was removed from slides by wiping edges with a tissue and then slides were mounted with buffered glycerol mounting media (section 2.1.1.5.3) and the edges of slides were sealed with a clear nail varnish. Cells were examined immediately and photographed at 20x magnification using a Fluorescence Olympus BX50 Microscope with filter Chroma 31001 at excitation 450-495nm, Dichroic 505 and emission 515-555nm for the green fluorescein of TUNEL and, Chroma 31002 at excitation 515-550nm, Dichroic 565 and emission 575-615nm for the red fluorescence of the PI staining, in the Flinders Microscopy and Image Analysis Facility in the Flinders Medical Centre.

2.2.4 Dissection, Isolation & Characterization of Bioactive Compounds from Muricid Molluscs

2.2.4.1 Dissection and Isolation of Bioactive Compounds from Specimens of D. orbita

2.2.4.1.1 Dissection & isolation of crude compounds from the Hypobranchial glands and egg masses

Bioactive compounds from the marine whelk, *Dicathais orbita* were extracted from the hypobranchial glands of frozen whelks collected in September 2008, from an abalone farm in Pt Lincoln, South Australia, and transported frozen to Flinders

University. Bioactive compounds were also isolated from the egg masses layed by specimens of live *D. orbita* from re-circulating tanks at Flinders University, South Australia. Frozen hypobranchial glands were dissected from whelks according to Westley and Benkendorff (2008). Briefly, the hypobranchial glands were removed from the frozen whelks (-20^{o}C), first by removing the shell by cracking it gently in a vice. The columnar muscle was then cut away from the shell with a sharp scalpel and the whole visceral mass gently removed from the shell. The three regions of the hypobranchial gland were cut away from the remaining tissue; easily distinguished as a yellow to greenish region. The hypobranchial gland tissue was masticated in a motar and pestle and then placed immediately into HPLC grade chloroform and methanol (1:1 v/v) in the dark with stirring at 4^{o}C; the solvent was replaced four times over a 48h period. The solution was then filtered through a Büchnel funnel under water pressure, followed by glass wool to remove and residue of tissue. The filtrate then added to a separating funnel with MilliQ water (~50mL), to facilitate separation of the aqueous and organic layers. The lower organic chloroform layer was collect and filtered through glass wool to remove any traces of remaining tissue. The chloroform solvent was then removed under reduced pressure (474mbar; 40^{o}C) on a Buchi Rotary evaporator to give an oily red/brown residue; which was re-dissolved in dichloromethane (DCM; 100%). The DCM was removed under a stream of N_2 gas and the final oily red product was stored in aliquots in amber vials at -20^{o}C to protect from light and oxygen until required.

The bioactive compounds were extracted from the egg capsules according to Benkendorff *et al.,* (2000). Briefly, the egg capsules were cut open and soaked immediately in HPLC grade chloroform and methanol (1:1 v/v) in the dark with

stirring at 4^{o}C for 4h. The solvent mix was then decanted and replaced another two times over a 24h period. A small amount of MilliQ water (~50-100mL) was then added to the combined extracts and the non-polar chloroform layer was separated from the polar water layer. The chloroform layer was then filtered under water pressure through a Büchnel funnel and then through glass wool to remove any unwanted tissue. The yellow chloroform layer was then evaporated to dryness under reduced pressure on a rotary evaporator as above (474mbar; 40^{o}C) to give a brown/red oil, and stored in amber vials under N_2 at -20^{o}C in aliquots until semi-purified.

Thin layer chromatography (section 2.2.4.4.1) was performed on aluminium silica gel 60F254 plates (Merck) with DCM (100%) to identify compounds of interest in the crude samples and retention factor (R_f) values were recorded and chemical analysis was performed by LC/MS (section 2.2.4.4.2).

2.2.4.2 Concentration of the Bioactive compounds by Flash Silica Chromatography

To facilitate separation of the bioactive compounds tyrindoleninone, tyrindolinone and 6-bromoisatin, crude samples were semi-purified using flash silica chromatography under N_2 pressure according to Benkendorff *et al*., (2000). A crude extract (~1g per column) was separated through silica mesh (60g silica; Merck) with increasing solvent polarity using DCM (100%), DCM and CH_3OH (10%) and DCM and CH_3OH (20%). Compounds of interest were collected by visual identification in coloured fractions and the solvents were removed under reduced pressure as described previously (section 2.2.4.1.1). Thin layer chromatography (section 2.2.4.4.1) was performed on aluminium silica gel 60F254 plates (Merck) with DCM (100%) to identify compounds of interest and the R_f values recorded. Chemical

analysis by LC/MS (section 2.2.4.4.2) was also performed. The collected fractions were stored in aliquots in amber vials under N_2 at -20^{o}C until required for cell culture assays.

2.2.4.3 Concentration of the Bioactive compounds Tyrindoleninone and Tyrindolinone

To concentrate and separate the bioactive compounds 6-bromo-2,2-dimethylthioindolin-3-one (Tyrindolinone) and 6-bromo-2-methylthioindoleninone (Tyrindoleninone) from a crude *Dicathias orbita* sample, an adaption from Baker and Duke (1973) with methanethiol (2%) in benzene was employed using alumina chromatography under N_2 (Appendix III).

2.2.4.4 Identification of Bioactive compounds

2.2.4.4.1 Identification by Thin layer Chromatography (TLC)

Thin layer chromatography (TLC) analysis using aluminium silica gel 60F 254 plates (Merck) was performed on all crude and purified compounds where specified to identify the bioactive compounds from *D. orbita.* The solvent system consisted of 100% dichloromethane (DCM). A small amount of compound dissolved in 100% DCM was added to the baseline of a TLC sheet. The bottom of the TLC was then placed into DCM (100%) in a glass beaker to just above baseline. The TLC was left for 1-2 minutes to allow separation of the compounds. The solvent front was then marked and the retention factor (R_f) values were determined by dividing the distance moved by the substance by the distance moved by the solvent. There are no commercial standards available for TLC, however this was used as a monitoring tool to identify any the potential bioactive compounds in the isolated extract which have been identified in the literature.

2.2.4.4.2 Identification by Liquid chromatography/mass spectrometry

Chemical analysis using liquid chromatography mass spectrometry (LC/MS) was performed on crude (section 2.2.4.1.1), semi-purified concentrated *D. orbita* samples

(section 2.2.4.1.2) and 5-bromoisatin (section 2.1.7.1) by dissolving 1mg of compound in acetonitrile (1mL). Analysis of the samples by LC/MS employed an electrospray ionisation mass spectrometry (ESI) and UV detection. The analysis was performed on a Micromass Quattro Micro (Waters, Manchester, UK) tandem quadrupole mass spectrometer. LC separation was provided by a Waters liquid chromatographer (Waters, Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector. Liquid chromatography incorporated a HPLC Phenomenex, Synergi Hydro-RP, 250 x 4.6 mm, 4µm particle column. Two solvents were employed; Solvent A, 0.1% aqueous formic acid and Solvent B, 0.1% formic acid in acetonitrile at a flow rate of 1.0mL/minute and injection volume of 0.02mL. The mobile phase was split after the UV detector using a low volume T-piece; 300µL was directed to the electrospray source of the mass spectrometer and the rest to waste. Data was acquired by the Masslynx Software (v4.0) for both the MS (negative ion mode) and UV data. UV detection wavelengths were 300 and 600nm. The pump gradient used in the LC/MS is shown in Table 2.3 and the two alternating scans performed on the mass-spectrometer are shown in Table 2.4.

Table 2.3: The pump gradient employed in the LC/MS (Jardine, 2008)

Time	A %	B %
0	70	30
1	70	30
3	40	60
15	0	100
25	0	100
30	70	30
35	70	30

Table 2.4: Two alternating scans employed on the mass spectrometer used in the LC/MS (Jardine, 2008).

Function	1	2
Polarity	+ve	-ve
Capillary (kV)	3.5	3.5
Cone (V)	35	20
Source Temperature (^{o}C)	80	80
Desolvation Temperature (^{o}C)	350	350
Desolvation Gas Flow (L/Hr)	600	600
Scan duration (secs):	1	1
Mass range scanned:	150-800	150-800

2.2.4.5 Preparation of D. orbita *Compounds for Treatment on Cells*

The semi-purified fraction aliquots were thawed, weighed and prepared fresh on the day of each experiment. To facilitate solubility of organic compounds in culture medium, fraction extracts were dissolved in dimethylsulphoxide (DMSO; 100%) at 100 x the final working concentration (from 0.005-1mg/mL). The fractions were then prepared in complete cell culture medium (2.1.2.1.3 and 2.1.2.6.4) at the final working concentrations (0.005-1mg/mL), which were then filtered using a sterile 0.22µm (Sartorius) filter. 0.1mL of each fraction in cell culture medium were added per well in the treatment plates, giving a final DMSO concentration of 1%.

2.2.4.6 Stability of D. orbita *Compounds in Medium at 37^{o}C*

This experiment was performed to test the degradation of the bioactive compounds from *D. orbita* in cell culture plates at 37^{o}C in the absence of cells. A crude sample of *D. orbita* compounds (section 2.2.4.1.1) was prepared in DMSO (100%) at 100x the final working concentration as per procedures used to prepare samples for cell treatments (section 2.2.4.5). As complete medium could not be added to the HPLC column for analyses, and the main constituent of cell culture medium is water, this experiment was performed in sterile water. The crude sample was diluted in sterile MilliQ water (Baxter) to a working concentration of 1mg/mL. Controls of 1% DMSO prepared in sterile MilliQ water (Baxter) and sterile MilliQ water (Baxter) only, along with the 1mg/mL crude sample prepared in sterile water were then added

to a sterile flat bottom 96-well plate (Interpath) at 0.2mL per well in 12 replicates each. The plate was incubated for 1 and 24h at 37^{o}C + 5% CO_2 in a cell culture incubator. At the end of each designated incubation period samples were taken for all wells and injected directly into a HPLC column and analyzed by LC/MS (section 2.2.4.4.2) for the presence of the *D. orbita* compounds. LC/MS was also performed on untreated crude sample (Appendix IV).

2.2.5 Hormone Assays

2.2.5.1 Enzyme Immunoassay (ELISA) for the Measurement of Progesterone

To identify progesterone produced by the JAr cells (section 3.2.3) an ELISA assay was performed and the results compared to the RIA assay (section 3.2.3). The CL425 primary antibody (section 2.1.4.1.5) was diluted in coating buffer to a working concentration of 1:10,000 and then 0.05mL per well was added to a NUNC maxisorb ELISA plate (Interpath). The plate was covered with acetate plate sealer and left to incubate overnight at 4^{o}C. The following day the ELISA plate was washed 5 times with wash solution (section 2.1.4.1.10); and the plates was dried on paper towel in between each wash. Then immediately 0.05mL per well of standards (0-4ng/mL) (section 2.1.4.1.6) and controls (Lyphochek Immunoassay Plus Controls; section 2.1.4.2.6) were added in duplicate and samples were added in triplicate to the ELISA plate containing the bound primary antibody. After which 0.05mL per well HRP conjugate (section 2.1.4.1.2) was immediately added at a working concentration of 1:40,000. The plate was then incubated at room temperature for 2h. The plate was then rinsed 5 times with wash buffer (section 2.1.4.1.10); drying on paper towel in between each wash on paper towel. The substrate was prepared by adding 0.5M H_2O_2, 40mM ABTS (section 2.1.4.1.1) to substrate buffer and then 0.1mL per well was added to all wells of the ELISA plate and plate was incubated with shaking at room temperature for 1h. The plate was read on a spectrophotometer with KC Junior

Software at 405nm, with reference absorbance 540nm. The concentration of progesterone was determined from a standard curve (Appendix V).

2.2.5.2 Radioimmunoassay (RIA) for the Measurement of Progesterone and Estradiol in Assay Medium

A competitive radio ligand assay was adopted for the measurement of the steroid hormones progesterone and estrogen (Travis, 1979).^{125}I labelled progesterone (section 2.1.4.2.11) and ^{125}I estradiol (section 2.1.4.2.4) tracer (MP Biomedicals) were measured on a Packard RIASTAR Gamma counter to determine activity and then diluted in Nafam buffer (section 2.1.4.2.7) to 10,000 counts per minute. Progesterone standards (0-2,000ng/mL; section 2.1.4.2.10) and estradiol standards (0-2,000pg/mL; section 2.1.4.2.3) were prepared in cell culture media (section 2.1.2.1.3 and 2.1.2.6.4). Standards and controls (section 2.1.4.2.6) were measured in duplicate and samples in triplicate. Nafam buffer (100µL; section 2.1.4.2.7) was added to all treatment 3DT tubes with the exception of the total count tubes, followed by 25µL per tube of standards, controls and samples. Progesterone or estradiol ^{125}I tracer (100µL per tube; sections 2.1.4.2.11 and 2.1.4.2.4, P_4 and E_2 respectively) was then added followed by 100µL of progesterone (section 2.1.4.2.1) or estradiol antiserum (section 2.1.4.2.5). Tubes were incubated at 37^{o}C in a water bath for 1h for progesterone assay and at 4^{o}C overnight for estradiol assay. Normal rabbit serum (NRS; 100µL; section 2.1.4.2.8), followed by secondary anti rabbit antibody or donkey anti-sheep/goat (SAR; 100µL; section 2.1.4.2.12 for P_4 and DAG; 100µL; section 2.1.4.2.2 for E_2) and 14% polyethylene glycol (PEG; 200µL; section 2.1.4.2.9) were then added and the contents of tubes were mixed by vortexing. The tubes were then incubated at room temperature for 30 minutes. After which, the tubes were centrifuged at 4^{o}C, 20002xg (3000rpm) for 30 minutes. The supernatant was aspirated off under suction at the tap and the radioactivity in pellet was determined

using the Packard RIASTAR gamma counter for 2 minutes per tube. Concentrations of progesterone and estrogen were determined from a standard curve (Appendix V).

Chapter 3 Optimisation of JAr Cells *In Vitro* Culture Conditions

3.1 Introduction

In vitro high through-put cell-based screening assays are an essential tool in the early preclinical stage of anti-cancer drug discovery (Seiler, *et al*., 2004). These assays are an effective way of screening large numbers of compounds, eliminating highly toxic and/or ineffective compounds and generating additional data to support animal studies on potential new cancer drug candidates (Ponsoda, *et al*., 1995; Hamid, *et al*., 2004). *In vitro* assays not only provide a profile on the effects and safety of a drug, but can also give an insight into the understanding of cell growth and development, and signalling pathways (Stockwell, *et al*., 1999).

There are a multitude of cell-based viability assays available that can measure the cytotoxicity of new drug candidates. The well-known enzymatic assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which relies on the reduction of a yellow substrate to formazan, a purple precipitate, by the mitochondrial succinate dehydrogenase enzymes, is an ideal assay for measuring functionality of the cells in culture (Mosmann, 1983). Based on the activity of the mitochondrial dehydrogenase enzymes, the MTT assay only detects metabolically active viable cells *in vitro*. The MTT assay, although developed over 15-years ago, is still considered today to be a fast, effective and reliable high through-put cell-based assay, as it has been well-documented and optimised for different cell lines (Mosmann, 1983; Twentyman and Luscombe, 1987; van der Loosdrecht, *et al.*, 1991; Hussain, *et al.*, 1993; Sladowski, *et al.*, 1993; Ahmed, *et al.*, 1994; Tosetti, *et al.*, 2003; Mueller, *et al.*, 2004; Young, *et al.*, 2005b).

A number of modifications to the original MTT assay have been made including the development of two analogues XTT and MTS (Scudiero, *et al*., 1988; Cory, *et al*., 1991). Modification of the original MTT assay to a water-soluble MTS, was found to reduce the number of steps required in the assay, therefore reducing the chance of errors and overall variability of the assay (Cory, *et al*., 1991). However, recent studies have shown that the MTT is a more sensitive and reproducible assay for different cell lines (Pabbruwe, *et al*., 2005).

Cell staining assays such as the crystal violet assay are an alternative to enzymatic assays (Saotome, *et al*., 1989; Bechtel and Bonavida, 2001; Siddiqui, *et al*., 2006). The crystal violet dye dissolved in methanol (50%) simultaneously stains the nuclei of live cells only and fixes the cells to culture plates (Saotome, *et al*., 1989). Consequently, the crystal violet assay does not rely on the metabolic function of specific enzymes and is therefore an effective way of determining final cell number in a cell-based assay, as opposed to cell metabolic functionality assays.

Selecting an appropriate cell-based assay ultimately depends on the endpoint required. An understanding of the parameters of each assay and how these measurements correlate with cell viability are essential (Freshney, 2005). Furthermore, the differences between cell lines and conditions in culture can vary enormously hence optimum cell culture conditions need to be established for individual cell lines.

Human reproductive cell lines have been used extensively to examine aspects of reproduction *in vitro* (Sullivan, 2004). Cell lines from the placenta have been used to

study growth factors, cell to cell interaction, signalling pathways, luteinizing hormone/human choronic gonadotrophin (LH/hCG) receptors and steroid hormone synthesis (Sullivan, 2004). The human choriocarcinoma cell lines JAr, BeWo and JEG, were developed over 40-years ago from a layer of cells, the trophoblastic cells, which form part of the placenta during pregnancy (Patillo and Gey, 1968; Pattillo, *et al*., 1971; Sullivan, 2004). These cells have been studied extensively *in vitro*, often to give some insight into normal trophoblastic cell functions by modelling endocrine, metabolic and biosynthetic processes (Patillo and Gey, 1968; Pattillo, *et al*., 1971; Sullivan, 2004).

Choriocarcinoma cell lines exhibit both endocrine and placental functions *in vitro*, including the synthesis of small amounts of progesterone (P_4), estradiol (E_2) and human chronic gonadotrophin (hCG) (King, *et al*., 2000; Sullivan, 2004). Furthermore, these cell lines have the ability to import nutrients and export waste products from mother to foetus (Sullivan, 2004; Serrano, *et al*., 2007). Studies have shown that choriocarcinoma cells exhibit transporters, receptors and export pumps similar to those of liver cells, which enable these cells to metabolise compounds and excrete them from the cells rapidly (Serrano, *et al*., 2007). This makes them an ideal model for studying the uptake and elimination of novel compounds *in vitro* which may be indicative of the *in vivo* response (Serrano, *et al*., 2007). JAr cells are also known to proliferate rapidly in culture, with a doubling rate of 15h, and they exhibit invasive characteristics, changes that are known to occur in the early placenta (Adler, *et al*., 1995; Plessinger and Miller, 1999). Consequently, JAr cells are a fast and reliable model for testing the immediate cytotoxic effects of a drug candidate i*n vitro*.

Marine whelks from the family Muricidae, are the source of the vibrant purple dye Tyrian Purple (Cooksey, 2001; Naegel, 2004). Chemical analysis of the hypobranchial glands of specimens of Muricidae has identified the precursor compound to Tyrian purple, tyrindoxyl sulphate (Baker and Sutherland, 1968). Subsequent analysis of tyrindoxyl sulphate has shown that when it is hydrolysed by an aryl sulfatase enzyme, a series of chemical reactions occur involving intermediate indole and isatin compounds which undergo further chemical reactions, including dimerization and oxidation, to produce Tyrian purple (Figure 1.3 Chapter 1; Baker and Duke, 1973; Cooksey, 2001; Benkendorff *et al*., 2001; Westley and Benkendorff, 2008).

Tyrian purple has no biological activity however, extracts containing a number of the precursor compounds, including tyrindoleninone and 6-bromoistain have demonstrated anticancer properties both *in vitro* and *in vivo* (Westley, *et al.*, 2010; Benkendorff, *et al.*, 2011). Chemical synthesis of these Muricidae compounds has proven difficult however several structurally similar synthetic compounds are commercially available; indirubin, 6'6-dibromoindirubin and 5-bromoisatin.

Indirubin, the active ingredient in some Traditional Chinese Medicine which is from a plant source, was effective against chronic myelogenous leukaemia in clinical trials (Xiao, *et al*., 2002). Research has also shown that indirubin induced apoptosis in a number of cancer cell lines, such as lung and prostate cancers *in vitro* (Kim, *et al*., 2007). Furthermore, indirubin and its brominated derivatives, including 6'6-dibromoindirubin target cyclin-dependent kinases (CDK's) and inhibit DNA synthesis in cancer cells by interrupting the cell cycle at the late-G1 and G2/M

phases (Hoessel, *et al.*, 1999; Meijer, *et al.*, 2003; Nam, *et al.*, 2005; Meijer, *et al.*, 2006).

The cytotoxicity of isatins and brominated isatins has also been evaluated (Cane, *et al.*, 2000; Vine, *et al.*, 2007a; Vine, *et al.*, 2007b; Matesic, *et al.*, 2008). Isatin induces apoptosis and prevents the proliferation of the human leukaemia cell line, HL60 by disrupting the extracellular signal-related protein kinase (ERK) pathway *in vitro* (Cane, *et al.*, 2000). A range of substituted isatins including 5 and 7-bromoisatin have been found to inhibit cell proliferation and induce apoptosis in several tumour cell lines (Vine, *et al.*, 2007a; Matesic, *et al.*, 2008). Furthermore, Vine *et al.*, (2007b) have shown that the substituted isatin, 5,7-dibromo-N-(p-methylbenzyl)isatin is greater than five times more toxic towards lymphoma and leukaemia cancer cell lines in comparison to primary human peripheral blood lymphocytes.

Therefore, the aims of this study were to:

1. Compare three colourmetric assays, MTT, MTS and crystal violet for use with the JAr cell line.
2. Determine the minimum time required for JAr cells to firmly attach to treatment cell culture plates.
3. Determine the optimum concentration of MTT substrate for the JAr cell line.
4. Determine the optimum human choronic gonadotrophin (hCG) concentration required to stimulate progesterone synthesis.
5. Comparison the ELISA and RIA assay for measurement of progesterone synthesis.

6. Establish an effective positive control for the JAr cell line that can then be used when testing novel compounds.
7. Examine the effects of synthetic brominated-indole compounds on the JAr cell line using the optimised MTT assay.

Given that indirubin and its analogues have a role in the treatment of human chronic myelogenous leukaemia in Traditional Chinese Medicine, and because isatin compounds inhibit the proliferation of cancer cells and induce apoptosis, the hypothesis in the present study was that the synthetic compounds 5-bromoisatin, indirubin and 6'6-dibromoindirubin would inhibit the proliferation of the human reproductive cancer cell line JAr.

3.2 Methods

3.2.1 Comparison between 1h MTS, 1h MTT, 24h MTT and Crystal Violet Assays

3.2.1.1 Procedure

The aim of this experiment was to select an *in vitro* cell-based screening assay that would produce the most accurate and sensitive standard curve for determining viable cell numbers for the JAr cell line. JAr cells grown to 80% confluence, were detached from flasks using trypsin-EDTA (section 2.2.1.2), pooled together and centrifuged at 78g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 5mL of assay medium (section 2.1.3.6.4) and viable cells counted using a haemocytometer and the trypan blue exclusion assay as previously described (section 2.2.2.3). Cell concentration was adjusted to $1.6x10^5$cells per 0.1mL and then plated into four sterile flat-bottom 96-well plates (Interpath) by serial 1:2 dilution of 80,000 cells to 0 cells per wells in six replicates. All outside wells of plate contained 0.1mL

of sterile 1x PBS (section 2.1.3.5) to prevent evaporation of inside wells and reduce the variability of the assay. Plates were incubated for 24h to allow cell adherence at 37^{o}C + 5% CO_2 because the optimum cell adherence concentration at this point was unknown.

After the 24h adherence period the spent medium was removed from the wells and 0.1mL per well of MTT (0.5mg/mL; section 2.1.4.2.) was added to the adherent cells on two of the plates and plates were incubated for 1 or 24h. After this 0.08mL of 20% SDS in 0.02M HCl (section 2.1.4.3.1) was added to each well to give a final volume of 0.18mL and the plates were incubated in the dark for 1h. The absorbance of each well was measured at 570nm, with reference absorbance 650nm, on an automatic spectrophotometer using KC Junior software.

The third plate had 0.02mL of MTS solution (0.32mg/mL; Promega) added to wells to give a final volume of 0.12mL per well. The MTS plate was incubated for 1h in the dark and then absorbance of each well was measured at 490nm, with reference 650nm, using an automatic spectrophotometer with KC Junior software.

The medium was removed from the fourth plate and the wells were rinsed with 1x PBS (section 2.1.3.5). Following which, 0.05mL of crystal violet stain (0.5% section 2.1.4.1) was added to all treatment wells and the plate was incubated at room temperature for 10 minutes. The plate was then rinsed extensively with deionised water, air dried and bound crystal violet stain re-crystallised with acetic acid (33%; section 2.1.4.1.2) for 10 minutes. The absorbance for each well was measured at

570nm, with reference absorbance 630nm, on an automatic spectrophotometer with KC Junior software. The assay was repeated on three separate occasions (n=3).

3.2.1.2 Statistical Analysis

To determine which assay gave the most reproducible standard curve with longest linear range, highest gradient and highest R^2 value, a linear regression model with 95% confidence interval was adopted using SPSS statistical software version 17 (Pallant, 2002). For all assays at the highest concentration (80,000 cells per well), the relationship between linearity and cell number decreased. Therefore, standard curves for each assay were recalculated and statistics were performed on cell concentrations of 0 to 40,000 cells per well. The lower and upper confidence intervals were defined as the range within which the true population mean would lie in 95 out of 100 hypothetical repeated experiments. Therefore, the assay that produced the lowest confidence intervals was considered to be the more accurate and reproducible assay.

3.2.2 Optimisation of Cell Adherence

3.2.2.1 Procedure

The aim of this experiment was to determine optimum adherence of JAr cells to treatment plates and optimum concentration of MTT for the 1h MTT assay. JAr cells at 80% confluence were detached from flasks, pooled together, counted by trypan blue exclusion assay (section 2.2.2.3) and the cell densities were adjusted to $1.6x10^5$ cells per 0.lmL of RPMI complete media (section 2.1.3.6.4). The JAr cells were seeded into four sterile flat-bottom 96-well microtitre plates (Interpath). Each plate contained 0 to 80,000 JAr cells per well, in 6 replicates, in a final volume of 0.1mL of assay medium per well (total of 8 columns in a 96-well plate). Two extra columns in the plate had 10,000 JAr cells per well in a final volume of 0.1mL per well of RPMI complete media (section 2.1.3.6.4) and were used to determine optimum MTT

concentration. All outside wells of plate contained 0.1mL of sterile 1x PBS (section 2.1.3.5.).

After the designated culture period (2, 4 and 6h) supernatant was removed from treatment wells and stored at -20^{o}C for the determination of progesterone by enzyme-linked immuno sorbent assay (ELISA) and radioimmunoassay (RIA respectively) (section 3.2.3.1.). A solution of MTT (0.5mg/mL; section 2.1.4.2) was added to columns 2-9 and plates incubated for 1h at 37^{o}C + 5% CO_2. MTT was also added to columns 10 and 11 (containing 10,000 cells per well) at the concentrations of 0, 0.1, 0.5 and 1mg/mL MTT (section 2.1.4.2) in triplicate wells. Cells in the 0h plate were seeded immediately into MTT (0.5mg/mL; section 2.1.4.2); they were not cultured in complete RPMI media before adding MTT, as occurred with the other three plates. The 1h MTT assay was then completed as previously stated (section 3.2.1). This experiment was repeated on four separate occasions (n=4).

3.2.2.2 The Intra- and Inter-Assay Coefficient of Variation (CV)

The intra and inter-assay coefficient of variation were calculated for the adherence assay and for the RIA and ELISA hormone assays, using the formula CV (%) = (Standard deviation/Mean)*100. The intra-assay coefficient of variation determined the variation within one experiment by measuring the standard deviation of six replicate optical density values at each concentration of cell number for one incubation period and then dividing by the mean of the same six optical densities. This was generated for each repeat of the assay therefore, generating four intra-assay CV values for each cell concentration. The final intra-assay CV was the mean of these four values. The inter-assay coefficient of variation measures the variation between the four repeat experiments. There were six replicates in each experiment

hence the standard deviation of 24 replicates was divided by the mean of 24 replicates.

3.2.2.3 Statistical Analysis

To determine the standard curve that produced the longest linear range, highest gradient of the line and highest R^2 values, absorbance values from the four plates were analysed by SPSS statistical software version 17 (Pallant, 2002). The number of cells per well changed according to the incubation period and the range of cell concentrations (0-80,000) were used to generate one standard curve for each incubation period. A linear regression model with 95% confidence intervals was developed for each standard curve and the lower the confidence interval the more accurate and reproducible the standard curve.

For a comparison of the effects of the four different concentrations of MTT substrate on absorbance values over the four different incubation periods, a one-way between groups analysis of variance (ANOVA) with Dunnett's T3 post-hoc was performed for each incubation period and the alpha value adjusted to significant level of $p < 0.01$ where data violated the homogeneity of variance (Pallant, 2002).

To compare the effect of the MTT substrate on JAr cell adherence at each incubation period, independent sample T-tests were conducted using SPSS statistical software version 17 (Pallant, 2002).

3.2.3 Comparison of the ELISA and RIA to Measure Progesterone Synthesis

3.2.3.1 Procedure

The aim of this experiment was to compare the ELISA and RIA for the measurement of progesterone secreted by the JAr cell line from the optimised assay (section 3.2.2.1). After the designated culture period (2, 4 and 6h) The supernatant from the 2,

4 and 6h treatment plates (section 3.2.2.1) was removed from treatment wells and progesterone concentration was determined by enzyme-linked immuno sorbent assay (ELISA) and radioimmunoassay (RIA respectively) (section 2.2.5)

3.2.3.2 Statistical Analysis

An independent sample T-test was conducted to compare the two different techniques for measurement of progesterone synthesis, RIA and ELISA, using SPSS statistical software version 17 (Pallant, 2002). The intra and inter-assay coefficient of variation was also calculated for both the RIA and ELISA assays as explained above.

3.2.4 Effects of hCG and H_2O_2 on Progesterone Synthesis

3.2.4.1 Procedure

To determine the optimum concentration of hCG to stimulate hormone production in steroidogenic cells and to determine the concentration of hydrogen peroxide that causes maximum cell death, JAr cells were detached from cell culture tissue flasks at 80% confluence with Trypsin EDTA and trypan blue cell count performed to determine number of cells per mL (section 2.2.2.3). Cells were then plated into four sterile flat-bottom 96-well plates (Interpath) at 20,000 cells per well in a final volume of 0.1mL of RPMI complete media. All outside wells contained 0.1mL of sterile PBS only (section 2.1.3.5). Plates were incubated for 2h as determined by optimisation of adherence assay at 37^oC + 5% CO_2 (section 3.2.2).

At the end of the adherence period, media was removed from all plates and hCG (section 2.1.6.2) in six replicates was added to the three plates at concentrations ranging from 0.1-10,000mIU/mL in a final volume of 0.1L RPMI complete media (section 2.1.3.6.4). A media-only control and a solvent control consisting of sterile MilliQ water (Baxter) in medium (section 2.1.3.6.4) were also added in six replicates. Hydrogen peroxide (section 2.1.7) at concentrations ranging from 0.1-

10,000µg/mL in 0.1mL of RPMI complete media (section 2.1.3.6.4) was added to the fourth plate in six replicates with a media-only control also in six replicates. The three hCG plates were incubated for 2, 4 and 24h, whereas the hydrogen peroxide plate was incubated for 1h at 37^{o}C + 5% CO_2. At the end of each incubation period the supernatant was removed and stored at -20^{o}C for the measurement of progesterone by RIA (section 2.2.5.2.) and the 1hr MTT assay performed as previous (section 3.1.1). The experiment was repeated on 4 separate occasions (n=4).

3.2.4.2 Statistical Analysis

A two-way analysis of variance (ANOVA) with Dunnett T3 post-hoc was conducted to explore the effects of the different concentrations of hCG on absorbance values and progesterone synthesis against the solvent control using SPSS statistical software version 17.

For the hydrogen peroxide dose dependent assay, a two-way analysis of variance (ANOVA) was conducted with Dunnett T3 post-hoc to compare the effects of the increase in hydrogen peroxide on cell viability and progesterone synthesis using SPSS statistical software version 17.

3.2.5 Effects of the Synthetic on JAr cells

3.2.5.1 Procedure

The aim of this experiment was to test the effects of the synthetic brominated-indole compounds on the choriocarcinoma JAr cell line viability and progesterone synthesis. Liquid chromatography mass spectrometry was conducted on the synthetic analogue 5-bromoisatin (section 2.1.8.1); 6'6-dibromoindirubin and indirubin were characterised as previously described by Westley and Benkendorff (2008).

For the *in vitro* cytotoxicity assays indirubin (APIN chemicals; section 2.1.8.1), 5-bromoisatin (Aldrich chemicals; section 2.1.8.1) and 6'6-dibromoindirubin (synthesized by Prof. A. L. Skaltsounis; section 2.1.8.1) were prepared in a drug transport vehicle, DMSO (100%) at 100 times the final concentration and then diluted to a range of working concentrations (0.01-100µg/mL) in RPMI complete cell assay medium (0.1mL per well; section 2.1.3.6.4) to give a final working concentration per well of 1% DMSO. All prepared solutions were filtered using a sterile Minisart 0.20µm (Sartorius stedim) before adding to cells.

The JAr cells were detached from flasks with 0.25% Trypsin-EDTA solution (section 2.2.1.2). Cell number and viability was determined using the trypan blue exclusion assay on a haemocytometer (section 2.2.2.3). JAr cells were seeded into three sterile 96-well plates (Interpath) at 20,000 cells per well in RPMI complete media (section 2.1.3.6.4) and incubated for 2h to cell adherence. A standard curve plate of 0-80,000 JAr cells per well in 6 replicate wells was generated and run concurrently with treatment plates.

At the end of the adherence period, the media was removed and 6'6-dibromindirubin, indirubin and 5-bromoisatin (0-100µg/mL) diluted in RPMI complete media (section 2.1.3.6.4) were added in a final volume of 0.1mL in 3 replicate wells for 2, 4, 6, 8, 10 or 24h at $37^{o}C$ + 5% CO_2. Controls consisted of a medium control only, 1% DMSO (solvent control) and hydrogen peroxide as a positive control (10,000µg/mL section 3.2.3). The standard curve plate was treated with the crystal violet assay (section 2.2.2.1). The absorbance of each well was measured on an automatic spectrophotometer at 570nm, with reference 630nm using KC Junior software.

At the end of each treatment period the supernatant was removed from the treatment plates and stored for the measurement of progesterone by RIA (section 2.2.5.2). Treatment wells were rinsed with sterile 1x PBS (section 2.1.3.5) and cell viability was determined by the crystal violet assay (section 2.2.2.1). The absorbance of each treatment well was converted to final number using the equation of the line from the standard curve plate. The experiment was repeated on 3 separate occasions using three independent cultures. The concentration of progesterone in cell culture assays (2, 4, 6, 8, 10 and 24h) was determined by radioimmunoassay (RIA), using a specific antibody as previously described (section 2.2.5.2).

3.2.5.2 Statistical Analysis

A two-way analysis of variance (ANOVA) using the sensitive contrast K Matrix analysis tests (Pallant, 2002) was conducted to examine the effects of the synthetic compounds on both cell viability and progesterone synthesis using SPSS software package version 17. The alpha value was adjusted for cell viability results for 6'6-dibromoindirubin at 2, 4, 8 and 24h, for indirubin at 2, 4, 8, 10 and 24h and for 5-bromoisatin at 4 and 24h, and also for all progesterone results to significant, if $p < 0.01$ as the data had violated the Levene's homogeneity of variance (n=3; ± 1 SEM).

3.3 Results

3.3.1 Comparison between 1h MTS, 1h MTT, 24h MTT and Crystal Violet Assays

The standard curves developed from 0-80,000 JAr cells per well for the crystal violet, 1h MTT, 1h MTS and 24h MTT all decreased in linearity as cell number increased (Figure 3.1A and Figure 3.2A, C, E & G). The 1h MTS produced the highest R^2 value 0.939, in comparison to the crystal violet ($R^2 = 0.868$), 1h MTT ($R^2 = 0.858$) and 24h MTT ($R^2 = 0.861$). However, when the same standard curves were

plotted at 0-40,000 cells per well (Figure 3.1B and 3.2B, D, F & H), the crystal violet standard curve generated the highest R^2 value (R^2 = 0.999; Figure 1B) and highest gradient of the line for determining final cell number.

The linear regression figures further indicated that the crystal violet standard curve was the most reproducible assay producing the lowest confidence intervals (Figure 3.2B). The 1h MTT standard curve of 0-40,000 cells per well generated a comparative R^2 value (R^2 = 0.998; Figure 3.1B & Figure 3.2D) to the crystal violet standard curve, however, the gradient of the line was not as steep. The 1hr MTT standard curve was the most sensitive at low cell numbers in comparison to the crystal violet, 24h MTT and 1h MTS. The 1h MTT also produced the least variation between replicates as can be seen in the standard deviations bars, specifically in comparison to the crystal violet assay (Figure 3.1B).

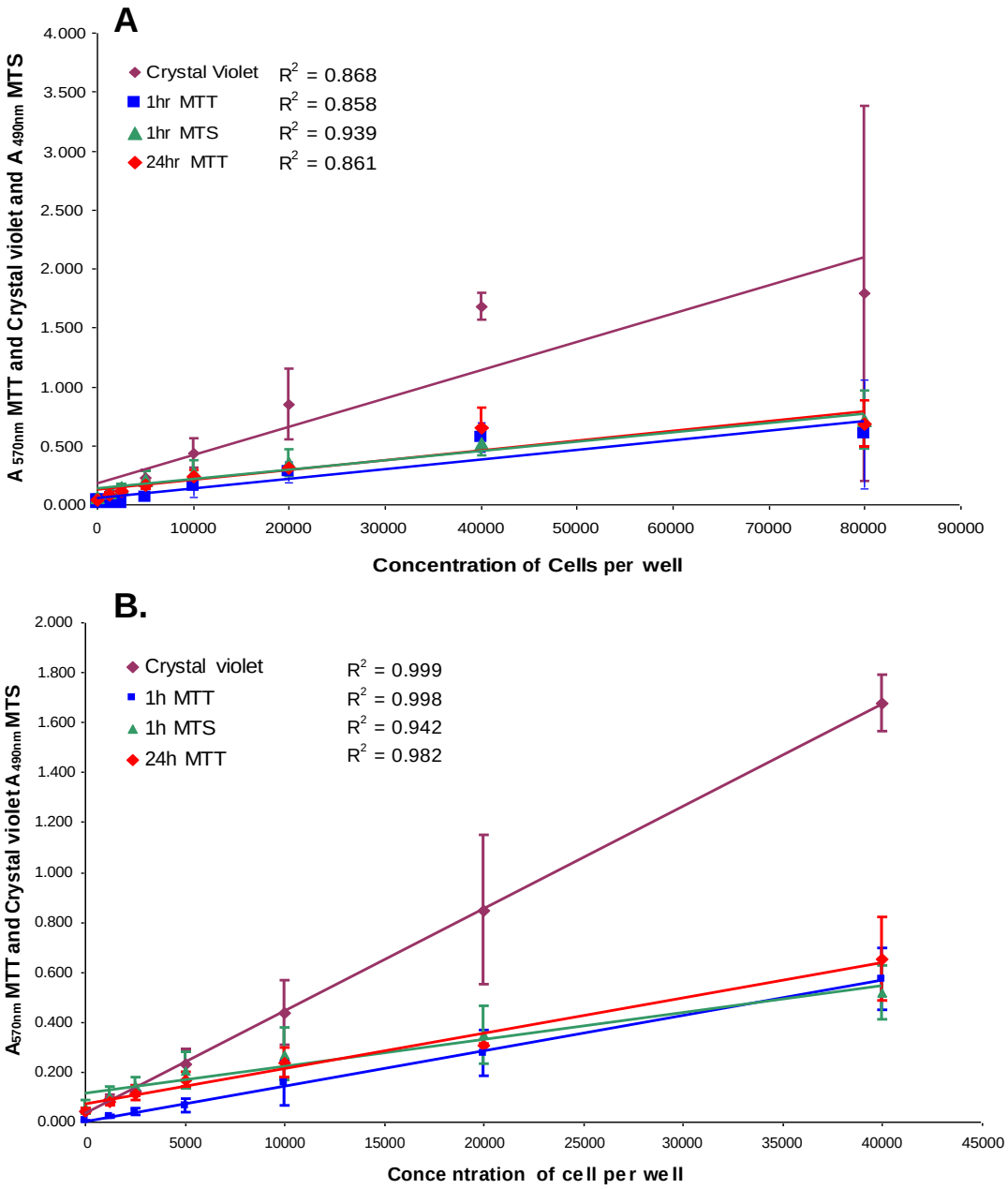


Figure 3.1: Comparison of Crystal Violet, MTT and MTS assays
Comparison of the Crystal Violet, MTT and MTS assays using JAr cells at (A) 0-80,000 cells per well and (B) 0-40,000 cells per well in six replicates. Cells were incubated for 24h for cell adherence to wells of plates. After this, the media was removed from the MTT plates and MTT (0.5mg/mL) was added to the adherent cells and plates were incubated for 1 or 24h, followed by the addition of 20% SDS in 0.02M HCl for a further 1h. A 3rd plate had 0.02mL of MTS solution (0.32mg/mL; Promega) added straight to the media and the plate was incubated for 1h in the dark. The media was removed from the 4th plate and 0.05mL of crystal violet stain (0.5%) was added to all treatment wells and the plate was incubated at room temperature for 10 minutes. The plate was then rinsed extensively with deionised water, air dried and bound crystal violet stain re-crystallised with acetic acid (33%) for 10 minutes. The absorbance for the MTT and crystal violet plates were measured at 570nm, and the MTS 490nm with reference absorbance 630nm, on an automatic spectrophotometer using KC Junior software (n=3; ± 1 SEM).

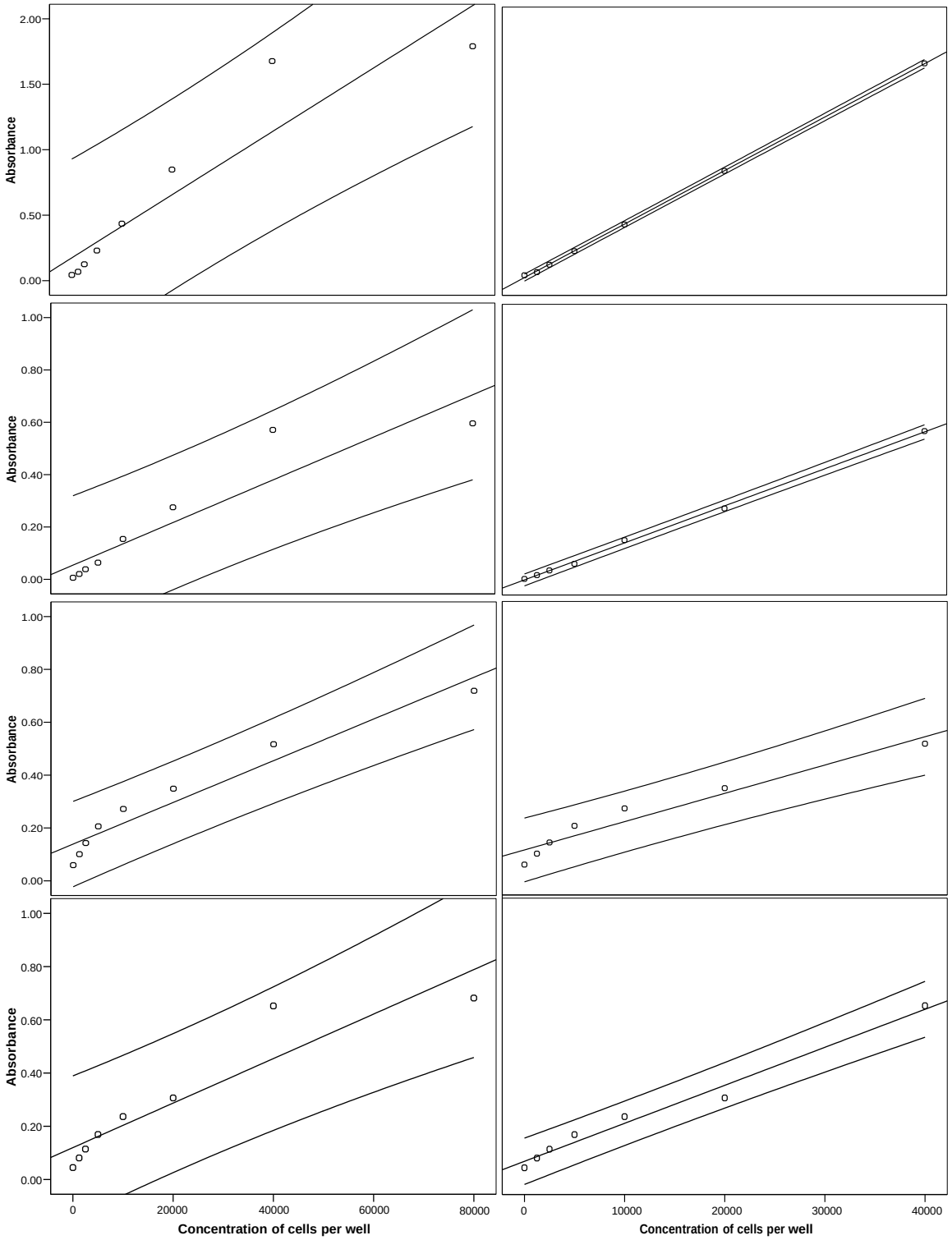


Figure 3.2: Regression lines for Crystal Violet, MTT and MTS assays.
Regression line and 95% confidence intervals for the crystal violet (A, B), 1h MTT (C,D), 1h MTS (E,F) and 24h MTT (G,H) assays of 0-80,000 cells per well (A, C, E, G) and 0-40,000 cells per well (B, D, F, H) calculated using SPSS statistical software version 17.

3.3.2 Optimisation of JAr Cell Adherence

At cell concentrations lower than 40,000 cells per well, absorbance values increased in proportion to incubation time (Figure 3.3). However, for the 4 and 6h incubation periods at 80,000 cells per well, the region of linearity decreased (Figure 3.3; Figure 3.4C and D) resulting in lower absorbance values and lower R^2 values (0.915 and 0.839 for 4 and 6h respectively), in comparison to the 2h incubation period (R^2 =0.962). The 95% confidence intervals for the 2h incubation period indicates this is the most reproducible and accurate standard curve (Figure 3.4B). The 0h standard curve generally produced the lowest absorbance values for all cell numbers, flattest gradient of the line and low R^2 value (Figure 3.3; R^2 = 0.876).

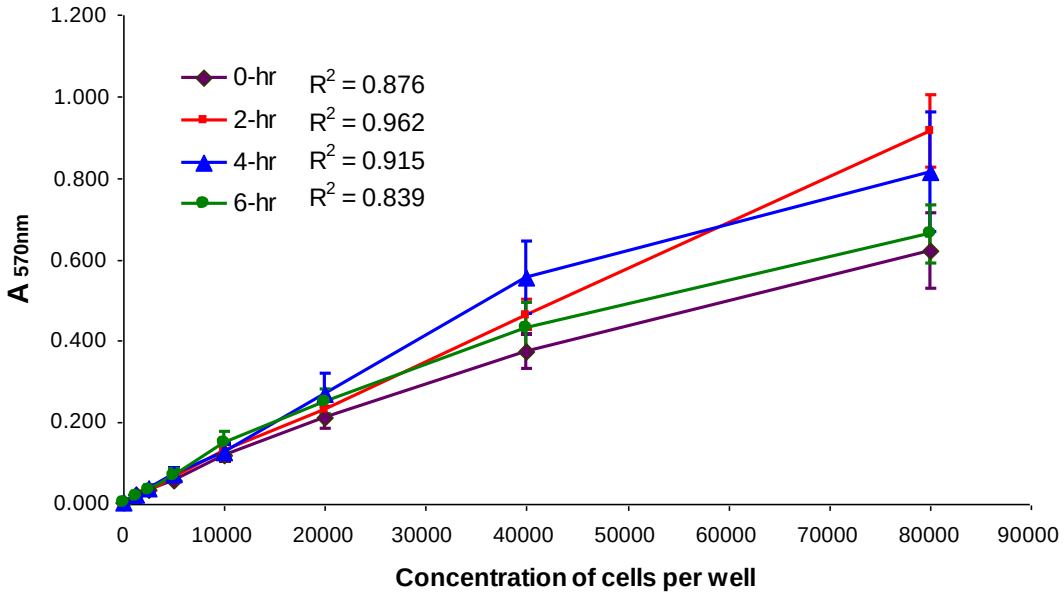


Figure 3.3: The effect of JAr cell incubation period on optical density.
JAr cells were plated into four flat-bottom 96-well plates from 0-80,000 cells per well in six replicates and incubated for 0, 2, 4 and 6h at 37°C + 5% CO_2. The media was removed and MTT (0.5mg/mL) was added for 1h incubation, followed by 20% SDS in 0.02M HCl for a further 1h. Plates were read at 570nm, with reference absorbance 630nm on an automatic spectrophotometer using KC Junior software (n=4; ± 1 SEM).

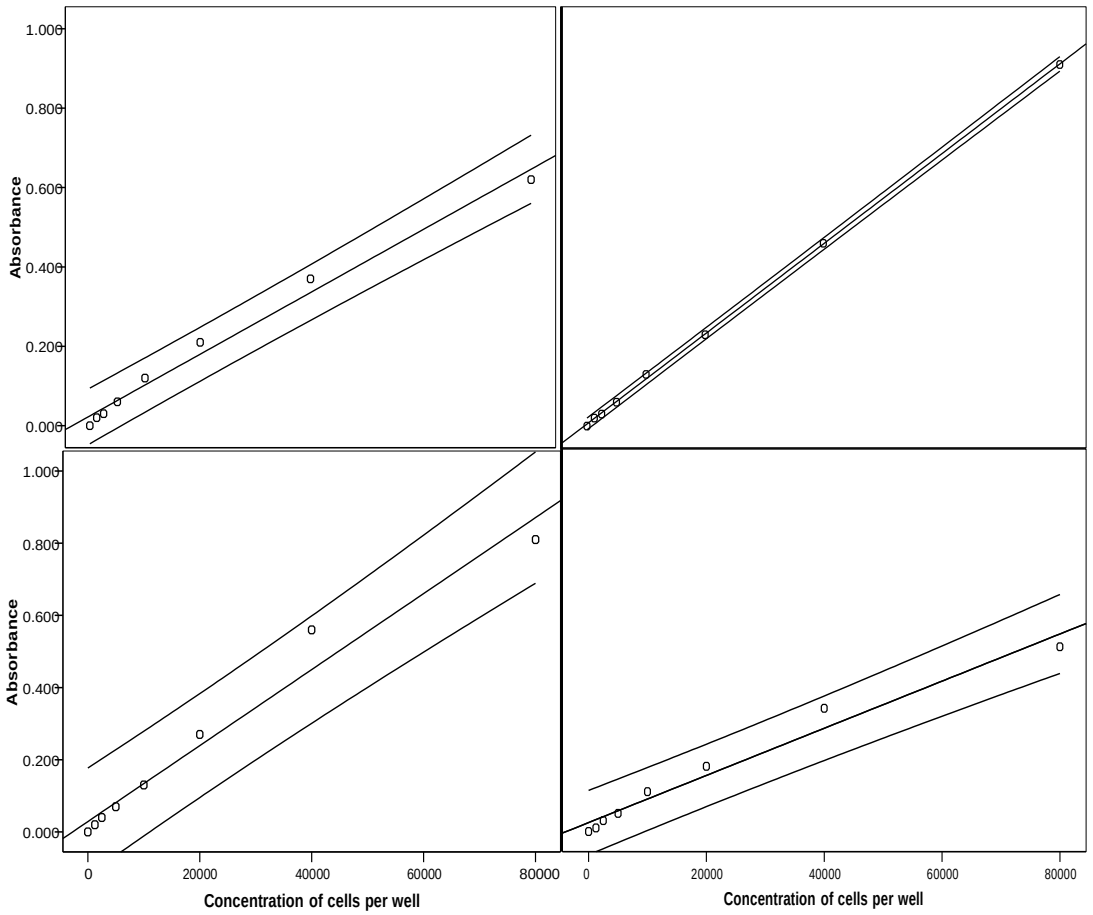


Figure 3.4: Regression lines for JAr incubation assay.
Regression line and 95% confidence intervals for the effects of JAr cell incubation on optical density for the 4 incubation periods 0, 2, 4 and 6h were determined using SPPS statistical software version 17.

3.3.3 Calculation of Final Cell Number per Well

The equations of the line obtained from the 0, 2, 4 and 6h cell incubations (Figure 3.3) were then used to calculate the final cell number per well to determine which standard curve was the most accurate for determining cell number (Figure 3.5) based on the premises that JAr cells have a doubling rate of 15h (Adler, *et al*., 1995). It was predicted that 20,000 cells per well would proliferated to 22,667 after 2h, 25,333 after 4h and 28,888 cells per well after 6h. Calculation of cell numbers using the 2h standard curve were 22,238 ± 2,599, from the 4h standard curve were 24,796 ± 5,189 and 24,348 ± 5,097 from the 6h standard curve (Figure 3.5). At ≥ 40,000 cells per

well the 2h standard curve was the most accurate in predicting cell number. For example it was predicted that 40,000 cells per well would proliferate to 45,333 after 2h, 50,667 after 4h and 56,000 after 6h and the results indicate that the 2h standard curve was the closest (45,785 ± 3,813), whereas the 4h standard curve (54,083 ± 8,937) and 6h standard curve (44,625 ± 8,589) both overestimated and under estimated final cell number respectively. Generally the 2 and 4h standard curves gave similar estimates of cell numbers at ≤ 20,000 cells per well and the 6h gave the lowest.

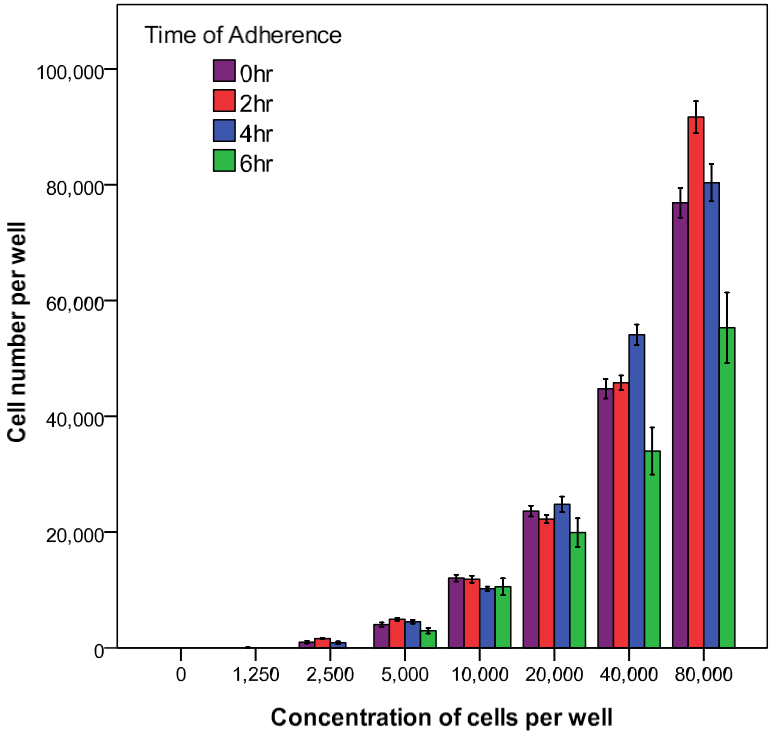


Figure 3.5: Final JAr cell numbers
Final JAr cell numbers per well calculated from the equation of the lines from the four standard curves (0, 2, 4 and 6h Figure 3.3).

3.3.3.1 The Intra- and inter-coefficient of Variation for the MTT assay

Both intra and inter-assay coefficients of variation were approximately the same for the 0h standard curve at all cell numbers per well (Figure 3.6). For the 2h standard curve the intra- and inter-assay coefficients were the highest at 10,000 cells per well (15% intra) and the lowest at 2,500 cells per well (7% inter). At 20,000 cells per well, the intra- and inter-assay coefficient of variation were both 11% for the 2h standard curve. The 4h standard curve generated the highest intra- and inter-assay coefficient of variation at 1,250 cell per well (35 and 43% respectively for intra- and inter). At 20,000 cells per well the 4h standard curve generated a 16% intra- and inter-assay coefficient of variation. The 6h standard curve generated higher coefficient of variations at $\geq$ 5,000 cells per well. For example at 20,000 cells per well the intra and inter-assay coefficient of variation were 19 and 18% respectively (Figure 3.6).

Overall the 2h attachment followed by 1h MTT (0.5mg/mL) and cell concentration of 20,000 cells per well proved to be optimum for the JAr cell line. The lower and upper 95% confidence intervals for the 2h attachment period confirmed that it was a reliable and reproducible assay. In comparison, after 4 and 6h incubation the relationship between absorbance and cell number was no longer linear, resulting in a lower R^2 value. Furthermore, the intra- and inter-assay coefficient of variation for the 2h incubation period were lower than for the 4 and 6h periods, confirming that the 2h attachment period was the most reproducible for the JAr cell line and was therefore adopted for all further experiments.

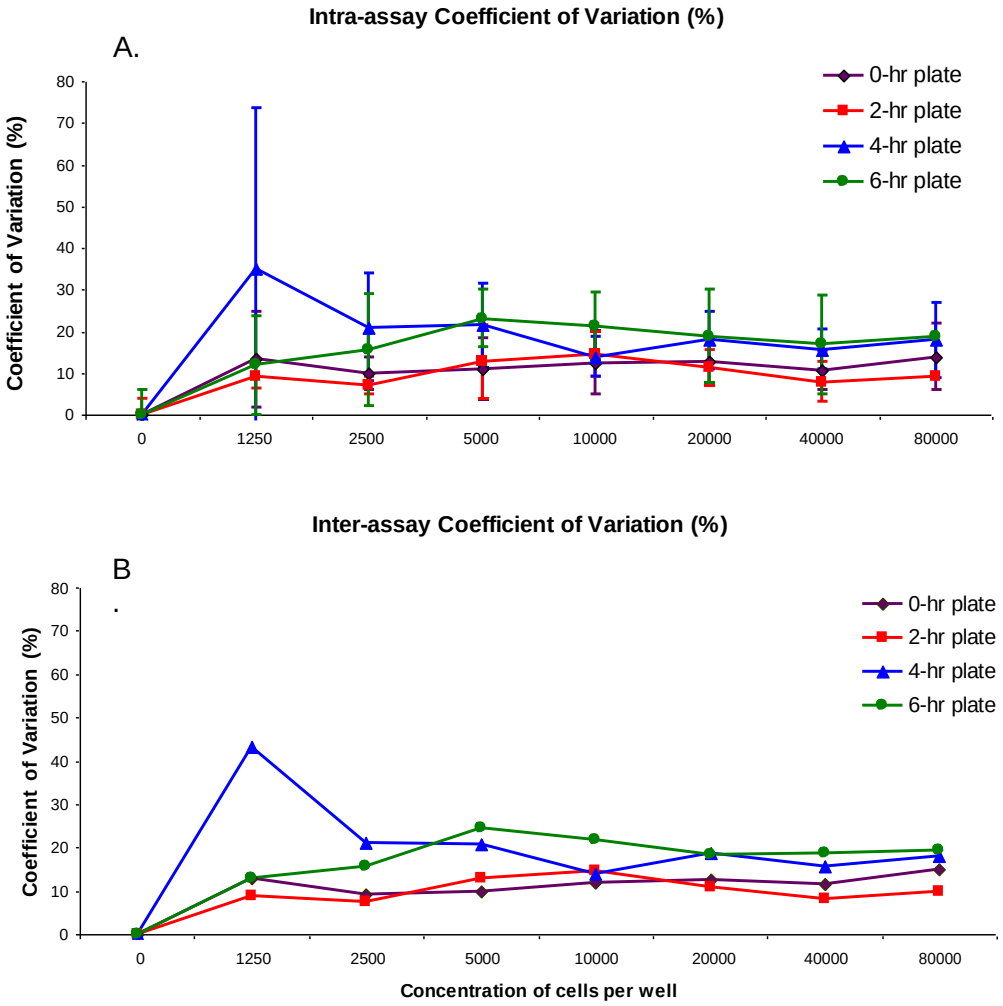


Figure 3.6: The intra and inter-assay coefficients of variation
The intra-assay coefficient of variation (A) for the four standard curves (0, 2, 4 & 6h) was calculated by applying the formula (standard deviation/mean)*100 to each six replicates in each repeat assay shown as the mean ± standard deviation (n=4). The inter-assay coefficient of variation (B) measured from the four different experiments, each experiment containing six means and six standard deviations. Therefore, the standard deviations of 24 replicates were divided by the mean of 24 replicates.

3.3.4 The Effects of MTT Concentration on Production of Formazan by the JAr Cell line

JAr cells (10,000 cells per well) were incubated for 0, 2, 4 and 6h to allow cell adherence, then exposed to concentrations of MTT at 0 (control), 0.1, 0.5 and 1mg/mL for 1h (Figure 3.7). The absorbance values measured at 570nm for the 0mg/mL control were all $\leq$ 0.01. Absorbance values increased gradually with incubation for cells exposed to 0.1mg/mL; 2h (0.04 ± 0.012), 4h (0.06 ± 0.013) and 6h (0.07 ± 0.015). When incubated with 0.5 and 1mg/mL MTT substrate, there was an increase in formazan production and hence, absorbance values increased significantly compared to control ($p < 0.001$) at all incubation periods. However, at each incubation period there was no significant difference between the absorbance values of 0.5 and 1mg/mL MTT (Figure 3.7).

3.3.5 The Effect of MTT Substrate on JAr Cell Adherence

10,000 JAr cells per well were exposed to 0.5mg/mL MTT substrate in triplicate for 2, 4 and 6h. At the end of each incubation period the supernatant was removed and fresh MTT was added for 1h (section 3.1.1). The absorbance values measured at 570nm, from these cells were compared with control cells (10,000 cells per well) which were incubated in the absence of MTT for 2, 4 and 6h before MTT was added for 1h MTT (section 3.1.1). At all three incubation periods tested it was found that there was no statistically significant difference in cell adherence on plates that did or didn't include MTT substrate (0.5mg/mL) at the beginning of the assay (Figure 3.8).

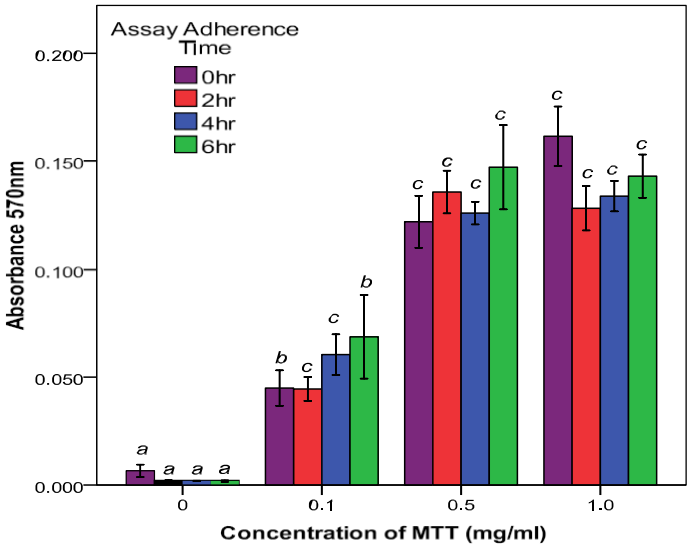


Figure 3.7: The effect of MTT concentration on the production of formazan by the JAr cell line.
JAr cells (10,000 cells per well) were plated into flat-bottom 96-well plates for 0, 2, 4 & 6h. Media was removed and four different concentration of MTT (0, 0.1, 0.5 &1mg/mL) were added in triplicate for 1h. The 20% SDS in 0.02M HCl was added for a further 1h. Plates were read at 570nm, with reference absorbance 630nm on an automatic spectrophotometer using KC Junior software. A two-way analysis of variance (ANOVA) with the Robust Test of Equality of Means and Dunnett's T3 post-hoc was conducted to explore the effects of MTT on absorbance at different incubation periods. Statistical difference between the control and the concentrations at each incubation period are shown as a versus *b* $p < 0.01$** and a versus *c* $p < 0.001$*** (n=4; ± 1 SEM).

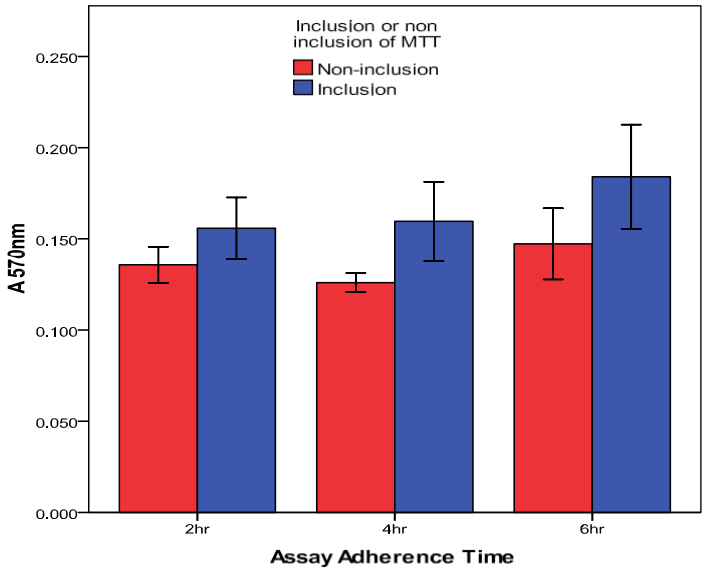


Figure 3.8: The effect of MTT substrate on JAr cell adherence.
JAr cells (10,000 cells per well) were plated into flat-bottom 96-well plates in the presence (inclusion) or absence (non-inclusion) of MTT (0.5mg/mL) for 2, 4 and 6h. Media was removed and MTT (0.5mg/mL) was added to all wells for 1h. The 20% SDS in 0.02M HCl was added for 1h in the dark. Absorbances were measured on an automatic spectrophotometer at 570, with reference absorbance 630nm, using KC Junior software.

3.3.6 The Effect of Cell Incubation on Progesterone Synthesis

Progesterone synthesis in the 2, 4 and 6h plates was measured by the RIA (section 2.2.5.2) and ELISA (section 2.2.5.1) assays and a comparison was made between the two assays to determine if the assays produced similar results. Progesterone concentrations determined by the RIA assay (Figure 3.9A) were generally much higher than the equivalent measurement obtained by the ELISA assay (Figure 3.9B), with a significance difference seen at each incubation period ($p < 0.001$).

The intra- and inter-assay coefficient of variation produced varying results at each incubation period for the RIA and the ELISA assay (Table 3.1). At 2h incubation and 10,000 cells per well the lowest intra-assay coefficient was 5% at for the RIA in comparison to 21% for the ELISA, and the inter-assay coefficient of variation was again 5% for the RIA and 10% for the ELISA. However, at 20,000 cells per well after 2h incubation the intra-assay coefficient was 15% for the ELISA and 45% for the RIA, and the inter-assay coefficient of variation was 15% for the ELISA and 54% for the RIA. After 4h incubation, the intra-assay coefficient of variation increased for the RIA with the lowest at 80,000 cells per well (23%) for the RIA in comparison to the ELISA (3%).

Table 3.1: Intra and inter-assay coefficient of variation for the ELISA and RIA
A comparison of the ELISA (E) and RIA (R) intra- and inter-assay coefficient of variations for each cell concentration at the three different adherence periods for progesterone synthesis by the JAr cell line

2h adherence														
Cell # per well	1,250		2,500		5,000		10,000		20,000		40,000		80,000	
	E	R	E	R	E	R	E	R	E	R	E	R	E	R
Intra	31	68	9	18	10	19	21	5	15	45	18	11	15	29
Inter	23	86	13	71	13	50	10	5	15	54	13	24	16	31
4h adherence														
	E	R	E	R	E	R	E	R	E	R	E	R	E	R
Intra	29	41	54	49	16	74	21	47	25	55	8	24	3	23
Inter	21	45	8	62	15	94	20	25	20	59	9	28	4	25
6h adherence														
	E	R	E	R	E	R	E	R	E	R	E	R	E	R
Intra	58	20	43	79	32	71	25	28	13	73	11	20	8	10
Inter	9	35	24	54	30	54	26	14	17	56	15	26	7	40

E = ELISA and R = RIA

The lowest inter-assay coefficient of variation for the RIA after 2h was at 10,000 cells per well (5%) while the ELISA at the same cell concentration was 10%. At 4h adherence the lowest inter-assay coefficient of variation was 4% at 80,000 cells per well for the ELISA and 25% for 10,000 cells for the RIA. Finally, after 6h incubation both the inter-assay coefficient was lowest for the ELISA assay at 80,000 cells per well (7%) compared to the RIA assay (14%) at 10,000 cells per well.

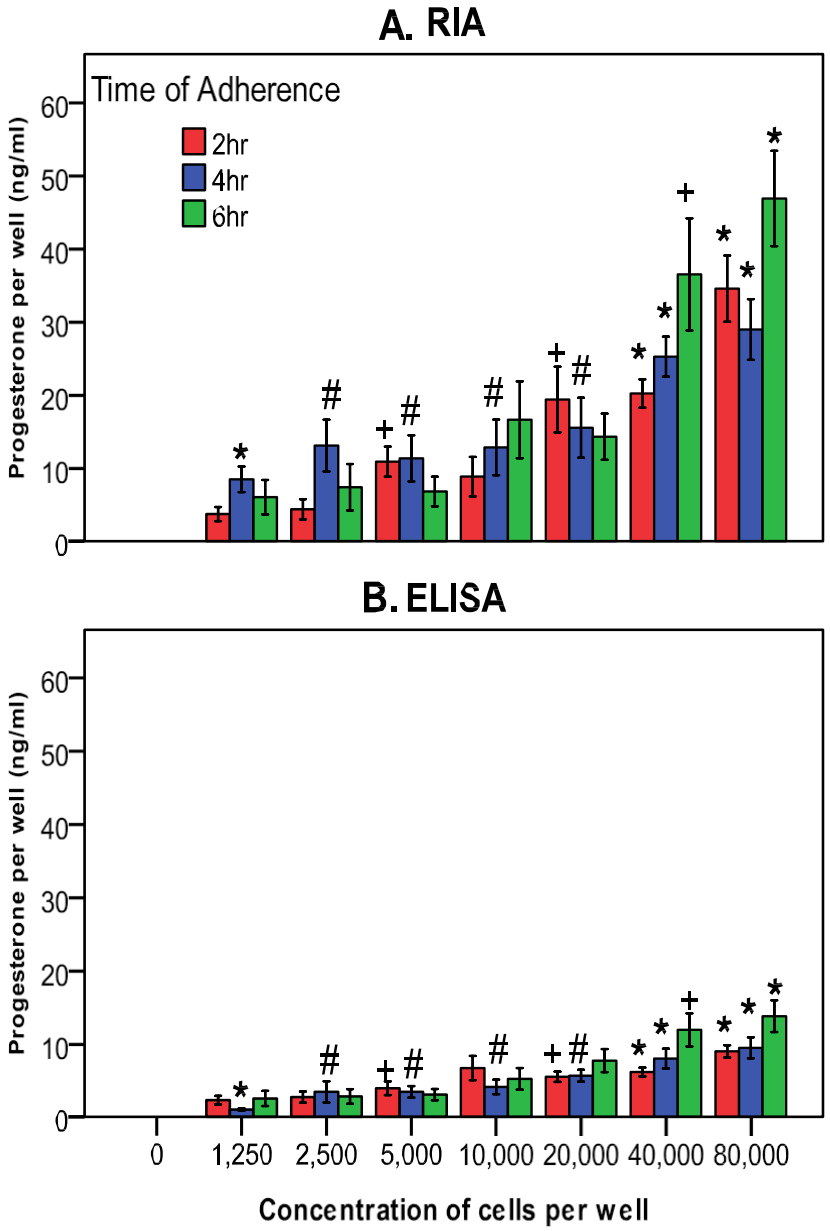


Figure 3.9: The effect of JAr cell incubation period on progesterone synthesis. JAr cells were plated into four flat-bottom 96-well plates from 0-80,000 cells per well in six replicates and incubated for 0, 2, 4 and 6h at 37^{o}C + 5% CO_2. The media was removed and stored for the measurement of progesterone by (A) radioimmunoassay (RIA) and (B) enzyme-linked immune-sorbent assay (ELISA), and the 1h MTT (0.5mg/mL) was performed (n=4; ± 1 SEM). An independent sample T-test was conducted at each cell concentration for each incubation period to compare the measurement of progesterone synthesis by JAr cells by RIA and ELISA using SPSS statistical software version 17 and significant difference between the RIA and ELISA assay at each cell concentration for each incubation period shown as $p < 0.05$ #, $p < 0.01$ +, $p < 0.001$ *.

3.3.7 Effects of hCG on Progesterone synthesis

hCG was found to have no effect on cell viability at ≤ 24h in comparison to the control (Figure 3.10A). However, there was a significant increase in progesterone production after exposure to ≥ 1,000 mIU/mL hCG ($p < 0.05$ for 2h and, $p < 0.01$ for 4 and 24h; Figure 3.10B).

3.3.8 Effects of Hydrogen Peroxide as a Positive Control

JAr cell viability decreased to 85% of control when treated with H_2O_2 at ≥ 100µg/mL (2.9mM) after 1h exposure ($p < 0.05$; Figure 3.11), with a significant decrease in cell viability at 1,000 and 10,000µg/mL (10% percent of control; $p < 0.001$; Figure 3.11). However, progesterone production was affected by H_2O_2 exposure at concentrations as low as ≥ 0.1 µg/mL (50%) in comparison to the control ($p < 0.001$; Figure 3.11).

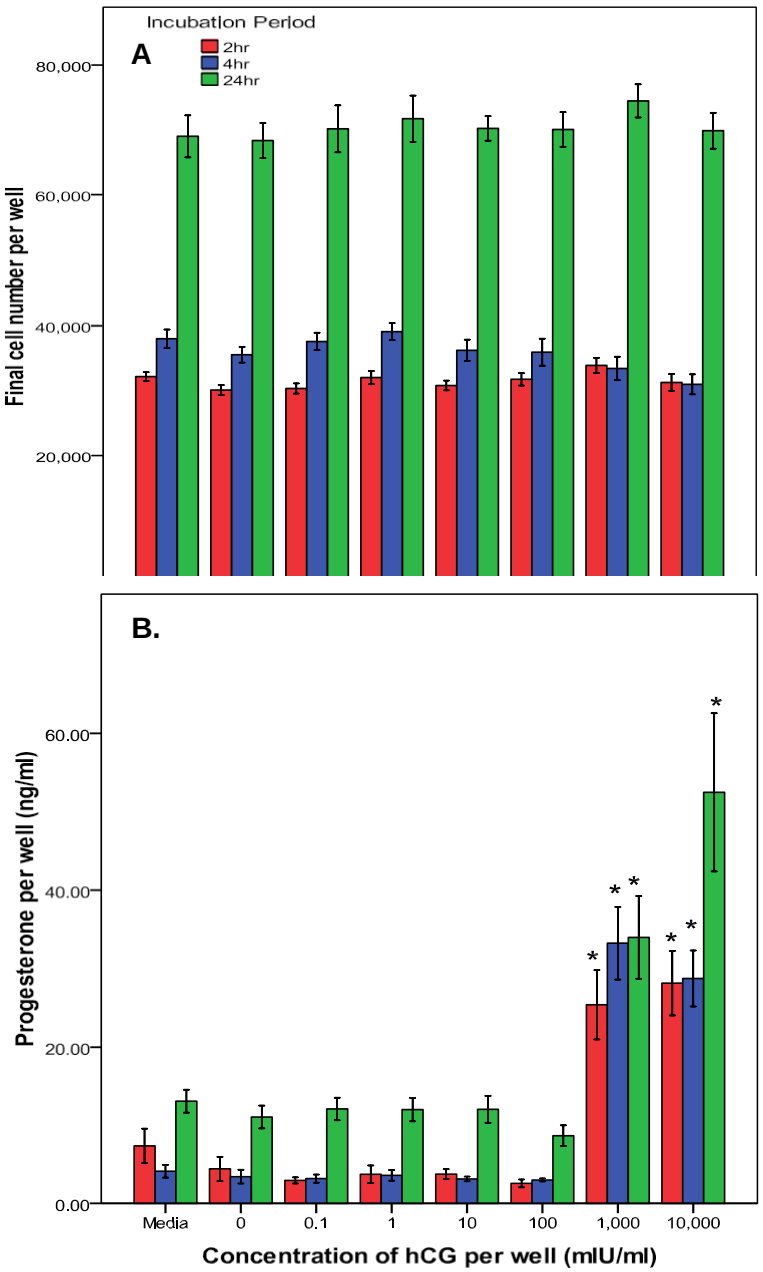


Figure 3.10: The effect of hCG on JAr cell line.
The effect of human chorionic gonadotrophin (hCG) on (A) JAr cell viability and (B) progesterone synthesis. JAr cells (20,000 cells per well) were plated into flat-bottom 96-well plates for 2h to allow cell adherence. hCG (0-10,000mIU/mL) was then added for 2, 4 and 24h. The supernatant was subjected to the radioimmunoassay (RIA) for progesterone concentration and the cells in the wells were treated for 1h with MTT (0.5mg/mL) followed by 20% SDS in 0.02M HCl for a further 1h. Cell number was calculated from a standard curve based on the absorbance measured at 570nm with reference absorbance 630nm. Two-way analysis of variance (ANOVA) with Dunnett T3 post-hoc were conducted to explore the effects of the different concentrations of hCG on absorbance values against the solvent control for progesterone synthesis using SPSS statistical software version 17 and significant difference between 0 control shown as $p < 0.001$ (*) (n=4; ± 1 SEM).

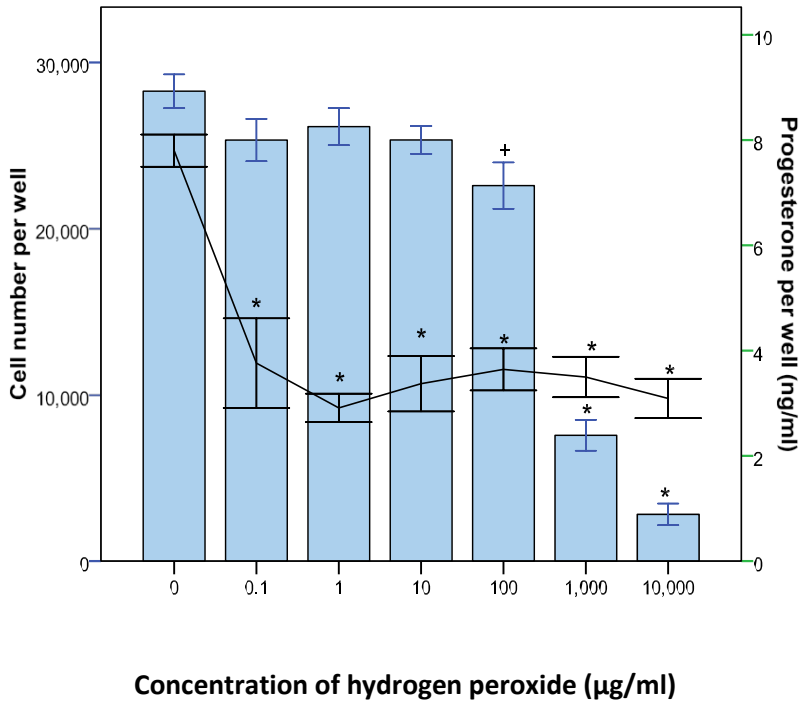


Figure 3.11: The effect of hydrogen peroxide on the JAr cell line.
JAr cells (20,000 cells per well) were plated into flat-bottom 96-well plates for 2h adherence period. Media was then removed and hydrogen peroxide (0-10,000µg/mL) was added for a further 1h. The supernatant was then removed and assayed by the RIA for progesterone concentration shown by the line. The cells in the wells were treated with MTT (0.5mg/mL) for 1h; followed by 20% SDS in 0.02M HCl for a further 1h shown by the bars. Two-way analysis of variance (ANOVA) were conducted with Dunnett T3 post-hoc to compare the effects of the increase in hydrogen peroxide on cell viability and progesterone synthesis using SPSS statistical software version 17 and significant difference between 0 control shown as $p < 0.01(^{+})$ and $p < 0.001(*)$ (n=4; ± 1 SEM).

3.3.9 Chemical Analysis

The purity of the synthetic 5-bromoisatin was confirmed by the molecular weight and characteristic fragment ions: LC/MS retention time (r.t.) = 6.5 minutes ESI-MS major ion peak at m/z 224, 226, corresponding to the molecular ions of 5-bromoisatin (Br^{79}, Br^{81}; Figure 3.12 B & C).

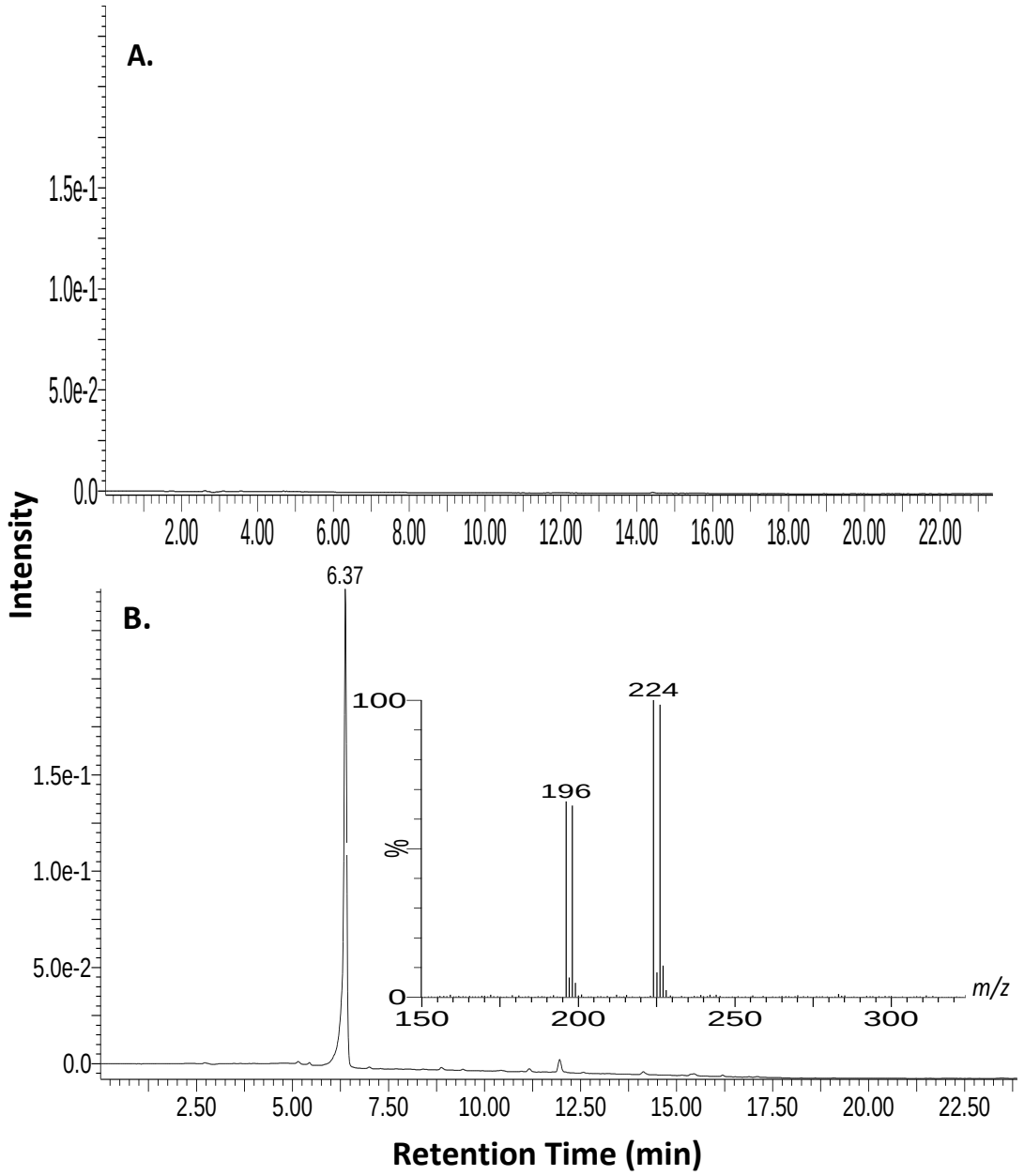


Figure 3.12: LC/MS of the synthetic 5-bromoisatin.
Liquid chromatography mass spectrometry analysis of the synthetic compound 5-bromoisatin which shows the retention time (t_R) at 6.52 minutes; and insert the electrospray ionisation mass spectrometry (ESI-MS) of the peak at t_R 6.52 minutes which corresponds to the molecular mass of 5-bromoisatin (*m/z* 224, 226; Br^{79} Br^{81}). Samples were prepared in HPLC grade acetonitrile (100%) at a concentration of 10µg/mL and total volume injected into the HPLC Column was 20µL.

3.3.10 Effects of the Synthetic compounds on JAr cells

DMSO at 1% had no significant effect on either JAr cell viability or progesterone synthesis compared to media control (Figures 3.13-15). In contrast the brominated indole compounds were found to have a significant effect on both cell numbers and progesterone synthesis (Table 3.2).

Treatment of JAr cells with 6'6-dibromoindirubin (0.1, 10 and 100µg/mL) resulted in a significant reduction in cell viability after 6h ($p < 0.01$; Figure 3.13A). However, longer exposure times of 8 and 10h, were less cytotoxic; 6'6-dibromoindirubin (100µg/mL) was only significantly cytotoxic at the highest concentration tested ($p < 0.001$ and $p < 0.01$, 8 and 10h respectively; Figure 3.13A). In fact, after 24h JAr cells were able to completely overcome the toxicity of 6'6-dibromoindirubin, with a

notable increase in cell numbers at 0.01, 0.1 and 10µg/mL ($p < 0.001$; Figure 3.13A) compared to control. With the decrease in cytotoxicity to cells after 8h treatment, 6'6-dibromoindirubin (0.01µg/mL), increased progesterone synthesis by the JAr cells at the lowest concentration tested ($p < 0.001$; Figure 3.13B). Progesterone synthesis by the JAr cells was also significantly higher than the 1% DMSO control after 4, 10 and 24h after treatment with 6'6-dibromoindirbuin (0.01µg/mL).

Indirubin significantly reduced JAr cell viability after a 6h exposure at 100µg/mL, the highest concentration tested ($p < 0.01$; Figure 3.14A). After 8h, a reduction in cell number was seen at 0.01µg/mL indirubin ($p < 0.01$) with a further significant decrease at 100µg/mL ($p < 0.001$). In a similar manner to 6'6-dibromoindirubin, JAr cell numbers increased after 24h when treated with indirubin at concentrations of 0.01, 0.1 and 1µg/mL ($p < 0.01$; Figure 3.14A), with a significant increase in progesterone synthesis at 0.01µg/mL ($p < 0.01$; Figures 3.14B) also noted as cell numbers increased.

In contrast, 5-bromoisatin (100µg/mL) was found to be cytotoxic to JAr cells at $\geq$ 4h incubation (Figure 3.15A; $p < 0.001$) with LC_{50} noted after just 4h (equal to 442µM). With longer exposure periods to 5-bromisatin (100µg/mL), JAr cell numbers were significantly reduced to 20 and 10% of the control after 8 and 24h respectively (Figure 3.15A; $p < 0.001$). In a similar manner to indirubin and 6'6-dibromoindirubin, JAr cell numbers increased after 24h exposure, but only at the lower concentrations tested 0.1 and 1µg/mL ($p < 0.01$; Figure 3.15A). A significant increase in progesterone synthesis was also noted after 24h in the presence of 5-bromoisatin at the lowest concentration 0.01µg/mL ($p < 0.001$; Figure 3.15B).

Table 3.2: Effects of 6'6-dibromoindirubin, indirubin and 5-bromoisatin on JAr cell viability and progesterone (P_4) synthesis
Cell viability represented as LC_{50} and hormone data shown as concentration that was significantly different to the 1% DMSO control.

JAr cell line						
	Time (h)					
	2	4	6	8	10	24
6'6-dibromoindirubin Concentrations tested 0.01, 0.1, 1, 10, 100µg/mL						
Cell viability	>100	>100	>100	>100	>100	>100
P_4	n/e	0.01 (+)	n/e	0.01 (+)	0.01 (+)	0.01 (+)
Indirubin Concentrations tested 0.01, 0.1, 1, 10, 100µg/mL						
Cell viability	>100	>100	>100	>100	>100	>100
P_4	n/e	n/e	n/e	n/e	n/e	0.01 (+)
5-bromoisatin Concentrations tested 0.01, 0.1, 1, 10, 100µg/mL						
Cell viability	>100	100	10-100	10-100	10-100	10-100
P_4	n/e	n/e	0.01 (-)	n/e	n/e	0.01 (+)

P_4 = progesterone; (+) stimulated; (-) inhibited; n/e = no significant effect.

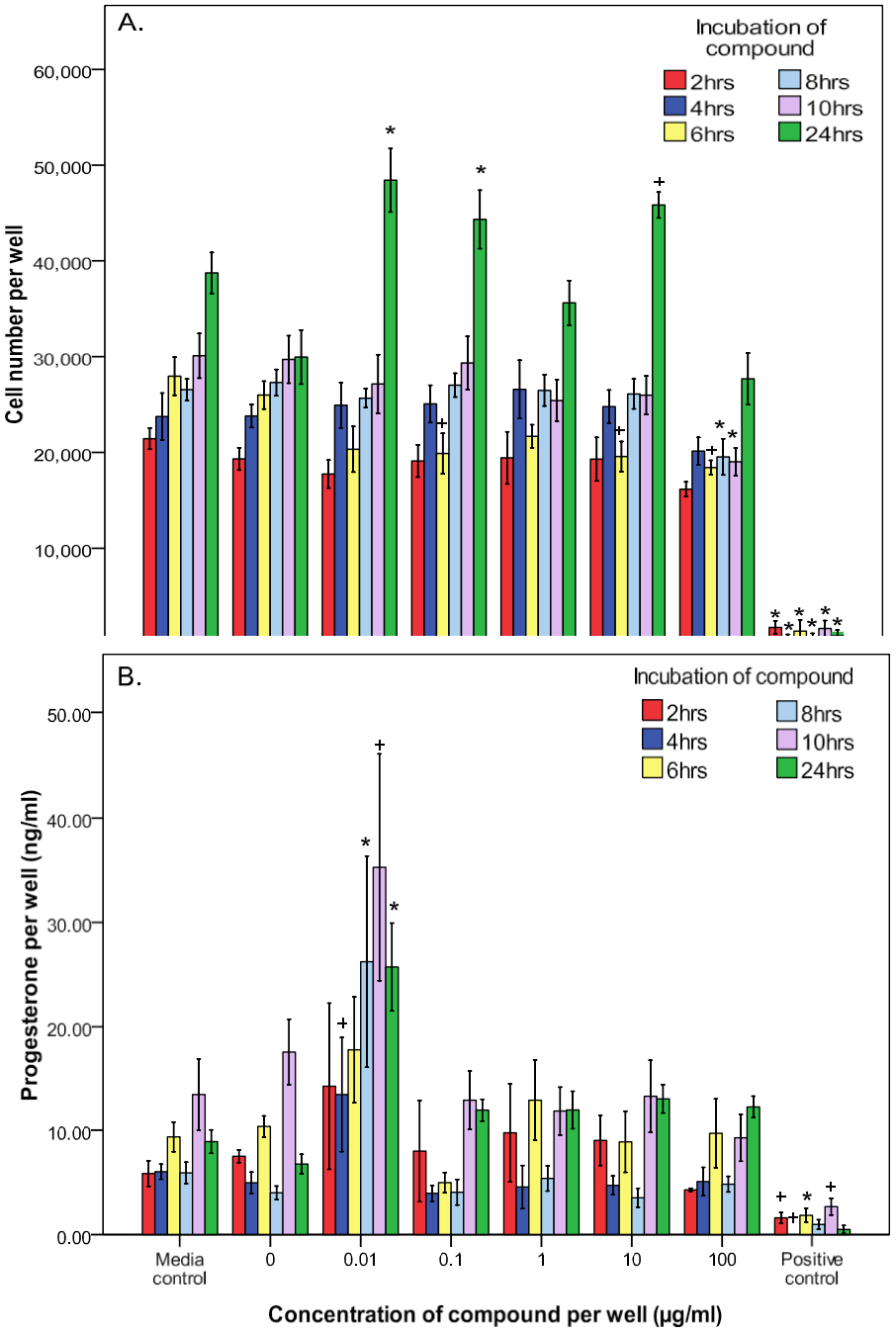


Figure 3.13: The effect of 6'6-dibromoindirubin on JAr cell viability and progesterone synthesis

After a 2h attachment period JAr cells (20,000cells/well) were treated with 6'6-dibromoindirubin (0.01-100µg/mL) for 2, 4, 6, 8, 10 and 24h. Cell viability (A) was determined by the uptake of crystal violet (0.5%) stain in the nuclei of live cells and progesterone synthesis (B) by radioimmunoassay. The positive control represents 1,000µg/mL H_2O_2. Univariate analysis of variance was conducted with the contrast (K Matrix) sensitive test and the p values were adjusted for 2, 4, 8 and 24h cell viability data and all progesterone results as the data had violated the Levene's homogeneity of variance. Significance shown against 0 control as $p < 0.01$ ($^+$) and $p < 0.001$ (*) (n=3; ± 1 SEM).

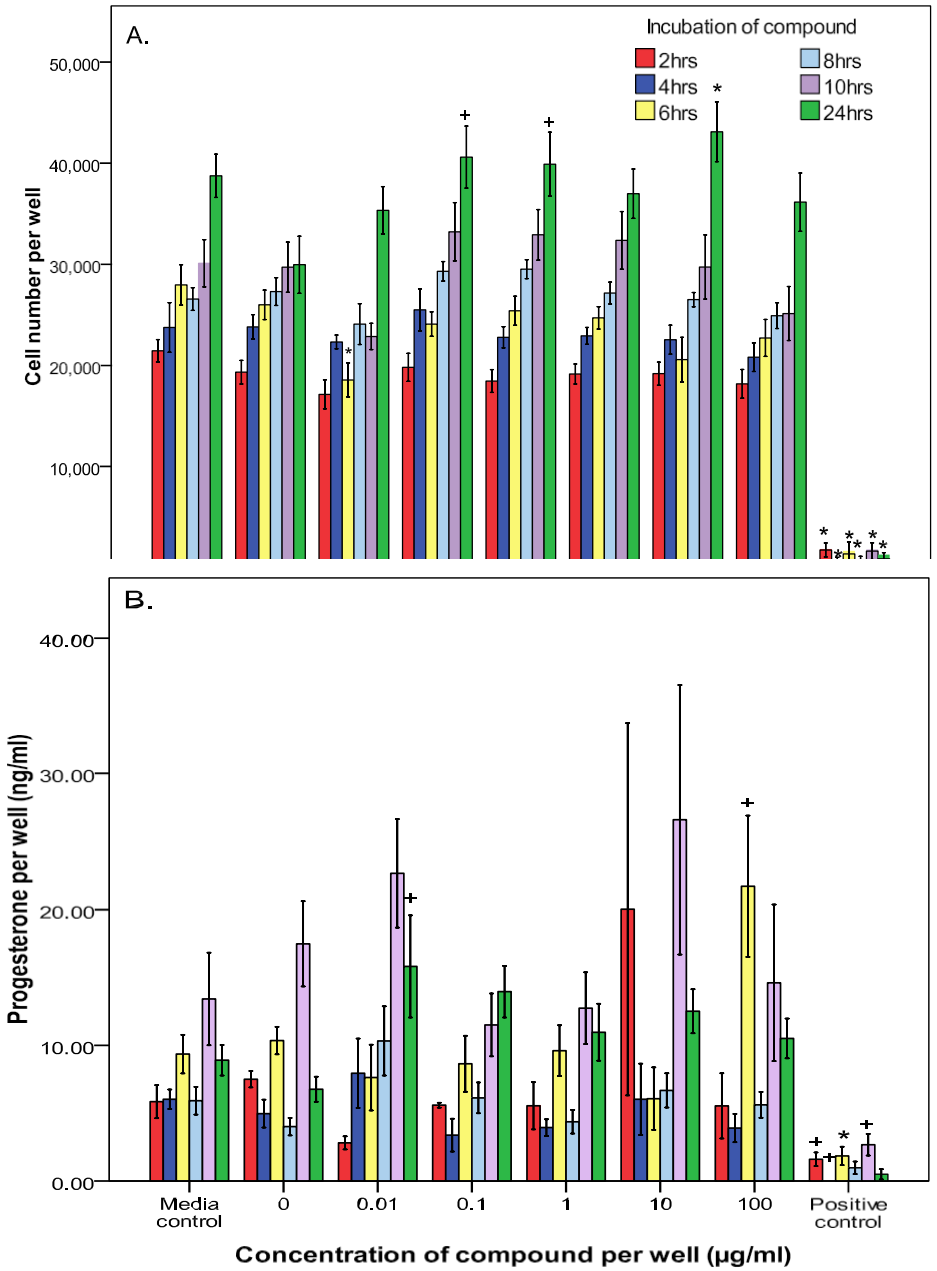


Figure 3.14: The effect of indirubin on JAr cell viability and progesterone synthesis

After a 2h attachment period JAr cells (20,000cells/well) were treated with indirubin (0.01-100µg/mL) for 2, 4, 6, 8, 10 and 24h. Cell viability (A) was determined by the uptake of crystal violet (0.5%) stain in the nuclei of live cells and progesterone synthesis (B) by radioimmunoassay. The positive control represents 1,000µg/mL H_2O_2. Univariate analysis of variance was conducted with the contrast (K Matrix) sensitive test and the p values were adjusted for 2, 4, 8, 10 and 24h cell viability results and all progesterone results as the data had violated the Levene's homogeneity of variance analysis. Significance shown against 0 control as $p < 0.01$ ($^{+}$) and $p < 0.001$ (*) (n=3; ± 1 SEM).

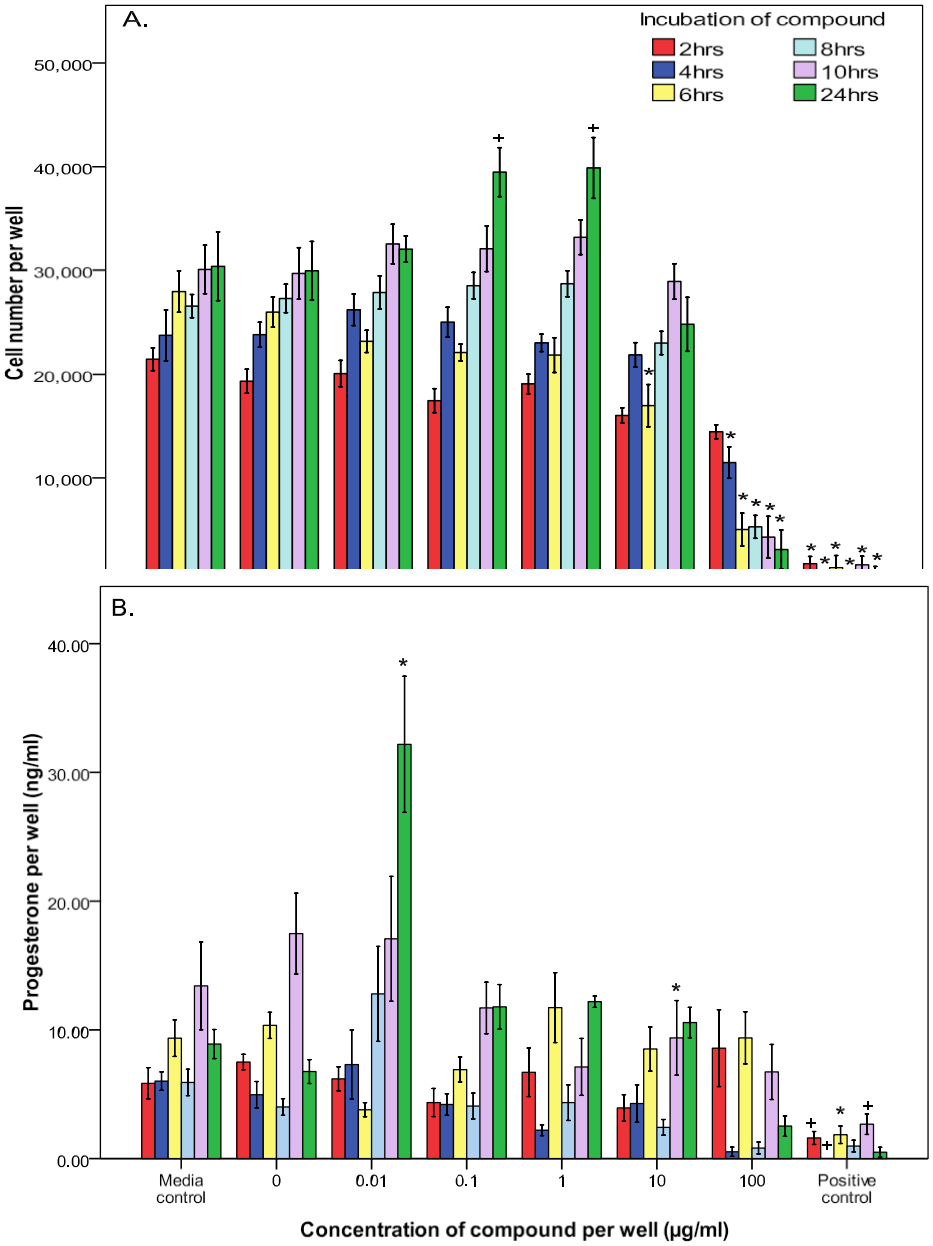


Figure 3.15: The effect of the 5-bromoisatin on JAr cell viability and progesterone synthesis

After a 2h attachment period JAr cells (20,000cells/well) were treated with 5-bromoisatin (0.01-100µg/mL) for 2, 4, 6, 8, 10 and 24h. Cell viability (A) was determined by the uptake of crystal violet (0.5%) stain in the nuclei of live cells and progesterone synthesis (B) by radioimmunoassay. The positive control represents 1,000µg/mL H_2O_2.Univariate analysis of variance was conducted with the contrast (K Matrix) sensitive test and the p values were adjusted for 4 and 24h cell viability data and all progesterone results as the data had violated the Levene's homogeneity of variance analysis. Significance shown against 0 control as $p < 0.01$ ($^{+}$) and $p < 0.001$ (*) (n=3; ± 1 SEM).

3.4 Discussion

The present study was undertaken to optimise a high through-put cell culture assay for the JAr cell line. This optimised assay was then used to investigate the cytotoxicity effects and the effects on hormone synthesis of the synthetic brominated indole compounds, indirubin, 6'6-dibromoindirubin and 5-bromoisatin. To increase reproducibility of the *in vitro* assay, cell culture conditions were optimised using the MTT assay, which was found to be suitable for determining direct cell viability. The optimised MTT assay in the present study adopted a 2h attachment using 20,000 cells per well, followed by 1h MTT (0.5mg/mL) incubation. Previous studies have shown that the MTT assay requires extensive incubation periods of anything from 4-17h (Cory, *et al.*, 1991; van der Loosdrecht, *et al.*, 1991; Young, *et al.*, 2005b). However, more recently our research has demonstrated that MTT incubation periods can vary greatly depending on the cell line (Edwards, *et al*., 2008).

Hydrogen peroxide as a positive control was found to be effective after only 1h exposure; it reduced cell viability by 85% at a concentration equivalent to 2.9mM, and reduced progesterone production at a concentration as low as 0.1µg/mL. Hallmann *et al*., (2004), found that JAr cells were resistant to H_2O_2-induced oxidative stress after a 24h exposure to H_2O_2 (1mM), with only a 12% reduction in cell viability noted. The authors suggested that over-expression of N-acetylglucosaminyltransferase (GnT-III) may suppress H_2O_2-induced apoptosis in JAr cells and their research further showed that JAr cells do have a high expression of both GnT-III and GnT-IV as opposed to cells in the human placenta (Hallmann, *et al.*, 2004). Our study also found no significant reduction in cell viability after 1h exposure to 1mM (34µg/mL) H_2O_2. However, it seems likely that the higher

concentration (2.9mM = 100µg/mL) tested was toxic enough to overcome the ability of the JAr cells to suppress H_2O_2-induced apoptosis by the GnT-III and GnT-IV enzymes. Consequently, this result suggests that H_2O_2 is effective as a positive control on the JAr cell line at concentrations ≥ 2.9mM (100µg/mL).

JAr cells are known to synthesize progesterone, hCG and pregnenolone and to a lesser degree 17β-estradiol, constitutively in culture (Bahn, *et al*., 1981). In this research we analysed the synthesis of progesterone by JAr cells using two different hormone assays, the RIA and ELISA assay and found the ELISA was a more sensitive assay. The RIA has predominately been used in the past for determining hormone concentrations and is often still used today (Edwards, *et al*., 2008; Young, *et al*., 2008; Pierre, et al., 2009). However, the main disadvantage of the RIA assay is the use and disposal of radioactive material (Graham, *et al.*, 2001; Ohno, *et al*., 2004). Therefore the added benefit of the sensitivity of the ELISA assay is that it is a non-radioactive cell based assay (Ohno, *et al.*, 2004). However, the disadvantage of the ELISA assay in our research was that the assay was unable to measure high quantities of progesterone as synthesised by primary reproductive cells (Chapter 4) Therefore, we could not use this technique to directly compare the results of progesterone synthesis by cell lines against primary-derived human granulosa cells (Chapter 4) and as such the RIA was adopted in further experiments.

Research has shown that the gonadotrophins, human choronic gonadotrophin (hCG) and luteinizing hormone (LH) play a key role in the development and proliferation of reproductive cancer cells and in particular, ovarian cancer cells (Mandi, *et al*., 2007). For example, Konishi *et al.*, (1999) have shown that hCG increases the proliferation

of ovarian surface epithelium cells (OSE) in a dose-dependent manner. However, in the present study, hCG (≤ 10,000mIU/mL) was found to have no effect on JAr cell viability. Other studies have also shown that hCG has no effect on cell proliferation. When the OVCAR-3 and SKOV-3 cell lines were treated with 0.1µg/mL hCG there was no significant effect on cell numbers (Kuroda, *et al*., 1998). Furthermore, Tourgeman *et al*., (2002) have demonstrated that hCG (200mIU/mL) actually decreases cell proliferation in ovarian epithelial cells (OEC). Hence, these differences between cell lines towards hCG stimulation emphasizes the importance of optimising culture conditions for all cell lines.

A number of studies have been conducted to investigate hCG synthesis by choriocarcinoma cell lines using dibutyryl cyclic AMP and gonadotrophin-releasing hormone (Hussa, *et al.*, 1975; Sibley, *et al*., 1991; Ertl, *et al*., 1993), however, very little research, if any has been undertaken to establish the effects of hCG on progesterone synthesis by JAr cells. As progesterone is secreted mainly from reproductive cells of the corpus luteum, previous research using hCG has predominately focused on cultured cells of the corpus luteum, such as the granulosa and theca cells. When stimulated with hCG (10-100mIU/mL) cultured theca cells have been shown to secrete progesterone in a dose-dependent manner (Bergh, *et al*., 1993). The present study found that low concentrations of hCG (≤ 100mIU/mL) did not significantly increase progesterone synthesis by the JAr cell line. Only at higher concentrations of hCG (≥ 1,000mIU/mL) was there a significant increase in progesterone synthesis in the absence of cell proliferation.

Previous studies have demonstrated that both indirubin and isatin compounds have anti-proliferation activity towards different cancer cell lines (Meijer, *et al.*, 2003; Kim, *et al.*, 2007; Vine, *et al.*, 2007a; Benkendorff, *et al.*, 2011). Kim *et al*., (2007) have shown that indirubin reduces cell viability in the lung carcinoma cell line A549 after a 24h period (31μM/L; IC_{50}). In the present study, indirubin only had a significant effect on cell viability at 8h exposure. In fact at 10 and 24h JAr cell numbers increased. This increase in cell proliferation may be characteristic of the excretory role associated with these choriocarcinoma cells, enabling the cells over time to metabolise and excrete the indirubin thereby preventing the cytotoxic effects of the compound and allowing cell division and consequently an increase in JAr cell numbers to occur (Sullivan, 2004; Serrano, *et al*., 2007). 6'6-dibromindirubin, an analogue of indirubin was also only found to have cytotoxic effects on JAr cell viability at the shorter incubation periods (6 and 8h) however, progesterone synthesis by JAr cells was significantly up-regulated in the presence of 6'6-dibromindirubin at the lowest concentration tested (0.01μg/mL).

The most promising anti-cancer compound screened was 5-bromoisatin, which significantly reduced JAr cell viability by 50% after a 4h exposure at the highest test concentration (442μM). In comparison to other cell lines, JAr cells appear to be more resilient to the toxic effects of this isatin compound. After 24h the JAr cells were able to overcome this initial toxic effect with an increase in cell numbers (Figure 3.16). Vine *et al*., (2007) have also shown that 5-bromoisatin is cytotoxic to the human monocyte-like, histiocytic lymphoma cell line U937 after 24h exposure at a concentration of just 65μM (IC_{50}). The difference in cytotoxicity between cell lines may be a result of the JAr cells ability to metabolise and utilise compounds thereby,

requiring much higher concentration of 5-bromoisatin to initiate any significant cytotoxic effect in comparison to other cell lines, such as U937 (Serrano, *et al.*, 2007).

5-bromoisatin affected cell proliferation but hormone synthesis was not affected at the same concentration, suggesting that progesterone production in JAr cells may not be linked to cell proliferation. Plessinger and Miller (1999) noted a similar finding with JAr cells treated with the HIV agents, zidovudine (AZT) and dideoxyinosine (ddI). They found a reduction in JAr cell number at high concentrations of AZT and ddI (7.6mM) after 24h exposure, but at the same time an increase in progesterone, estradiol and hCG secretion suggested that hormone regulation could be indicative of cellular defence mechanisms (Plessinger and Miller, 1999). Interestingly, with all four compounds tested in the present study, progesterone synthesis was only significantly increased at the lowest concentration tested (0.01µg/mL). In comparison, there was no effect on progesterone synthesis at the higher concentrations tested. It is difficult to speculate why this peak occurred however, this phenomenon known as 'hormesis' has been proven to commonly occur in cells and organisms (Calabrese, 2008). This unforeseen increase in progesterone synthesis could be advantageous in the treatment of infertility if the results are at all indicative of the *in vivo* response. Furthermore, in humans, a deficiency of hormones and/or an imbalance in hormone synthesis has been associated with a number of gynaecological disorders, including chronic endometriosis and polycystic ovary syndrome (PCOS), disorders which are often the underlining cause of sub-fertility (Meletis and Barker, 2004; Kocak and Üstün, 2006; van Kaam, *et al.*, 2007). Hence,

this increase in progesterone synthesis at low concentrations may be beneficial for the treatment of a range of gynaecological disorders.

In conclusion, we have shown that the synthetic bioactive compounds, indirubin and 6'6-dibromoindirubin, which are components of the Tyrian purple molluscan secretion were not cytotoxic to the human choriocarcinoma JAr cell line in culture. It is hypothesised that the JAr cells are able to metabolise and eliminate these compounds from the cell before cytotoxicity could occur. However, further investigations would be required to investigate this hypothesis. The most promising anti-proliferative compound tested was the isatin compound, 5-bromoisatin, which was found to significantly decrease cell-proliferation in the human choriocarcinoma JAr cell line without affecting progesterone synthesis. Further examination of the cytotoxicity of the synthetic indole and isatin compounds on female human primary-derived reproductive cells were investigated in the following chapter.

Chapter 4 The Effects of Synthetic Analogues of Muricid Extracts on Primary-Derived Human Granulosa Cells

4.1 Introduction

There are many causes of infertility, including both anatomical and hormonal dysfunctions, however the underlining cause is still often unknown (Chapter 1.20; Raine-Fenning, *et al.*, 2004). Research indicates the polycystic ovarian syndrome (PCOS) is one of the major causes of anovulatory infertility in women (Chapter 1.20.1; Amer, 2006). The exact cause of PCOS is still unclear, however abnormal folliculogenesis and steroidogenesis are often related to the condition (Chapter 1.20.1; Amer, 2006). Other research also suggests that endometriosis is the underlining cause of infertility and miscarriage in some women (Chapter 1.20.2; Raine-Fenning, *et al.*, 2004). Furthermore, low mid-luteal plasma progesterone levels (Chapter 1.20.3) below the threshold of 32nmol/L, are often related to ovarian disorders, abnormal cycles, spontaneous abortions and infertility (Abdulla, *et al.*, 1983; van Zonneveld, *et al.*, 1994; Baird, *et al.*, 2000; Jose-Miller, *et al.*, 2007).

Muricidae are the source of a range of bioactive compounds which have demonstrated anticancer properties both *in vitro* and *in vivo* (Westley, *et al.*, 2010; Benkendorff, *et al.*, 2011). Chemical synthesis and/or purification of the muricid compounds has proven difficult (Vine, *et al.*, 2007a). However, the structurally related indole and isatin derivatives, 5-bromoisatin and indirubin are commercially available (Chapter 3). Indirubin, the active ingredient in Traditional Chinese Medicine, has shown to be effective against chronic myelogenous leukaemia in clinical trials (Xiao, *et al.*, 2002). The isatin compound, 5-bromoisatin, has been found to inhibit cell proliferation in a range of tumour cell lines *in vitro* (Vine, *et al.*, 2007a). In our preliminary studies (Chapter 3), indirubin and 5-bromoisatin,

significantly increased basal progesterone synthesis in the JAr cell line, but only at the lowest concentrations tested (0.001 and 0.01µg/mL).

Therefore, in light of the positive results obtained previously (Chapter 3) on progesterone synthesis by the choriocarcinoma JAr cell line in the presence of the synthetic compounds 5-bromoisatin and indirubin, we further aimed to investigate the effects of the synthetic compounds on primary-derived human granulosa cells.

Primary-derived human granulosa cells were donated by women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatments at the Flinders Medical Centre due to various infertility issues. The follicular fluid containing the granulosa cells donated from women were subdivided into two main groups based on the infertility factor of the women (section 2.2.1.1 and Appendix II). The fertile group or cells from women with normal reproductive physiology were based on male infertility (sperm defects, decreased motility and erectile dysfunctions) and women who had previously had ectopic pregnancies or a tubular blockage. The infertile group or cells from women with abnormal reproductive physiology included women with polycystic ovarian syndrome (PCOS), single ovarian cysts, endometriosis, and idiopathic infertility. Granulosa cells cannot be considered either fertile or infertile, however for the benefit of this thesis they were classified into two groups, either fertile or infertile based on the original infertility factor of each woman. As a large percentage of the infertile cases undergoing assisted reproductive technology (ART) are often due to unknown infertility factors, it was of interest to establish whether these synthetic compounds could stimulate steroidogenesis in granulosa cells derived from a range of infertile women. This

would therefore establish whether these synthetic compounds and homeopathic remedy could have general applications in treating infertility.

The aims of this research therefore, were as follows:

1. Examine the effects of the synthetic compounds indirubin and 5-bromoisatin on primary-derived human granulosa cell viability (sub-divided into two groups from women classified as either, fertile and infertile).
2. To investigate the effects of indirubin and 5-bromoisatin on hormone secretion by primary-derived human granulosa cells from women classified as either fertile or infertile.

Since the synthetic compounds indirubin and 5-bromoisatin increased progesterone synthesis at low concentrations before cell death occurred in the JAr cell line (Chapter 3), the hypothesis in the following experiment was that these compounds would stimulate steroid hormone synthesis by primary-derived human granulosa cells at low concentrations without effecting cell viability.

4.2 Methods

4.2.1 Isolation of Primary-Derived Human Granulosa Cells

Primary-derived human granulosa cells were isolated from follicular aspirates donated by 14 women undergoing IVF or ICSI treatments at the Flinders Medical Centre with their informed consent (number 260/067; Appendix II; cases 1-10 and 16-19). Donated aspirates were divided into two groups classified as fertile (cases 1, 5, 6, 7, 8, 9 & 10; 3 decreased sperm motility cases, 3 tubular blockage cases and 1 sperm defect and tubular blockage case) or infertile (cases 2, 3, 4, 16, 17, 18 & 19; 2 ovarian cysts cases, 2 idiopathic cases, 2 PCOS cases and 1 endometriosis case)

under the criteria previously specified based on the donors infertility factor (section 2.2.1.1; Appendix II). Granulosa cells were isolated from the follicular aspirates (section 2.1.7.3) and cell number was determined by the trypan blue exclusion assay (Freshney, 2005). Note: physiologically normal and infertile in the results and discussion refers to the original infertility factor of the donors of the follicular aspirates and not the granulosa cells.

4.2.2 Compound Preparation for Cell Culture Assays

The synthetic compounds 5-bromoisatin and indirubin (section 2.1.8.1) were prepared as described previously (section 3.2.5.2) in DMSO (100%), and then diluted in DMEM/F12 + 10% FBS complete cell medium (section 2.1.7.3) at working concentrations of 0.01, 0.1, 0.1, 10 and 100µg/mL (giving a final DMSO concentration of 1% in each concentration).

4.2.3 Cell Culture Assay of the Synthetic Compounds

To determine the effects of indirubin and 5-bromoisatin, 10,000 granulosa cells were plated per well into three sterile 96-well flat bottom microtitre plates (Interpath) in a final volume of 0.1mL per well of complete DMEM/F12 + 10% FBS cell culture medium (section 2.1.7.3). A standard curve plate was also seeded by 1:2 serial dilutions of 0-40,000 cells per well, in quadruplicate, which was used to determine final cell numbers in test plates. The outside wells of all plates contained 0.1mL of sterile PBS only (section 2.1.3.5) to prevent evaporation of all internal treatment wells. Plates were incubated for 24h in a humidified CO_2 incubator at 37^{o}C to allow cell adherence to the bottom of the wells, and cell differentiation and luteinisation.

After 24h, the supernatant was removed from all treatment wells and discarded. Indirubin and 5-bromoisatin were then added at concentrations of 0.01-100µg/mL in triplicate wells to three treatment plates. Controls consisted of 1% DMSO,

DMEM/F12 + 10% FBS cell culture medium only (negative control; section 2.1.7.3) and DMEM/F12 base cell culture medium only without serum (positive control; section 2.1.7.1) all in triplicate wells. The treatment plates were then incubated for 24, 48 and 72h respectively at 37^{o}C and 5% CO_2.

At the end of the designated incubation periods for each of the test plates, the supernatant was removed from all treatment wells and stored at -20^{o}C for the determination of progesterone and estradiol by the radioimmunoassay (section 2.2.5.2). The cells in the plates were rinsed with sterile 1x PBS and then the test plates were subjected to the crystal violet assay as previously described (section 2.2.2.1). Cell viability in the standard curve plate was determined by the crystal violet assay (section 2.2.2.1) The absorbance in the wells was then measured at 570nm, with background absorbance 630nm, using an automatic spectrophotometer with KC Junior software. The final cell number in test plates was calculated from the standard curve plate. The experiment was repeated on fourteen different occasions (n = 7 fertile women and n = 7 infertile women).

4.2.4 Statistical Analysis

Two-way analyses of variance (ANOVA) using the sensitive contrast K Matrix test were conducted to examine the effects of the synthetic compounds on cell viability and both progesterone and estradiol synthesis, relative to the 1% DMSO control, using SPSS software package version 17 (Pallant, 2002). The alpha value was adjusted when necessary to significant if $p < 0.01$ where homogeneity of variance was violated as explained in Appendix VIII.

4.3 Results

4.3.1 Controls

In general, in the absence of any treatment (media only control) there was a slight trend towards proliferation of the granulosa cells derived from the infertile group of women, in comparison to the cells from the fertile group (Table 4.1). This would suggest that the cells from the infertile group were still proliferating and had not yet luteinised. But the results clearly show that the granulosa cells from the infertile group produced equal amounts of hormones, if not more after 48 and 72h when compared to granulosa cells from the fertile group (Table 4.1).

The 1% DMSO control had no effect on primary-derived human granulosa cell viability, in cells derived from either the fertile or infertile groups of women (Table 4.1). In the absence of serum, there was a significant decrease in viability of primary-derived human granulosa cells derived from both the infertile and fertile groups of women in comparison to the media and 1% DMSO control (Table 4.1 and Figures 4.1-2A & D).

Progesterone (≥ 48h incubation) and estradiol (≥ 24h incubation) synthesis were both inhibited in granulosa cells derived from the physiologically fertile group of women in the presence of the 1% DMSO control compared to the media control (Table 4.1 and Figures 4.1-2A). Progesterone synthesis was also inhibited by 1% DMSO at 24h in granulosa cells derived from the physiologically infertile group of women ($p < 0.01$; Figures 4.1-2D). However, after 48h there was an increase in progesterone produced by granulosa cells derived from the infertile group in the presence of 1% DMSO (Table 4.1).

In the absence of serum there was a decrease in progesterone synthesis by primary-derived human granulosa cells derived from women in both the fertile and infertile groups (Table 4.1). In a similar manner estradiol synthesis by granulosa cells derived from the fertile group of women was inhibited in the absence of serum (Table 4.1). Estradiol synthesis by granulosa cells derived from the infertile group of women was also inhibited in the absence of serum, however, after 48 and 72h estradiol synthesis gradually increased (Table 4.1).

Table 4.1: Primary-derived granulosa cell viability and hormone results
Cell viability and hormone synthesis in primary-derived human granulosa cells from women with normal or abnormal reproductive physiology (based on the infertility factor of the women; sections 2.2.1.1 and 4.2.1 and Appendix II and classified as either fertile or infertile), treated with 1% DMSO (-ve control) and no serum (+ve control) relative to media control. Cell number is shown as mean ± 1 SEM for final cell number per well, progesterone as ng/mL and estradiol as pg/mL.

	Fertile			Infertile		
	Time (h)					
	24	48	72	24	48	72
Media control						
Cell number	10,522 (± 2159)	9,593 (± 1868)	8,960 (± 1996)	11,207 (± 2936)	12,339 (± 3820)	10,609 (± 4369)
Progesterone	1190 (± 654)	1221 (± 782)	1408 (± 972)	804 (± 1014)	3,516 (± 3019)	1,776 (± 2280)
Estradiol	576 (± 335)	672 (± 479)	687 (± 628)	554 (± 298)	817 (± 550)	575 (± 413)
1% DMSO control						
Cell number	10,322 (± 2519)	8,950 (± 2064)	8,750 (± 2877)	10,534 (± 2217)	11,383 (± 3954)	8,500 (± 5663)
Progesterone	1053 (± 609)	560 (± 331)	510 (± 423)	363 (± 534)	1,593 (± 1385)	1,699 (± 2436)
Estradiol	339 (± 132)	296 (± 265)	273 (± 174)	725 (± 496)	549 (± 416)	513 (± 349)
No serum control						
Cell number	3,734 (± 1622)	2,601 (± 1583)	1,137 (± 2659)	6,254 (± 3642)	2,761 (± 6736)	3,856 (± 4566)
Progesterone	410 (± 196)	168 (± 106)	75 (± 51)	396 (± 257)	655 (± 631)	521 (± 601)
Estradiol	220 (± 215)	209 (± 298)	114 (± 171)	154 (± 119)	266 (± 177)	333 (± 393)

4.3.2 Indirubin

Indirubin had no effect on primary-derived granulosa cell viability derived from the fertile (Figure 4.1A) or infertile (Figure 4.1D) groups of women, at all treatment periods (Table 4.2). After 48 and 72h, however progesterone synthesis by granulosa cells from the normal group of women slightly increased (48h at 0.01 and 100µg/mL; 72h at 10µg/ml; Figure 4.1B; Table 4.2) in the presence of indirubin. Conversely, indirubin (0.01, 0.1, 1 and 10µg/mL) inhibited progesterone synthesis by granulosa cells from the infertile group of women after 72h treatment (Figure 4.1E; Table 4.2).

In a similar manner to the Murex remedy, estradiol synthesis was only inhibited by granulosa cells derived from the infertile group, in the presence of indirubin (at all concentrations; Figure 4.1F; Table 4.2) after 24h.

4.3.3 5-Bromoisatin

5-bromoisatin (LC_{50} between 10-100µg/mL) was cytotoxic to granulosa cells sourced from the physiologically fertile (Figure 4.2A; Table 4.2) and infertile (Figure 4.2D; Table 4.2) groups at 24, 48 and 72h. At the lower concentrations of 5-bromoisatin (0.1 and 1µg/mL) after 48h, progesterone synthesis by granulosa cells from the fertile group increased (Figure 4.2B; Table 4.2), whereas the highest concentration of 5-bromoisatin (100µg/mL) inhibited progesterone synthesis at 24 and 48h. 5-bromoisatin (0.01, 1, 10 & 100µg/mL) also inhibited progesterone synthesis in granulosa cells derived from the infertile group of women after 72h (Figure 4.2E; Table 4.2).

In a similar manner to indirubin, estradiol synthesis was only inhibited in the presence of 5-bromoisatin by granulosa cells from the infertile group (Figure 4.2F; Table 4.2). After 24h treatment with 5-bromoisatin estradiol synthesis was inhibited at all concentrations tested at 72h treatment (Figure 4.2F; Table 4.2).

Table 4.2: The effects of indirubin and 5-bromosiatin on female human primary-derived granulosa cells

Effects of indirubin and 5-bromoisatin on primary-derived human granulosa cell viability, progesterone and estradiol synthesis. Granulosa cells were derived from women undergoing IVF and ICSI treatments for infertility and classified as fertile or infertile (based on the infertility factor of the women sections 2.2.1.1 and 4.2.1 and Appendix II). Cell viability is represented as LC_{50} and hormone data shown as concentration (µg/m) that was significantly different to the 1% DMSO control. (+) stimulated; (-) inhibited; n/e = no significant effect.

	Fertile n = 7			**Infertile n =7**		
	Time (h)					
	24	48	72	24	48	72
Indirubin Concentrations tested 0.01, 0.1, 1, 10, 100 µg/mL						
Cell viability	> 100	> 100	> 100	> 100	> 100	> 100
Progesterone	n/e	0.01 & 100 (+)	10 (+)	n/e	n/e	≤10 (-)
Estradiol	n/e	n/e	n/e	≤100 (-)	n/e	n/e
5-bromoisatin Concentrations tested 0.01, 0.1, 1, 10, 100 µg/mL						
Cell viability	16	11	13	44	25	16
Progesterone	100 (-)	0.1,1 (+) 100 (-)	n/e	n/e	n/e	0.01,1, 10 & 100 (-)
Estradiol	n/e	n/e	n/e	≤100 (-)	n/e	100 (-)

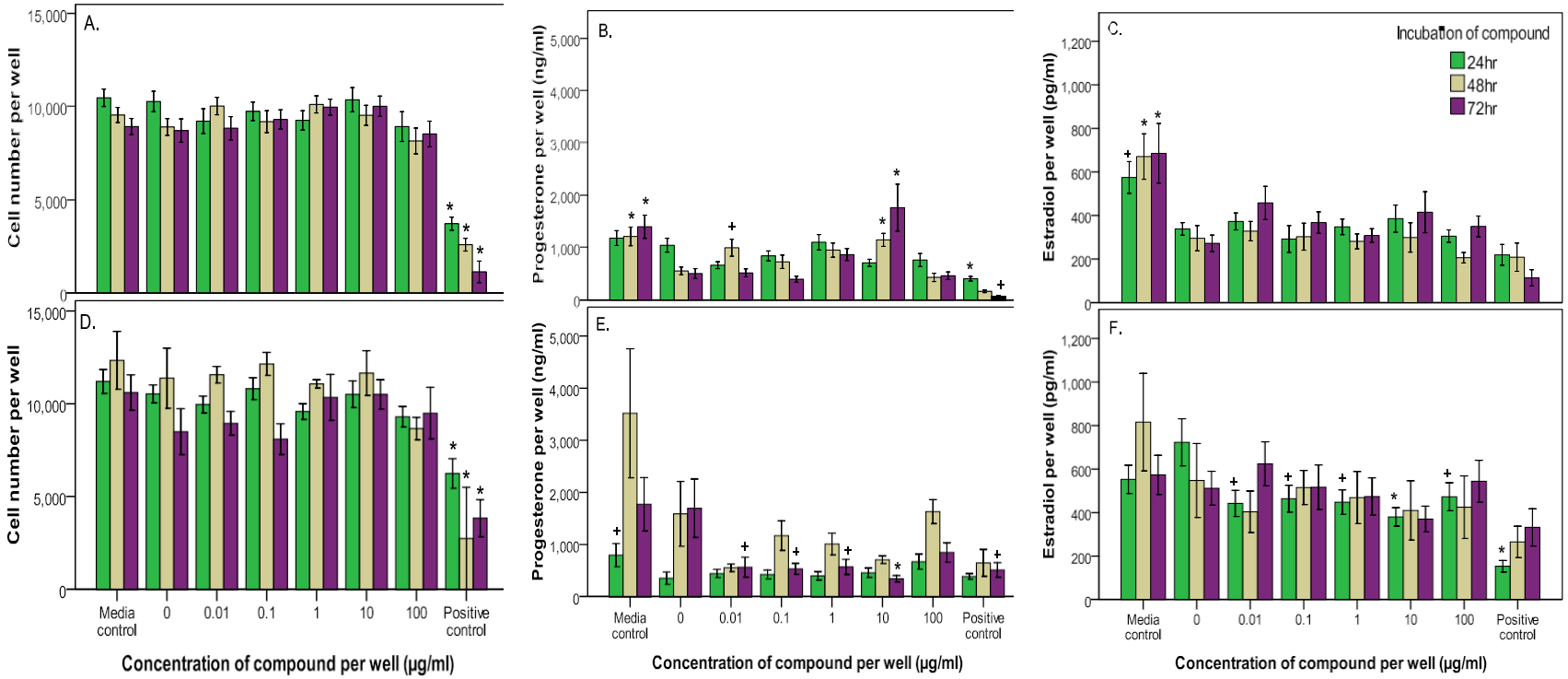


Figure 4.1: The effect of indirubin on primary-derived human granulosa cells

The effect of indirubin on primary-derived granulosa cell viability, progesterone synthesis and estradiol synthesis in cells from women with normal/fertile (A, B, C) and abnormal/infertile (D, E, F) reproductive physiology. After an initial 24h cell attachment period, granulosa cells (10,000 cells/well) were treated with indirubin (0-100µg/mL) for 24, 48 and 72h. Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis by radioimmunoassay. The results are the mean for seven separate repeat assays for both fertile and infertile groups of women (n = 7; ± 1 SEM). The positive control represents cells treated with base DMEM/F12 media only in the absence of serum. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of indirubin on cell number per well, and hormone synthesis, against the 1% DMSO control (shown as 0 concentration) at different incubation periods. Significant difference between the 1% DMSO control and each treatment concentration at each incubation period shown $p < 0.01$ (+) and $p < 0.001$ (*)

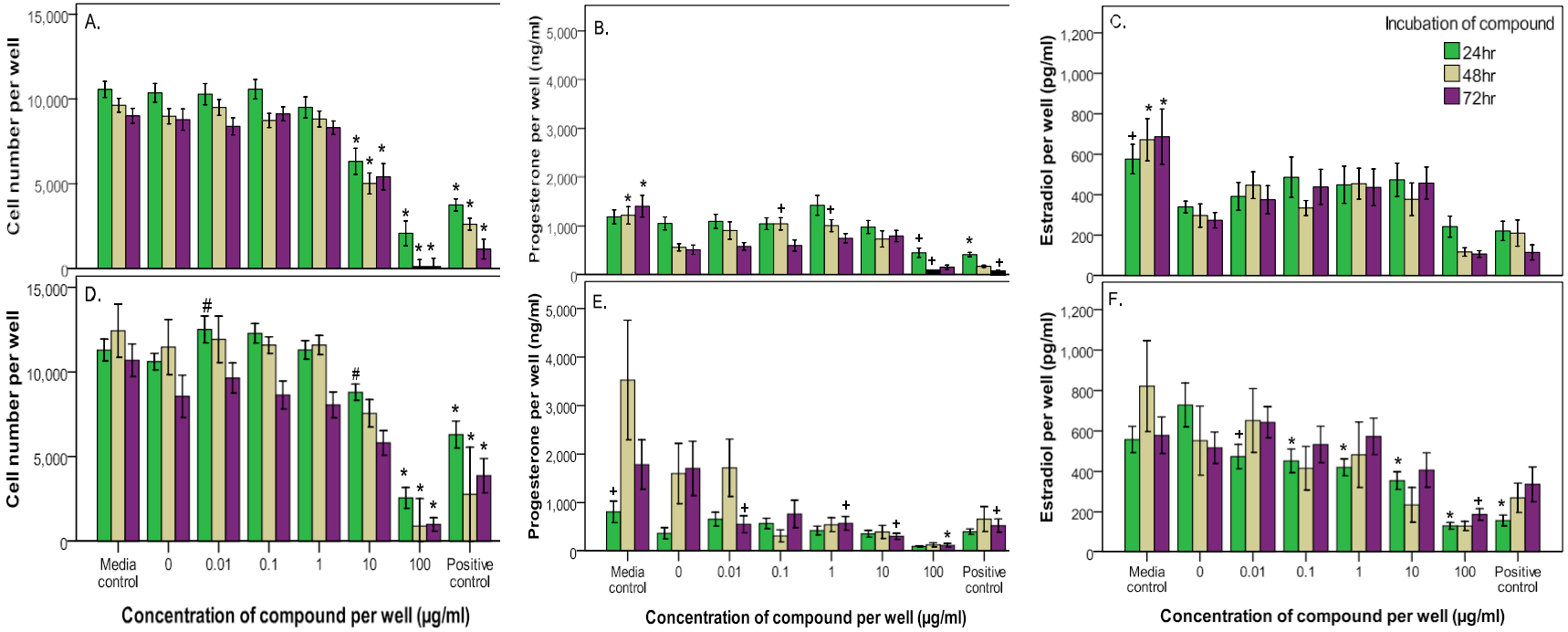


Figure 4.2: The effect of 5-bromoisatin on primary-derived human granulosa cells

The effect of 5-bromoisatin on primary-derived granulosa cell viability, progesterone and estradiol synthesis in cells from women with normal/fertile (A, B, C) and abnormal/infertile (D, E, F) reproductive physiology. After an initial 24h cell attachment period, granulosa cells (10,000 cells/well) were treated with 5-bromoisatin (0-100µg/mL) for 24, 48 and 72h. Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis by radioimmunoassay. The results are the mean for seven separate repeat assays for both fertile and infertile groups of women (n = 7; ± 1 SEM). The positive control represents cells treated with base DMEM/F12 media only in the absence of serum. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of 5-bromoisatin on cell number per well, and hormone synthesis, against the 1% DMSO control (shown as 0 concentration) at different incubation periods. Significant difference between the 1% DMSO control and each treatment concentration at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$

4.4 Discussion

Synthetic indole derivatives including 6'6-dibromoindirubin, indirubin and 5-bromoisatin have been recognised as potent antitumor agents due to their ability to arrest cell growth and induce apoptosis (Hoessel, *et al.*, 1999; Kim, *et al.*, 2007; Vine, *et al.*, 2007b), however this is the first study known to examine any beneficial effects of these compounds on human reproductive cells. 5-bromoisatin significantly reduced cell numbers at concentrations greater than 10µg/mL. Both indirubin and 5-bromoisatin stimulated progesterone synthesis at low concentrations (0.01µg/mL) after 48h in granulosa cells derived from women with normal reproductive physiology. Indirubin and 5-bromoisatin (100µg/mL) did, however inhibit estradiol synthesis by granulosa cells derived from women with abnormal reproductive physiology after 24h exposure.

The importance of high progesterone levels in regulating ovarian functions, normal ovulation and fertility has been well documented (Robker and Richards, 1998; Peluso, *et al.*, 2009; Zhang, *et al.*, 2009). Insufficient serum progesterone and estradiol *in vivo* has been associated with a number of gynaecological disorders, which are often linked to infertility (Erickson, *et al.*, 1992; Mason, *et al.*, 1994; McAllister, 1995; Harlow, *et al.*, 1996; Amer, 2006; Wu, *et al.*, 2010). For this research primary granulosa cells were sourced from women undergoing ART due to a number of different infertility factors. These cells where then sub-divided into two groups based on the donors infertility factor and classified as either fertile or infertile. Interestingly, the untreated granulosa cells from the infertile group in our study produced comparable quantities of both basal progesterone and estradiol levels, if not more than the granulosa cells from the fertile group (Table 4.1; Figures 4.1, 4.2 & 4.3). Cells from the infertile group in this study were sourced from a mixed cohort of women with PCOS, endometriosis, ovarian cysts

or unexplained infertility and the results combined. Combining the results may have obscured the true results for each group.

Research shows while sufferers of PCOS have normal FSH serum levels, they also have abnormally high levels of androgens, LH and $P450_{arom}$ expression and synthesize varying amounts of progesterone and estradiol (Amer, 2006). When the cohort of results from the infertile women were analysed separately, the granulosa cells from PCOS women produced substantially more progesterone and estradiol than the other three infertility groups (Appendix VI). In fact, surprisingly, granulosa cells from the PCOS group produced more progesterone and estradiol than cells from fertile women. This high synthesis of estradiol could be related to the high levels of plasma androgens, which are the precursors for estradiol, that PCOS sufferers are known to have (Erickson, *et al.*, 1992; Amer, 2006), however the high levels of progesterone were not expected in the PCOS group.

The granulosa cells derived from women who suffer from endometriosis produced minimal basal progesterone and estradiol (Appendix VI), as expected from previous research (Harlow, *et al.*, 1996). Endometriosis has been linked to a long follicular phase, reduced expression of $P450_{arom}$ and LH, and low serum progesterone and estradiol levels (Harlow, *et al.*, 1996). When the infertile group were separated into individual infertility factors (Appendix VI) it became apparent that only the PCOS group had luteinized and was able to produce both basal progesterone and estradiol. Therefore, future research would benefit from testing these compounds on specific infertility groups (Appendix VI) rather than combining the data together, as the results

on hormone synthesis obtain herein may not be a true account of how these compounds would accurately effect hormone synthesis in each individual cohort of infertile women.

The inhibitory effect of 1% DMSO on progesterone and estradiol synthesis in the absence of treatment indicates that an alternative solvent system or decreased concentration of DMSO should be investigated. Otherwise any effect of the testing compounds on the cells maybe due to a synergistic interaction between the DMSO and the testing compounds. In fact, there was a slight stimulatory effect on both cell numbers and progesterone from the infertile group in the presence of indirubin (0.01, 0.1 and 10µg/mL) after 48h. This effect may have been attributed to the compounds minimising the toxic effect of the DMSO, rather than stimulation of progesterone by the compounds themselves. Previous research has shown that DMSO at 0.1% has no effect on progesterone or estradiol synthesis by human luteinized granulosa cells (Moran, *et al*., 2003). Further studies have also found that DMSO at 0.2% has no effect on porcine granulosa cells (Tiemann, *et al*., 2007). However, other research has shown that DMSO is estrogenic and stimulates estrogen synthesis. Studies in salmon hepatocytes demonstrated that DSMO at a concentration as low as 0.1% induces both estrogen receptors, ERα and ERβ mRNA expression in a dose-dependent manner over 48h (Mortensen and Arukwe, 2006). Therefore, in future studies it would worthwhile trying the DMSO solvent at a lower concentration to prevent inhibition of both progesterone and estradiol.

Both indirubin and 5-bromoisatin stimulated progesterone synthesis at low concentrations by granulosa cells from the fertile group but not in cells from the infertile group. As serum progesterone is essential for conception and throughout

pregnancy (van Zonneveld, *et al.*, 1994; Speroff and Fritz, 2005), the negative effect these compounds have on progesterone synthesis in cells from infertile women suggests that these synthetic indole and isatin compounds could be detrimental to women with a background of infertility problems who were trying to conceive or during pregnancy; but they may have beneficial effects or no effects on normal or fertile women. Estradiol synthesis was also inhibited by the synthetic compounds, but only by granulosa cells from the infertile group of women. The inhibition of both progesterone and estradiol in the cells from the infertile group and not the fertile group implies that granulosa cells from the infertile group may have defective receptor responses which prevents them responding to these compounds. In fact if we could define how these groups of cells respond differently it may shed some light on the mode of action of these compounds. Future assays to investigate the mode of action of these synthetic compounds on receptor binding in granulosa cells from specific infertility cohorts, would be beneficial.

The synthetic compound 5-bromoisatin inhibited cell viability in the JAr reproductive cancer cell line (Chapter 3) after 24h treatment at low concentrations (0.1 and 1µg/mL), whereas these same conditions did not affect the primary-derived human granulosa cells. Further still, 5-bromisatin had no inhibitory effects on hormone synthesis by granulosa cells from women with normal reproductive physiology at these low concentrations; in fact an increase in progesterone synthesis was noted after 48h. Low serum progesterone levels are known to be associated with infertility, failed embryo implantation into the uterus and recurrent miscarriages (Banerjee, *et al*., 2001; Check, 2008; Kamel, 2010). Research has demonstrated that progesterone therapy prevents miscarriage in women who are prone to having recurrent miscarriages by supporting the development of the embryo (Check, 2008; Daya, 2009; Qureshi, 2009). In fact,

progesterone supplementation is common practise in pregnant women whose serum progesterone level is < 30ng/mL during pregnancy (Check, 2008). However the US Food and Drug Administration (FDA) have warned that synthetic progesterone or natural exogenous progesterone maybe associated with heart abnormalities and neural tube defects (Andrews, 1979). Consequently, a therapy which maintains a woman's own endogenous progesterone serum levels of ≥ 30nmol/L during pregnancy would be considered beneficial for these women, rather than having to supplement with synthetic or even natural exogenous progesterone. However, progesterone synthesis by granulosa cells from the infertile group in the presence of 5-bromoisatin was inhibited even at low sub-lethal concentrations. Therefore, it must be concluded from these results that 5-bromoisatin does not confer any beneficial effects on progesterone synthesis *in vitro*.

Alternatively, the inhibitory effects that indirubin and 5-bromoisatin have on estradiol synthesis by granulosa cells from infertile women suggests that these extracts may be beneficial as a natural product for hormone dependent cancers, such as estradiol sensitive breast cancer. High endogenous estrogen has been associated with hormone-dependent cancers including breast, endometrial and prostate cancers (Dı´az-Cruz, *et al*., 2005). As breast cancer is the most common cancer in women, only secondary to lung cancer (Dı´az-Cruz, *et al*., 2005), a therapy which regulates endogenous estrogen levels could be beneficial for the prevention of hormone dependent breast cancers. The structurally similar indole compound isolated from cruciferous vegetables, indole-3-carbinol (I3C) and its biologically active metabolite diindoylmethane (DIM), have been shown to interfere with the estrogen receptor, ERα resulting in the inhibition of estrogen signalling (Auborn, *et al.*, 2003). Other studies have shown that, while I3C blocks

ovulation in rats resulting in decreased plasma progesterone levels, plasma estradiol concentrations are not affected (Gao, *et al.*, 2002).

Based on our preliminary data of cell cytotoxicity and hormone synthesis the mode of action of these synthetic compounds could not be determine, however we could speculate that maybe the synthetic compounds act by binding the estrogen receptor or at least, compete for binding of the receptor in granulosa cells from the infertile group and thereby inhibit estradiol biosynthesis. However, as both estradiol and progesterone synthesis by granulosa cells from the fertile group was not inhibited under the same conditions, it would be more probable to argue that the granulosa cells from the infertile group just have a impaired ability to synthesize hormones in culture in the presence of the synthetic compounds, compared to the granulosa cells from the fertile group. The cells from the infertile group used in this study were sourced from a cohort of women with disorders which are known to cause impaired follicular and luteal cycles, along with reduced LH and FSH surges and often reduced expression of the $P450_{arom}$ enzyme, thus, resulting in a reduced secretion of both estradiol and progesterone both *in vitro* and *in vivo* (Mason, *et al.*, 1994; McAllister, 1995; Harlow, *et al.*, 1996). Possibly, when the cells from the infertile group were challenged with the toxic effects of the synthetic compounds, hormone synthesis was down-regulated, while the cells were trying to metabolise the compounds.

In conclusion, from the findings herein, neither 5-bromoisatin nor indirubin displayed any beneficial effects for primary-derived human granulosa cells. Progesterone synthesis was up-regulated in the granulosa cells from the fertile group at low sub-lethal concentrations but these compounds either had no effect, or inhibited progesterone

synthesis in the granulosa cells from the infertile group. These data suggest that if these results are at all indicative of an *in vivo* response, these compounds could be detrimental to many infertile women trying to conceive or throughout pregnancy. The inhibitory effect of these compounds on estradiol synthesis showed potential benefits for the treatment of estrogen dependent cancers (as demonstrated in Chapter 3 on JAr cells), however this effect again was only displayed in one group, the infertile group. There is no substantial evidence from our findings that the synthetic indole derivatives are beneficial for female reproductive cells, however these results do not negate any potential effects of the structurally similar muricid compounds.

Chapter 5 The Cytotoxic Effects of Bioactive Compounds from *D. orbita* on Primary-derived Human Reproductive Cells and Human Reproductive Cancer Cell lines

5.1 Introduction

The chemical compounds from the tissues of the marine predatory whelk, *Dicathais orbita* (Chapter 1) have been extensively investigated (Friedländer, 1909; Baker, 1974; Benkendorff, *et al*., 2000; Benkendorff, *et al*., 2001; Cooksey, 2001; Westley and Benkendorff, 2008). These compounds are brominated indoles and isatins and include the historical purple dye, Tyrian purple (6'6-dibromoindigo) (Friedländer, 1909; Baker, 1974). Tyrian purple is also the source of 'Murex purpurea', (Dunham, 1864; Cazalet, 2008) the natural homeopathy remedy sold for the treatment of a range of gynaecological disorders including uterine cancer (Chapters 3 & 4).

Tyrian purple itself does not show any biological activity (Westley, *et al*., 2006) however, several of its precursor metabolites including tyrindoleninone and 6-bromoistain have demonstrated therapeutic effects both *in vitro* and *in vivo* (Vine, *et al.*, 2007a; Westley, *et al.*, 2010; Benkendorff, *et al.*, 2011). A mixture of these metabolites have been shown to induce apoptosis in Jurkat cells *in vitro* (Benkendorff, *et al*., 2011), and in a colorectal cancer mouse model, *in vivo* (Westley, *et al*., 2010). Tyrindoleninone in particular is cytotoxic against the human lymphoma cell lines, U937 and Jurkat (LC_{50} = 3.9µM; 1µg/mL) in comparison to the untransformed, human, mononuclear cells (MNC) (LC_{50} = 195µM; 50µg/mL) *in vitro* (Vine, *et al*., 2007a). A range of isatin compounds also demonstrate anticancer properties against the U937 cell line including 5- (LC_{50} = 65µM; 15µg/mL) and 6-bromoisatin (LC_{50} = 75µM; 20µg/mL) (Vine, *et al*., 2007a). Analogues of 5 and 7-bromoisatin not only demonstrate anticancer properties against several cancer lines,

including the breast MCF-7 (LC_{50} = 13µM; 5µg/mL) but have been shown to inhibit micro-tubular formation *in vitro* (Matesic, *et al*., 2008). Furthermore, substitution of 5'7-dibromoisatin compounds with the addition of electron withdrawing groups increases the potency of these compounds against lymphoma cell lines (Vine, *et al*., 2007b). In particular, 5'7-dibromo-N-(p-methylbenzyl)isatin is cytotoxic to both U937 and Jurkat cell lines at sub-molecular concentrations (LC_{50} = 0.49µM; 0.2µg/mL; Vine, *et al*., 2007b).

Despite the in depth investigation into the bioactive compounds from *D. orbita* both *in vitro* and *in vivo*, to date there still remains a lack of knowledge on the cytotoxic effects of these compounds on female human reproductive cells. Furthermore, there is no data on the effects of these bioactive compounds on female hormone synthesis. Therefore, the aims of this study were to:

1. Isolate, purify and identify the bioactive compounds from the hypobranchial glands of the marine mollusc *D. orbita*.
2. Investigate the cytotoxic effects of the semi-purified fractions containing the bioactive compounds on primary-derived human granulosa cells and compare with the female reproductive cancer cell lines, KGN (equivalent cancer granulosa cell line), JAr and OVCAR-3.
3. To examine the effects of the semi-purified fractions on basal progesterone and estradiol synthesis and human choronic gonadotrophin (hCG) and dibutyryl adenosine 3',5'-cyclic monophosphate (db-cAMP) stimulated progesterone and estradiol synthesis by the reproductive cells.

Since several of the *D. orbita* compounds have been shown to induce cytotoxicity in a range of cancer cell lines *in vitro* (Benkendorff, *et al.*, 2011), and in light of the results obtained from the synthetic 5-bromoisatin compound on the human reproductive cancer cell line, JAr (Chapter 3), the hypothesis in the present study was that the semi-purified bioactive compounds from *D. orbita* would inhibit proliferation of human reproductive cancer cell lines KGN, JAr and OVCAR-3 without affecting primary-derived human granulosa cells. Given that the synthetic isatin and indirubin compounds stimulated progesterone synthesis by the JAr cell line and primary-derived human granulosa cells (Chapter 3 & 4) at low concentrations we further hypothesized that these naturally derived brominated compounds would affect steroidogenesis by stimulating progesterone and estradiol synthesis at sub-lethal concentrations.

5.2 Methods

5.2.1 Extraction, Isolation & Semi-purification of Bioactive compounds from *D. orbita*

5.2.1.1 Dissection & Isolation of Bioactive Compounds from D. orbita *Specimens*

The hypobranchial glands were dissected from 300 frozen specimens of *Dicathais orbita* ranging in size from 1-5cm, collected in September 2008, from an abalone farm in Pt Lincoln, South Australia, as described previously (section 2.2.4.1.1). The compounds of interest were isolated from the gland tissue by soaking in chloroform and methanol (v/v) over a 48h period (section 2.2.4.1.1). The organic chloroform layer was separated from the aqueous layer and filtered under water pressure and then through glass wool, (to remove tissue residue) and concentrated on a rotary evaporator under reduced pressure (section 2.2.4.1.1). The resulting brown/orange oil

crude product produced a yield of 5.072g and was stored in amber vials at -20^{o}C under N_2 in five aliquots of 1g each.

5.2.1.2 Concentration of Bioactive Compounds from the Crude Extract by Flash Silica Chromatography

To facilitate the separation of the bioactive compounds tyrindoleninone, tyrindolinone and 6-bromoisatin, the crude samples extracted from the hypobranchial glands (section 5.2.1.1) were semi-purified using flash silica chromatography under N_2 pressure using a solvent system with increasing polarity of DCM, 5% methanol in DCM and 10% methanol in DCM (section 2.2.4.2). The collected fractions were dried on a rotary evaporator under reduced pressure and stored under N_2 at -20^{o}C in amber vials.

From the crude sample extracted from the hypobranchial glands, five separate silica columns were run and three main fractions were collected from each column by visual identification of colour. Fraction one and two were eluted with 100% DCM, and Fraction three was eluted with 5% methanol. These three fractions were pooled from each replicate column (Fraction one, 0.598g, Fraction two, 0.256g and Fraction three, 0.823g), and stored in amber vials at -20^{o}C under N_2 in six aliquots of each fraction. These three fractions were used for the MTT cell viability assays and hormone assays.

Thin layer chromatography with 100% dichloromethane (DCM) was performed on aluminium silica gel 60F254 plates (Merck) and R_f values recorded (section 2.2.4.4.1). Chemical analysis was also conducted using liquid chromatography coupled to a mass spectrometer (LC/MS) to identify the bioactive compounds in the crude and semi-purified samples (section 2.2.4.4.2).

5.2.1.3 Compound Preparation for Cell Culture Assays

For the cell viability assays, an aliquot of each fraction was thawed, weighed and prepared fresh on the day of the experiment. To facilitate the solubility of the organic compounds into the aqueous cell culture medium, each fraction was dissolved in 100% dimethyl sulfoxide (DMSO) at 100x the final concentration and serially diluted in DMSO (100%) to give six concentrations of 0.5, 1, 5, 10, 50 and 100mg/mL. For the cell culture experiments each sample was then diluted in complete cell culture medium at working concentrations ranging from 0.005-1mg/mL. All samples were sterilised using a Minisart 0.22µm (Sartorius) filter and sterile syringe (2.2.5.4) prior to cell exposure. The final experimental wells contained a working concentration of 1% DMSO.

5.2.2 Cytotoxic Effects of *D. orbita* Compounds on Reproductive Cells

5.2.2.1 Cell Culture

5.2.2.1.1 Primary Cell Isolation

Primary-derived human granulosa cells were obtained from women who were undergoing assisted reproductive technology (ART) *in vitro* fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI) at Flinders Reproductive Medicine, in the Flinders Medical Centre. These women had given their informed consent and the procedure was approved by the Flinders Clinical Research Ethics Committee (number 260/067; Appendix II). For the following experiment, aspirates containing granulosa cells were donated from three separate women (cases 22, 23 and 24; Appendix II) who were considered fertile, under the criteria previously specified (section 2.2.1.1 and Appendix II).

Primary granulosa cells were isolated from the follicular aspirates by density gradient (section 2.2.1.1) and re-suspended in DMEM/F12 + 10% FBS complete cell medium

(section 2.1.7.3). Viable cell number was determined by the trypan blue exclusion assay (section 2.2.2.3).

5.2.2.1.2 Reproductive Cancer Cell Lines

The KGN granulosa cell line (Nishi, *et al*., 2001) was kindly donated by Theresa Hickey, Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, and maintained in DMEM/F12 + 10% FBS complete cell medium (section 2.1.7.3). The JAr (Pattillo, *et al*., 1971) and OVCAR-3 cell lines (Hamilton, *et al*., 1983) were obtained from the Global Bioresource Centre™ American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium supplemented with 10 and 20% FBS respectively (section 2.1.22.4). All cell lines were maintained in 75cm^2 sterile tissue culture flasks (NUNC, Thermo Fisher Scientific) at 37^{o}C in a humidified atmosphere with 5% CO_2 and sub-cultured every 2-3 days as required.

5.2.2.1.3 Determination of Cell Number Prior to all Experiments

The adherent cell lines were detached from 75cm^2 flasks in their exponential growth phase at 80% confluence using sterile Trypsin-EDTA, pooled together and centrifuged at 75g (1500rpm). The cell pellet was re-suspended in complete cell culture medium (section 2.1.3.1.3 and 2.1.3.6.4) and viable cells were counted on a haemocytometer with trypan blue (2.2.2.3).

5.2.2.2 Cell Viability Assay to Test the Semi-purified D. orbita *Fractions*

Primary-derived human granulosa cells (10,000 cells per well), KGN, JAr and OVCAR-3 cell lines (20,000 cells per well) were seeded into sterile 96-well flat bottom plates (Interpath) in a final volume of 0.1mL per well of complete cell culture medium (section 2.1.3.1.3 and 2.1.3.6.4) and incubated at 37^{o}C + 5% CO_2 for 2h for JAr cells (Chapter 3) or 24h for primary granulosa cells (Young, *et al.*, 2005a), KGN cells (Matsuda, *et al*., 2008) and OVCAR-3 cells (Smith, *et al*., 2005), to allow cell

adherence to plates. All outside wells of plates contained 0.1mL of sterile PBS (section 2.1.3.5) to prevent evaporation in the inside treatment wells. Standard curve plates of 0-40,000 cells per well (primary granulosa cells) and 0-80,000 cells per well (KGN, JAr and OVCAR-3 cell lines) in doubling concentrations in six replicate wells were setup for each cell type and run in conjunction with each experiment (used to determine final cell number in the test plates).

After the initial cell adherence period, the spent medium was removed from all treatment wells and cells were exposed to 0.005, 0.01, 0.05, 0.1, 0.5 and 1mg/mL of the three semi-purified *D. orbita* fractions (section 5.2.1.2) prepared in cell culture medium (section 2.1.3.1.3 and 2.1.3.6.4) ± 1,000mIU/mL of hCG (for granulosa, JAr and OVCAR-3; section 2.1.6.2) or 1mM db-cAMP (for KGN; section 2.1.6.1), for 1, 4, 24, 48 or 72h in three replicate wells each at 37^{o}C + 5% CO_2. Controls consisted of a medium only negative control, 1% DMSO solvent control, base media without serum as a positive control (for primary granulosa, KGN and OVCAR-3 cells) or hydrogen peroxide (10,000µg/mL; section 2.1.7.1) as a positive control (for JAr cells). Additional controls of 1,000mIU/mL hCG (section 2.1.6.2) in 1% DMSO (for granulosa, JAr and OVCAR-3) or 1mM db-cAMP (section 2.1.6.1) in 1% DMSO (for KGN) were added as relevant.

At the end of each treatment period the supernatant was removed from all treatment wells and stored (-20^{o}C) for the measurement of both progesterone and estradiol by radioimmunoassay as described previously (section 2.2.5.2). The cells in the treatment wells were then rinsed with sterile 1x PBS (section 2.1.3.5). Cell viability was determined by the 1h MTT assay for JAr cells (section 2.2.2.2) and 2h MTT

assay (0.5mg/mL) for OVCAR-3 cells (Smith, *et al*., 2005), and 18h MTT assay (0.5mg/mL) for primary granulosa cells and KGN cells (Young, *et al.*, 2005b). The assay was repeated on three separate occasions and the results are the mean ± 1 SEM.

5.2.2.2.1 Microscopic Observation

Changes in cell morphology after exposure to the three semi-purified fractions were also visualised at the end of each incubation period and phase contrast images taken using Olympus IX71 Inverted Phase Contrast Microscope at 20x magnification.

5.2.2.3 Statistical Analysis

Two-way analysis of variance (ANOVA) using the sensitive contrast K Matrix analysis tests were conducted to examine the effects of the semi-purified *D. orbita* fractions on both cell viability and hormone synthesis using SPSS software package version 17 (Pallant, 2002). The alpha value was adjusted when necessary to a significant level of $p < 0.01$ where homogeneity of variance was violated using the Levene's Test of Equality of Error. The concentration that effectively inhibited the enzymes of the mitochondria by 50% (LC_{50}) was calculated using nonlinear regression and sigmoidal curves with GraphPad Prism software version 5 (GraphPad Software Inc).

5.3 Results

5.3.1 Extraction and Isolation of Bioactive compounds from *D. orbita*

5.3.2 Crude Extract for the Hypobranchial Glands

Chemical analysis by thin layer chromatography (TLC) of the crude extract from the hypobranchial glands of the *D. orbita* specimens using 100% dichloromethane (DCM) revealed five distinct coloured compounds in the sample (Table 5.1). A green/purple spot (R_f = 0.35), a light orange/yellow spot (R_f = 0.55), an

orange/yellow spot (R_f = 0.65), an orange spot (R_f = 0.77) and a dark brown/orange spot (R_f =0.95).

Table 5.1: Thin Layer Chromatography of *D. orbita* compounds
Thin layer chromatography analysis of coloured compounds from the crude and three semi-purified extracts from the hypobranchial glands (HG) of *D. orbita* specimens run on aluminium silica gel 60F254 plates (Merck) using dichloromethane (100%) showing retention times (Rf) in cm.

Probable compound	Colour	Rf value (cm)	Crude HG sample	Semi-purified Fractions HG		
				F1	F2	F3
Unidentified	Dark brown/orange	0.95	+			
Tyrindoleninone/Tyrinolinone	Orange	0.77	+	+	+	
Tyrindoleninone/Tyrindolinone	Orange/yellow	0.65	+	+	+	
6-bromoisatin	Light orange/yellow	0.55	+	+		+
Tyriverdin/Tyrian purple	Green/purple	0.35	+			

Further analysis of the crude extract using LC/MS identified a number of brominated precursor compounds consistent with published data (Westley and Benkendorff, 2008) (Figure 5.1). The major peak on the UV diode array chromatogram (Figure 1A) at retention time (t_R) 6.37 minutes corresponded to the molecular mass (MW) of 6-bromoindole-2,3-dione (6-bromoisatin). ESI/MS (Figure 5.1B) of the base peak which shows the duplet ion clusters of bromine (Br^{79} Br^{81}), confirmed the molecular weight of 6-bromoisatin (*m/z* 224, 226). The second largest peak on the diode array chromatogram (Figure 5.1A) t_R 9.45 minutes corresponded to the molecular mass of 6-bromo-2,2-dimethylthioindolin-3-one (tyrindolinone). The ESI/MS of the peak at t_R 9.45 minutes (Figure 5.1C) and the base peak 257 of the duplet ion clusters of bromine (Br^{79} Br^{81}) corresponds to a fragmented structure of tyrindolinone (*m/z* 302, 304) representing the loss of the functional group $–SCH_3$ from the structure and second peak 242 loss of a water molecule from the structure. Smaller peaks at t_R 11.17 and 5.52 minutes confirmed the presence of and 6-bromo-2-

methylthioindoleninone (tyrindoleninone; *mlz* 255, 257) and 6-bromo-2-methylthioindoxyl sulphate (tyrindoxyl sulphate; *mlz* 336, 338) in the sample (Figure 5.1A).

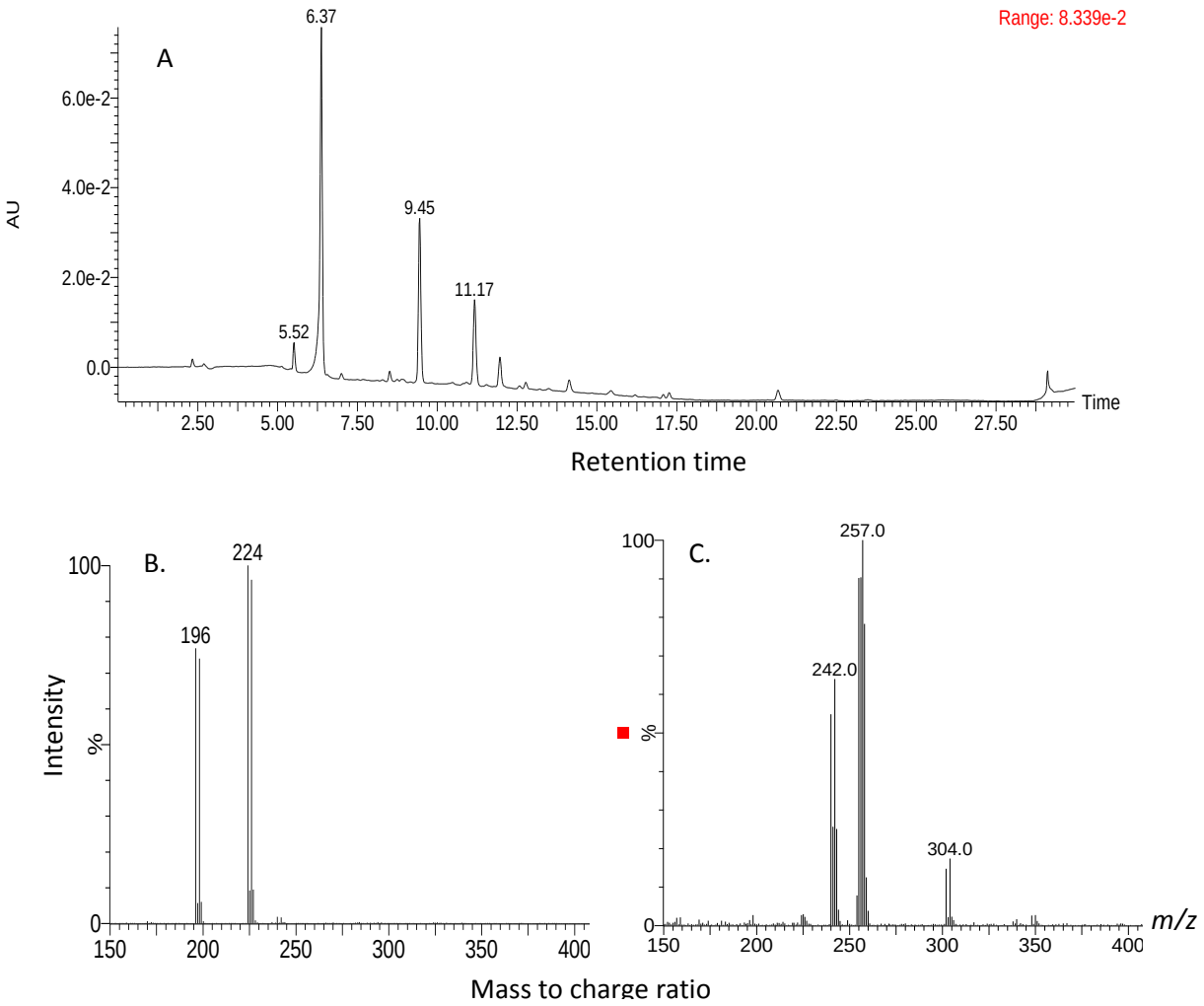


Figure 5.1: LC/MS of crude *D. orbita* hypobranchial gland sample
Liquid chromatography mass spectrometry analysis from a crude sample extracted from the hypobranchial glands of specimens of *D. orbita*. (A) The chromatogram from the Diode array at 300/600nm shows the retention times (t_R) and relative peaks of all compounds in the sample. The major peak at 6.37 minutes corresponds to the molecular mass of 6-bromoisatin (*m/z* 224, 226). The second largest peak at t_R 9.45 minutes corresponds to the molecular mass of tyrindolinone (*m/z* 302, 304). Smaller peaks at t_R 11.17 and 5.52 minutes correspond to the molecular mass of tyrindoleninone (*m/z* 255, 257) and tyrindoxyl sulphate (*m/z* 336, 338) respectively. (B) The electrospray ionisation mass spectrometry (ESI-MS) of the base peak at t_R 6.37 minutes (6-bromoisatin), (C) the ESI-MS of the t_R peak 9.45 minutes showing the base peak 257 of the duplet ion clusters of bromine (Br^{79} Br^{81}) corresponding to a fragmented structure of tyrindolinone (*m/z* 302, 304) with the loss of a $–SCH_3$ group. The second ion clusters at 242 corresponding to the loss of a H_2O group.

5.3.2.1 Semi-purification of the Crude Extract

Concentration of the bioactive compounds from the hypobranchial gland crude sample by flash silica chromatography produced three main fractions. TLC of these three fractions confirmed a number of coloured compounds in each (Table 5.1). Fraction one consisted of four main compounds, a green spot that turned purple with increased light exposure (R_f = 0.35), a light orange/yellow spot (R_f = 0.55), an orange/yellow spot (R_f = 0.65) and a darker orange spot (R_f = 0.77). LC/MS of fraction one confirmed that it consisted of a mixture of four main compounds (Figure 5.2A), the major peak at t_R 5.55 minutes corresponded to the molecular mass of tyrindoxyl sulphate with *m/z* base peak 336, 338 (Br^{79}, Br^{81}). The second largest peak corresponded to the molecular mass of 6-bromoisatin at t_R 6.43 minutes and *m/z* 224, 226. Two other peaks registered as tyrindoleninone t_R 11.30 minutes and *m/z* 255, 257 and tyrindolinone t_R 9.57 minutes and *m/z* 302, 304 (Figure 5.2A).

TLC of fraction two produced two main coloured spots (Table 5.1). An orange/yellow spot at (R_f = 0.65) and a darker orange spot (R_f = 0.77). Further chemical analysis by LC/MS confirmed the second fraction (Figure 5.2B) consisted of two main compounds. The major peak corresponded to the molecular mass of tyrindoleninone t_R 11.19 minutes; ESI/MS *m/z* 255, 257 (Figure 5.2B insert) and the smaller peak tyrindolinone t_R 9.47 minutes (Figure 5.2B) with *m/z* 302, 304. TLC of the third fraction produced one main coloured component, a light orange/yellow spot (R_f = 0.55; Table 5.1). LC/MS of the third fraction revealed that it contained only one major peak at t_R 6.39 minutes (Figure 5.2C), which was confirmed to be 6-bromoisatin by its molecular mass; ESI/MS *m/z* 224, 226 (Figure 5.2C insert).

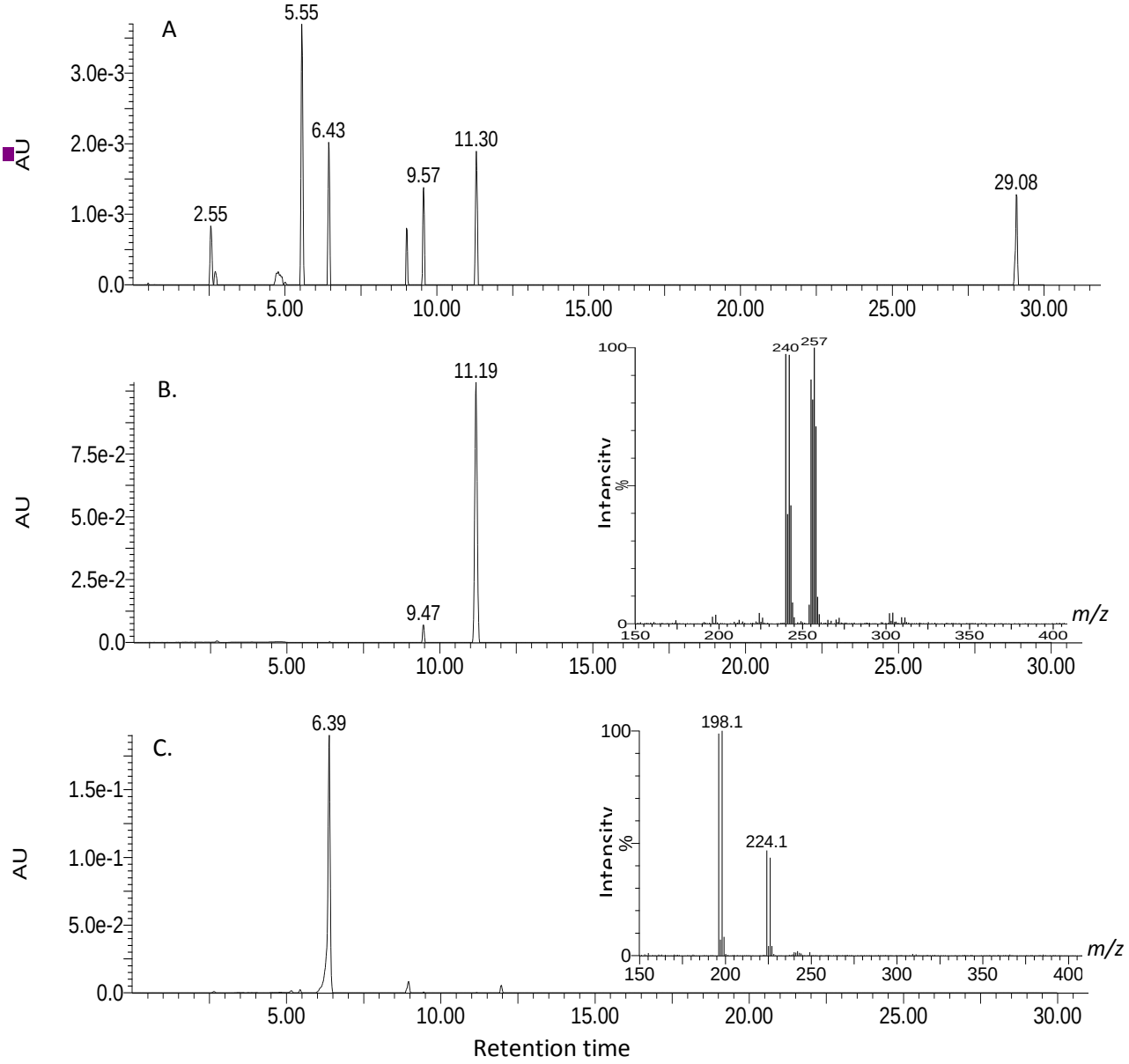


Figure 5.2: LC/MS of purified *D. orbita* extract.
Liquid chromatography mass spectrometry analysis of the three main fractions collected from semi-purification of the *D. orita* sample. The chromatograms from the Diode array at 300/600nm (A) Fraction one a mixture of brominated indole compounds, major peak at t_R 5.55 minutes corresponds to the molecular mass of tyrindoxyl sulphate. Smaller peaks at t_R 6.43, 9.55 and 11.30 minutes correspond to the molecular mass of 6-bromoisatin, tyrindolinone and tyrindoleninone respectively; (B) Fraction two, the major peak at t_R 11.19 minutes corresponds to the molecular mass of tyrindoleninone and the smaller peak t_R 9.47 minutes, tyrindolinone. The ESI/MS (insert) of major peak represents tyrindoleninone (*m/z* 255, 257); (C) Fraction three the major peak at t_R 6.39 minutes corresponds to the molecular mass of 6-bromoisatin and ESI/MS inset (*m/z* 224, 226) confirmed the molecular weight.

5.3.3 The Effect of hCG/cAMP on Cell Proliferation in Primary & Reproductive Cancer Cell Lines

Primary-derived human granulosa cells along with the reproductive cancer cell lines KGN, Jar and OVCAR-3 were treated with three semi-purified *D. orbita* fractions in the presence or absence of hCG (primary granulosa, JAr and OVCAR-3) or db-cAMP (KGN). hCG alone (in the absence of the compounds) had no proliferative or inhibitory effect on primary granulosa cell numbers (Table 5.2). Conversely, KGN, JAr and OVCAR-3 cell numbers were considerably lower at ≥ 24h in the presence of db-cAMP or hCG in comparison to media only control and the 1% DMSO control (Table 5.2). Neither hCG nor db-cAMP inhibited cell growth, however both progesterone and estradiol synthesis was higher in the presence of hCG or db-cAMP in comparison to un-stimulated cells.

Table 5.2: Final cell numbers of controls

Effect of controls, hCG (primary granulosa, JAr and OVCAR-3 cells) or db-cAMP (KGN cells) on cell viability in comparison to media only and 1% DMSO control; shown as mean cell number ± 1 standard deviation. GC = primary-derived human granulosa cells; DMSO = dimethyl sulfoxide; hCG = human chronic gonadotrophin; db-cAMP = dibutyryl adenosine 3‘,5‘-cyclic monophosphate; Positive control = no serum (primary granulosa, KGN and OVCAR-3 cells) of 1,000µg/mL hydrogen peroxide (JAr cells); N/T = not tested.

Cell Type	Time (h)	Mean Cell Number				
		Media	1% DMSO	hCG or db-cAMP	1% DMSO + hCG or cAMP	Positive control
GC	1	N/T	N/T	N/T	N/T	N/T
KGN		N/T	N/T	N/T	N/T	N/T
JAr		21557 (1400)	22310 (2765)	21507 (2117)	21322 (4723)	2501 1266)
OVCAR-3		N/T	N/T	N/T	N/T	N/T
GC	4	11523 (1467)	11342 (1069)	11451 (982)	10732 (1107)	4944 (2041)
KGN		26258 (4960)	25681 (3621)	19490 (2281)	20362 (2890)	8329 (1160)
JAr		28002 (3150)	26749 (3404)	22907 (1785)	23350 (3072)	1039 (667)
OVCAR-3		20385 (2364)	20867 (2776)	21702 (2399)	20847 (2359)	3010 (2830)
GC	24	11537 (1879)	11635 (1708)	11955 (2409)	10876 (1534)	5862 (2431)
KGN		29169 (4678)	29529 (3639)	22157 (3122)	24618 (3742)	14334 (2040)
JAr		38123 (5348)	39018 (6527)	38211 (3030)	37687 (6671)	1272 (556)
OVCAR-3		33237 (7490)	33339 (2570)	25510 (4752)	28876 (3739)	5595 (4237)
GC	48	12301 (1394)	11838 (2001)	10884 (2667)	11006 (1379)	3976 (2441)
KGN		45376 (6361)	47647 (6380)	18923 (8479)	24001 (2055)	4629 (5003)
JAr		65132 (12330)	63462 (9984)	38761 (5000)	45227 (9062)	1994 (1778)
OVCAR-3		45367 (7803)	45802 (12493)	29462 (3608)	29660 (3538)	4906 (4423)
GC	72	10277 (2030)	10499 (2950)	9413 (2245)	10236 (1729)	2662 (2711)
KGN		50507 (3089)	54655 (5174)	16993 (10420)	22421 (2693)	2193 (1327)
JAr		N/T	N/T	N/T	N/T	N/T
OVCAR-3		54820 (9204)	54348 (11408)	21432 (3755)	23422 (4952)	1754 (1175)

5.3.4 The Effects of the *D. orbita* Compounds on Reproductive Cells

5.3.4.1 Effects of Fraction One on Cell Viability & Hormone Synthesis

Overall, fraction one containing a mixture of four brominated indole compounds (Figure 5.2A) was cytotoxic at lower concentrations towards the KGN, JAr and OVCAR-3 cancer cell lines in comparison to the primary granulosa cells (Table 5.3). The concentration of fraction one which, killed half (LC_{50}) the cancer cells (KGN, JAr and OVCAR-3) was < 0.3mg/mL after 4, 24 and 48h in comparison to the primary granulosa cells (LC_{50} 1mg/mL). The cancerous granulosa cell line, KGN however, was more sensitive, with LC_{50} of 0.05, 0.09, 0.007 and 0.004mg/mL for all four exposure periods respectively. The JAr and OVCAR-3 cell lines were less sensitive than KGN, but still an order of magnitude more sensitive than the primary granulosa cells.

In the presence of hCG or db-cAMP, fraction one was not as cytotoxic as when it was tested in the absence of hCG or. db-cAMP on the reproductive cancer cells (LC_{50} < 0.4 for all time periods tested). However, the LC_{50}'s (>1mg/mL for 4, 24 and 48h, and 0.8mg/mL for 72h) for the primary granulosa cells were still an order of magnitude greater than the three cancer cell lines (Table 5.3).

Fraction one had no effect on progesterone synthesis by primary granulosa cells either in the absence or presence of hCG. In comparison, in the presence of hCG, fraction one generally inhibited progesterone synthesis by the three cancer cell lines. At 24 and 48h fraction one without hCG and 4h with hCG, stimulated estrogen synthesis by primary granulosa cells at 0.05 and 0.5mg/mL. In comparison, fraction one in the presence of hCG generally inhibited estrogen synthesis by primary granulosa cells at 24 and 48h treatment. Fraction one with and without hCG or db-

cAMP generally inhibited estrogen synthesis at 24 and 48h, whereas estrogen synthesis by the JAr cells was slightly stimulated at 24h and inhibited at 48h.

5.3.4.1.1 Primary-derived Human Granulosa Cells

In the absence of hCG, primary-derived granulosa cell numbers were only reduced to 90% of control at 0.05mg/mL after 4h treatment (Figure 5.3A). Higher concentrations $\geq$ 0.5mg/mL $\pm$ hCG, and $\geq$ 4h exposure to fraction one were significantly toxic to primary granulosa cells ($p < 0.001$; Figure 5.3A and D). Cells were more rounded after 4h treatment with fraction one (0.5mg/mL; Figure 5.4). However, very little change in cell structure and morphology was seen between the DMSO control and fraction one at 24 and 48h (Figure 5.4). Despite the decrease in cell numbers progesterone synthesis was not inhibited by fraction one $\pm$ hCG at all concentrations less than or equal to 0.5mg/mL or $\leq$ 48h exposure (Figure 5.3B & E).

In comparison, estradiol synthesis by primary granulosa cells increased after 24h exposure to 0.05mg/mL fraction one in the absence of hCG, with a significant increase also noted at 0.5mg/mL after 24 and 48h ($p < 0.001$; Figure 5.3C). However, when granulosa cells were treated with fraction one in the presence of hCG there was a decrease in estradiol synthesis at the lower concentrations of 0.005 and 0.05mg/mL after 48h of treatment ($p < 0.01$) but not at the highest concentration of 0.5mg/mL (Figure 5.3F).

Table 5.3: Results of Fraction one on Reproductive Cells

Effects of Fraction one (F1) ± hCG (primary-derived human granulosa, JAr and OVCAR-3 cells) or db-cAMP (KGN cells) on cell viability, progesterone and estradiol synthesis. Cell viability shown as LC_{50} concentration that had an effect on 50% of the cells, & hormone data shown as concentration that was significantly different to the 1% DMSO control. GC = primary-derived human granulosa cells; hCG = human chronic gonadotrophin; db-cAMP = dibutyryl adenosine 3‘,5‘-cyclic monophosphate; LC_{50} = concentration that had an effect on 50% of the cells; F1= Fraction one; F1 + hCG/db-cAMP = Fraction one in the presence of hCG or db-cAMP; (+) concentration that stimulated hormone synthesis; (-) concentration that inhibited hormone synthesis; N/T = not tested; n/e = no significant effect

	Time (h)	**LC_{50} (mg/mL)**		**Effect on hormone synthesis (mg/mL)**			
				Progesterone		Estradiol	
Conc. 0005, 0.01, 0.05, 0.1, 0.5, 1.0mg/mL		F1	F1 + hCG/db-cAMP	F1	F1 + hCG/db-cAMP	F1	F1 + hCG/db-cAMP
GC	1	N/T	N/T	N/T	N/T	N/T	N/T
KGN		N/T	N/T	N/T	N/T	N/T	N/T
JAr		0.2	0.2	N/T	N/T	N/T	N/T
OVCAR-3		N/T	N/T	N/T	N/T	N/T	N/T
GC	4	1	>1	n/e	n/e	n/e	0.05 (+)
KGN		0.05	0.2	n/e	0.05, 0.5 (-)	0.5 (+)	0.005 (-)
JAr		0.04	0.1	≤0.5 (-)	≤0.05 (-)	n/e	n/e
OVCAR-3		0.2	0.2	n/e	0.05, 0.5 (-)	0.05 (-)	n/e
GC	24	1	>1	n/e	n/e	0.05, 0.5 (+)	0.005, 0.05 (-)
KGN		0.09	0.2	0.005, 0.05 (-)	n/e	0.005 (-)	0.005 (-)
JAr		0.3	0.3	n/e	≤0.5 (-)	0.005, 0.5 (+)	0.5 (+)
OVCAR-3		0.2	0.2	n/e	0.05, 0.5 (-)	n/e	≤0.5 (-)
GC	48	1	>1	n/e	n/e	0.5 (+)	0.005, 0.05 (-)
KGN		0.007	0.1	≤0.5 (-)	≤0.5 (-)	0.5 (-)	0.5 (-)
JAr		0.1	0.4	n/e	≤0.5 (-)	0.005, 0.5 (-)	0.005 (-)
OVCAR-3		0.03	0.1	n/e	n/e	n/e	n/e
GC	72	>1	0.8	N/T	N/T	N/T	N/T
KGN		0.004	0.1	N/T	N/T	N/T	N/T
JAr		N/T	N/T	N/T	N/T	N/T	N/T
OVCAR-3		0.001	0.3	N/T	N/T	N/T	N/T

5.3.4.1.2 KGN Cell Line

Significant reductions in cell viability were observed in all three cancer cell lines when exposed to Fraction one. KGN cell viability was significantly inhibited at all concentrations tested and all incubation periods in the absence of db-cAMP ($p < 0.001$; LC_{50} 0.05mg/mL at 4h, 0.09mg/mL at 24h and 0.007mg/mL at 48h; Figure 5.5A). However, in the presence of db-cAMP, fraction one only inhibited the mitochondrial enzymes of the KGN cells at 0.05mg/mL and 4h exposure ($p < 0.001$; Figure 5.5D). At lower concentrations of 0.01mg/mL there was only a notable reduction in KGN cells treated with fraction one + db-cAMP after 24h ($p < 0.05$; Figure 5.5D), and at 0.005mg/mL the lowest concentration tested after 48h ($p < 0.001$; Figure 5.5D). Changes in KGN cell morphology after treatment with fraction one in the absence of db-cAMP indicated cell shrinkage as the concentration increased (Figure 5.6). The cell structure was notably different to the DMSO control at the lowest concentration of fraction one (0.005mg/mL) and at all incubation times. As the concentration increased to 0.5mg/mL the KGN cells no longer formed an organised pattern, they were more rounded and detached (Figure 5.6).

Even though KGN cell viability was greatly reduced after 4h exposure to all concentrations of fraction one without db-cAMP, basal progesterone synthesis was only inhibited after 24h of treatment at concentrations of 0.005 and 0.05mg/mL (Figure 5.5B). This effect was also noted after 48h at 0.005 and 0.5mg/mL ($p < 0.001$; Figure 5.5B). However, at the highest concentration of fraction one (0.5mg/mL) without db-cAMP there was no effect on progesterone synthesis at 4 or 24h exposure (Figure 5.5B). In the presence of db-cAMP fraction one had a significant negative effect on progesterone synthesis after 4h at 0.05 and 0.5mg/mL, and after 48h at all concentrations tested ($p < 0.001$; Figure 5.5E).

Estradiol synthesis was significantly inhibited after 4h (+ db-cAMP) and 24h treatment of fraction one (0.005mg/mL) and after 48h ± db-cAMP (0.5mg/mL; Figure 5.5C & F). Furthermore at higher concentrations of fraction one (0.5mg/mL without db-cAMP) estradiol synthesis by KGN cells was stimulated at 4h exposure relative to the control (Figure 5.5C).

5.3.4.1.3 JAr Cell Line

Fraction one without hCG was also found to be cytotoxic to the JAr cell line at all concentrations tested after 1 and 4h exposure (Figure 5.7A). In comparison, in the presence of hCG this toxic effect was only noted after 4h at all concentrations (Figure 5.7D). After 24h treatment with fraction one ± hCG, JAr cells were significantly reduced at ≥ 0.01mg/mL ($p < 0.001$; Figure 5.7A). Furthermore, after 48h, fraction one was found to significantly reduce JAr cell numbers at all concentrations tested in the absence of hCG (Figure 5.7A), whereas after 48h in the presence of hCG, fraction one was only cytotoxic to the JAr cells at ≥ 0.5mg/mL (Figure 5.7D). The images of cell morphology (Figure 5.8) indicate that fraction one without hCG caused significant shrinkage and rounding of the JAr cells even at the lowest concentration (0.005mg/mL) after 4 and 24h. The cells did not fill the intercellular spaces at confluence after 24h treatment even at the lowest concentration of fraction one (0.005mg/mL; Figure 5.8). However, after 48h of treatment with fraction one no cell changes were noted at any concentration (Figure 5.8).

Progesterone synthesis by the JAr cells was significantly inhibited in comparison to the 1% DMSO control in the absence of hCG only after 4h incubation at all concentrations of fraction one ($p < 0.01$ at 0.005mg/mL and $p < 0.001$ at 0.05 and 0.5mg/mL; Figure 5.7B). In comparison, fraction one in the presence of hCG

significantly inhibited progesterone synthesis by the JAr cells at all incubation periods (Figure 5.7E).

Estrogen synthesis by JAr cells treated with fraction one was only significantly inhibited at 0.005mg/mL in the presence of hCG after 48h (Figure 5.7F). However, after 24h, estrogen was stimulated slightly with and without hCG at all concentrations tested (Figure 5.7C and F). A significant increase was noted at 0.005 and 0.5mg/mL of fraction one without hCG and at 0.5mg/mL in the presence of hCG ($p < 0.01$; Figure 5.7C and F), although large error bars indicates variation in repeats of the assay.

5.3.4.1.4 OVCAR-3 Cell Line

Cell growth by the OVCAR-3 ovarian cancer cell line was also inhibited by fraction one. In the absence of hCG, fraction one inhibited the mitochondrial enzymes after 4h treatment at ≥ 0.05mg/mL (Figure 5.9A). In the presence of hCG OVCAR-3 cell proliferation were only inhibited by fraction one at ≥ 0.1mg/mL at 4h treatment (Figure 5.9D). After 24h OVCAR-3 cell viability was reduced significantly when treated with fraction one with and without hCG at concentrations as low as 0.01mg/mL ($p < 0.01$; Figure 5.9A and D). After 48 and 72h at the lowest concentration tested (0.005mg/mL), OVCAR-3 cell numbers were significantly reduced by fraction one without hCG ($p < 0.01$; Figure 5.9A). In comparison, fraction one in the presence of hCG only affected OVCAR-3 cell viability after 48h at 0.1mg/mL ($p < 0.001$; Figure 5.9D) and at the concentration of 0.01mg/mL after 72h ($p < 0.01$; Figure 5.9D). Changes in OVCAR-3 cell morphology (Figure 5.10) show cell damage and shrinking with possible cell blebbing and microspikes, indicating apoptosis.

Progesterone synthesis by OVCAR-3 cells was only inhibited with fraction one in the presence of hCG (Figure 5.9B). After 4 and 24hh treatment fraction one (0.05 and 0.5mg/mL) in the presence of hCG significantly decreased progesterone synthesis by OVCAR-3 cells (Figure 5.9E). After 48h treatment there was no effect on estradiol synthesis by the OVCAR-3 cells (Figure 5.9E). Fraction one without hCG had a slight inhibitory effect on estradiol synthesis by the OVCAR-3 cell line at the concentration of 0.05mg/mL after 4h treatment (Figure 5.9C). Whereas after 24h treatment, estradiol synthesis by the OVCAR-3 cell line was significantly inhibited at all concentrations tested of fraction one in the presence of hCG. However, this effect was not seen after 48h (Figure 5.9F).

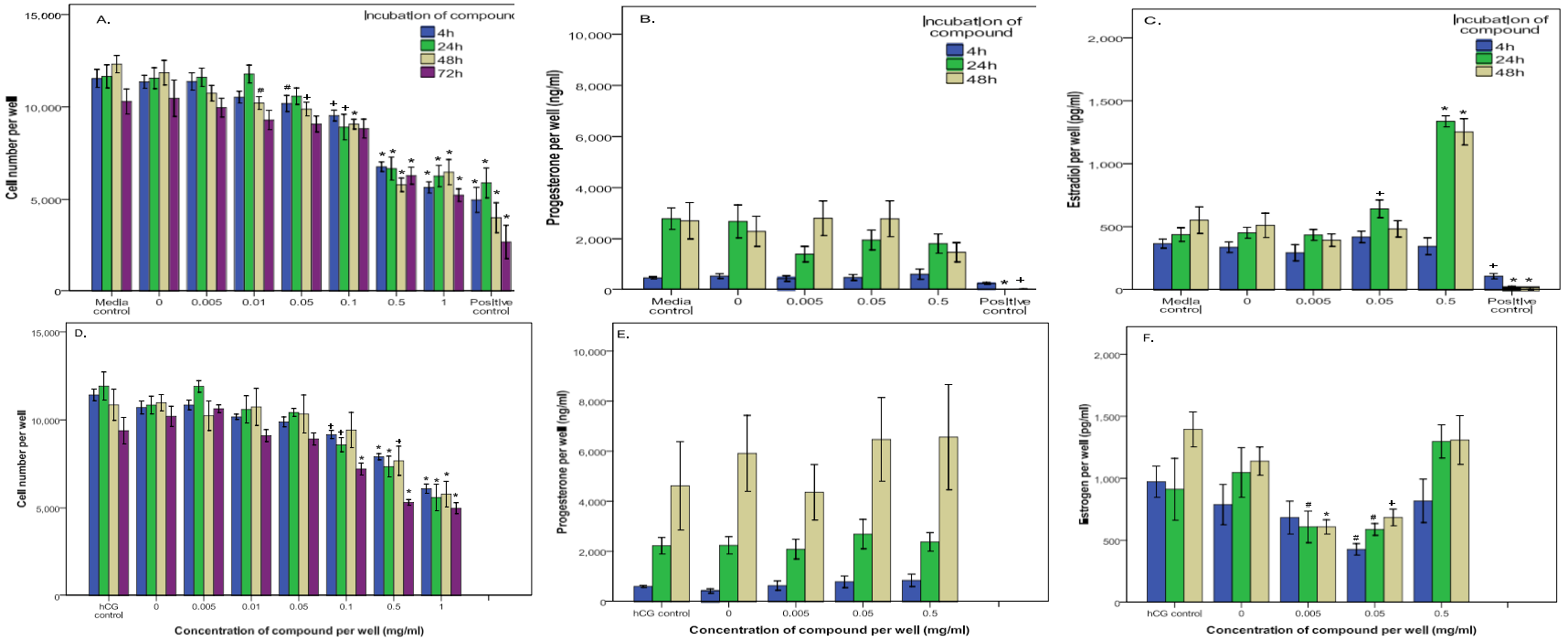


Figure 5.3: The effects of fraction one on primary-derived human granulosa cells

The effect of the semi-purified *D. orbita* fraction one on primary-derived granulosa cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction one ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm, with reference absorbance 630nm, and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

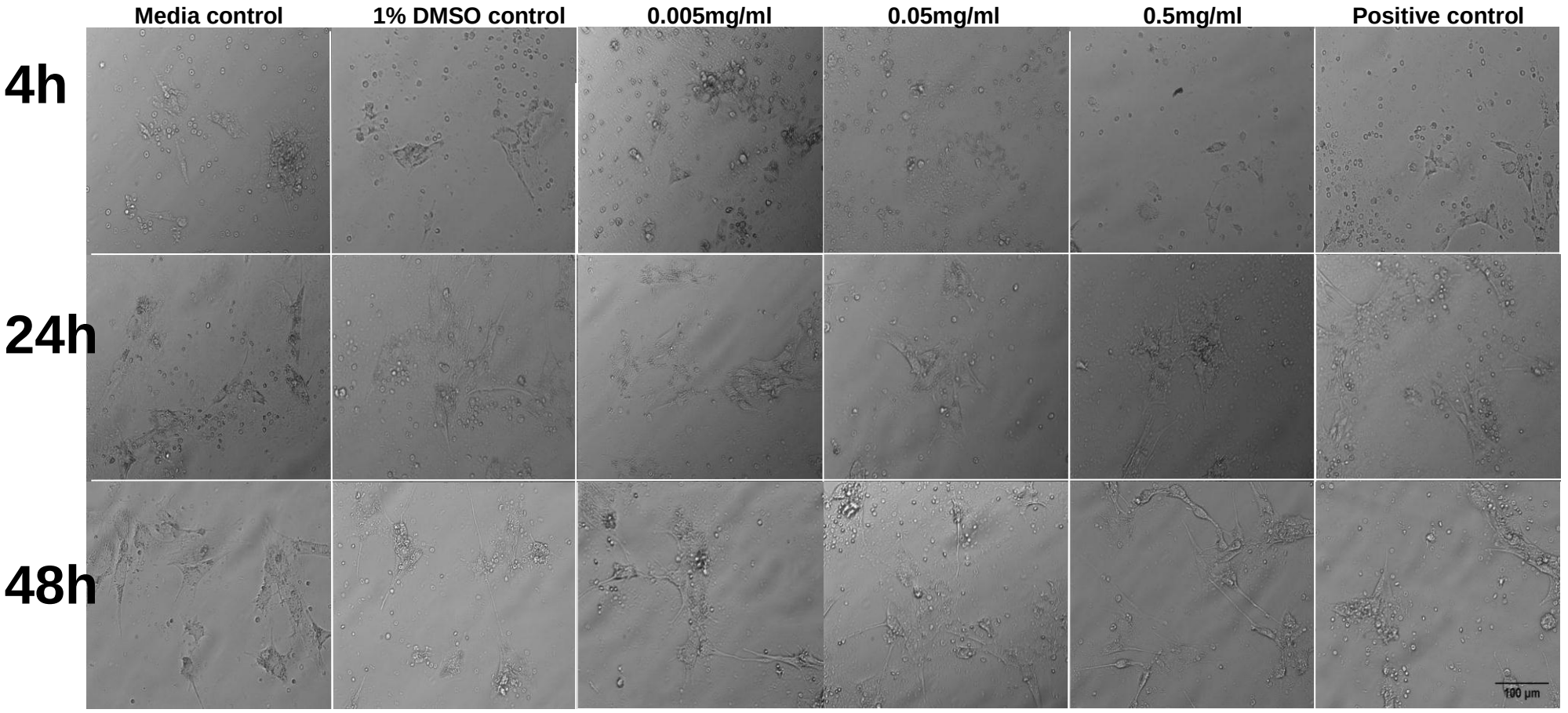


Figure 5.4: Morphological changes in primary-derived human granulosa cells after treatment with fraction one
Morphological changes in the primary-derived human granulosa cells after treatment with the semi-purified *D. orbita* fraction one without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

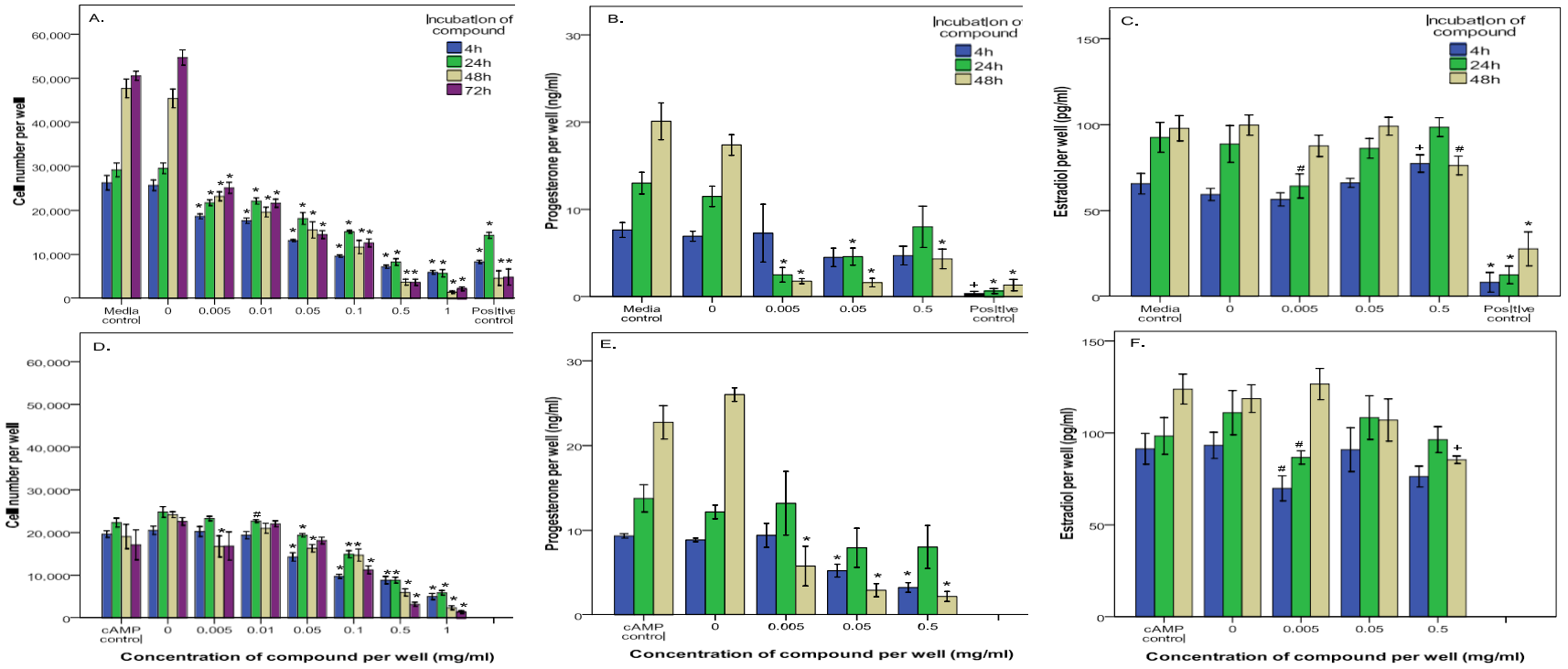


Figure 5.6: The effects of fraction one on KGN cells

The effect of the semi-purified *D. orbita* fraction one on KGN cell viability, progesterone and estradiol synthesis in the absence (A, B, C) and in the presence of db-cAMP (D, E, F). After an initial 24h cell attachment period KGN cells (20,000cells/well) were treated with fraction one ± db-cAMP for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

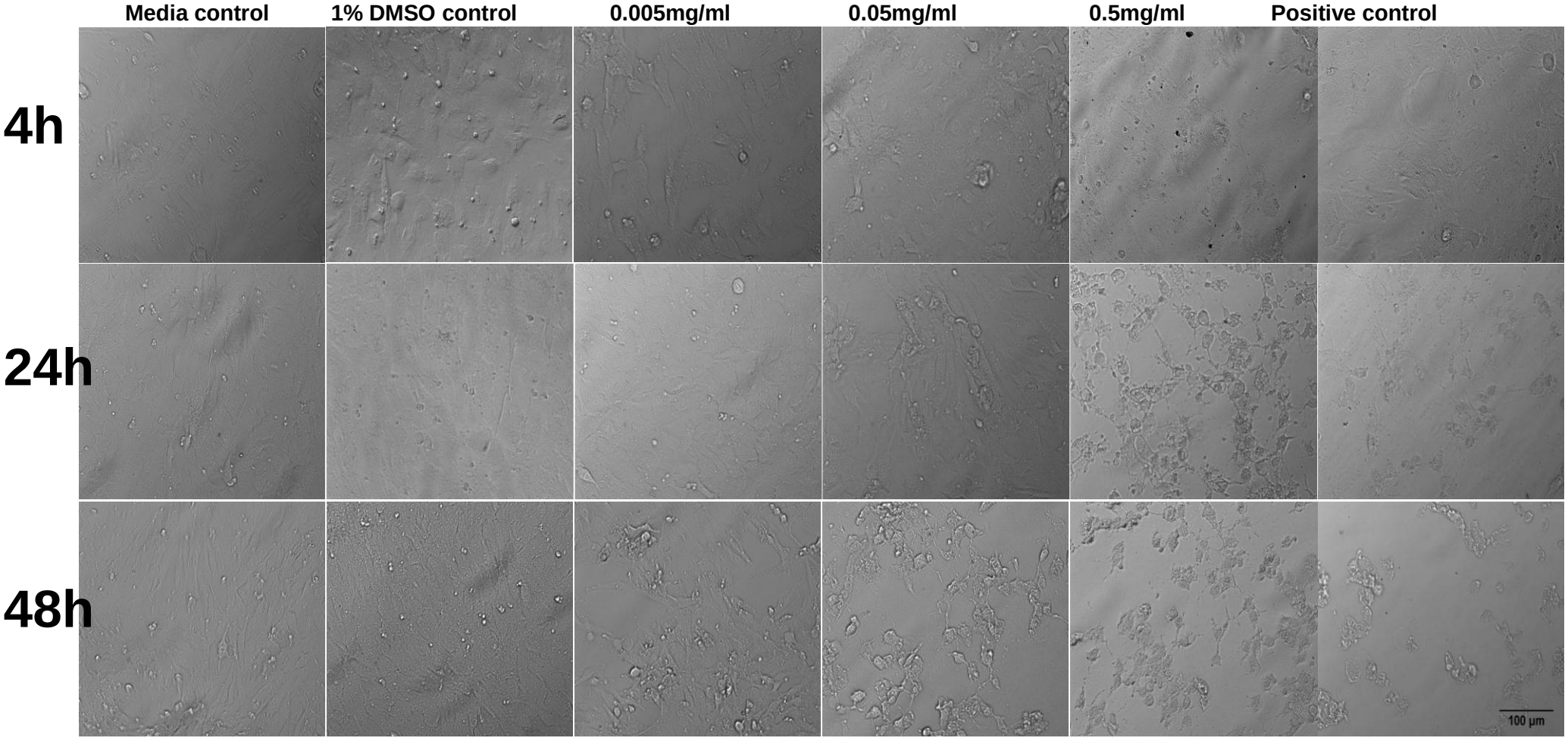


Figure 5.4: Morphological changes in the KGN granulosa cancer cell line after treatment with fraction one Morphological changes in the KGN cells after treatment with the semi-purified *D. orbita* fraction one without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

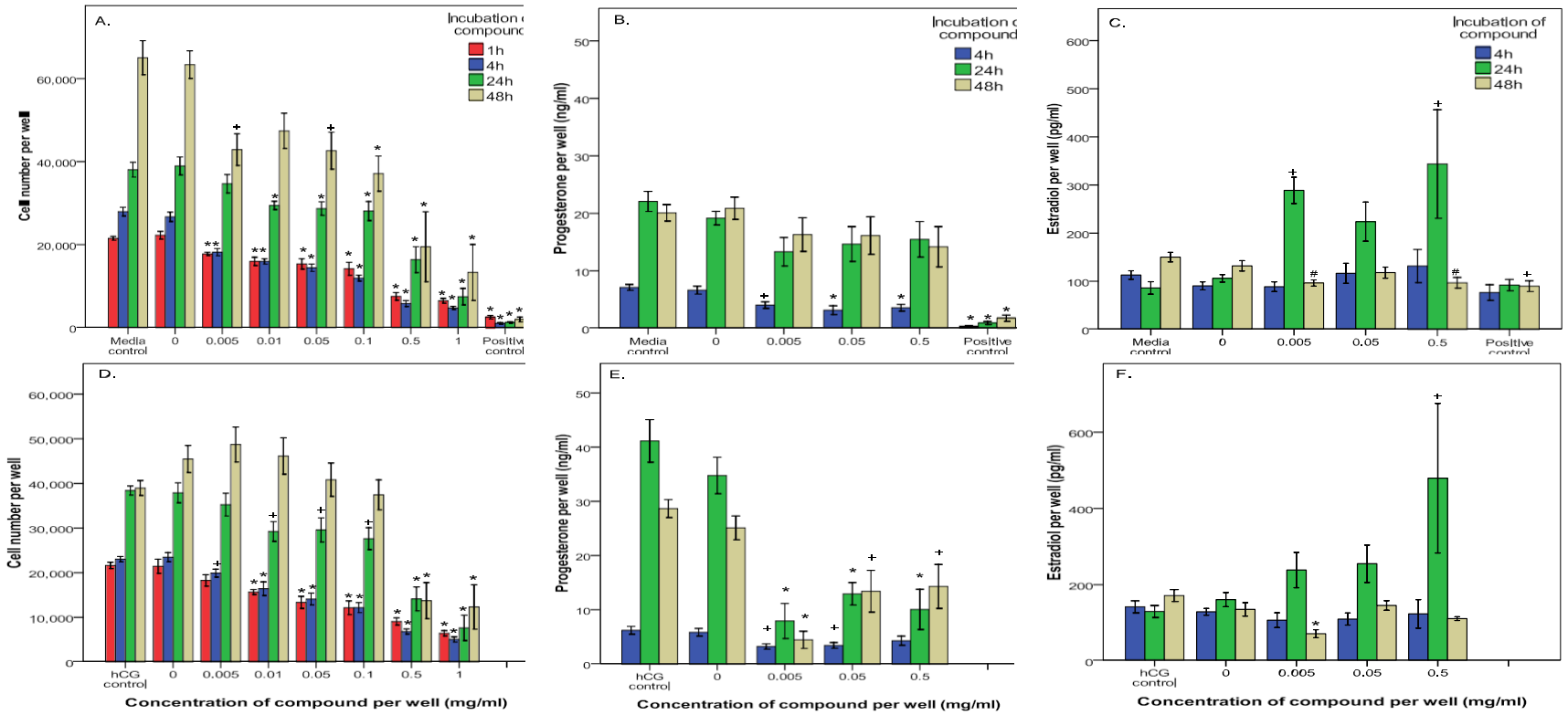


Figure 5.7: The effect of fraction one on JAr cells

The effect of the semi-purified *D. orbita* fraction one on JAr cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 2h cell attachment period JAr cells (20,000cells/well) were treated with fraction one ± hCG for 1, 4, 24 and 48h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

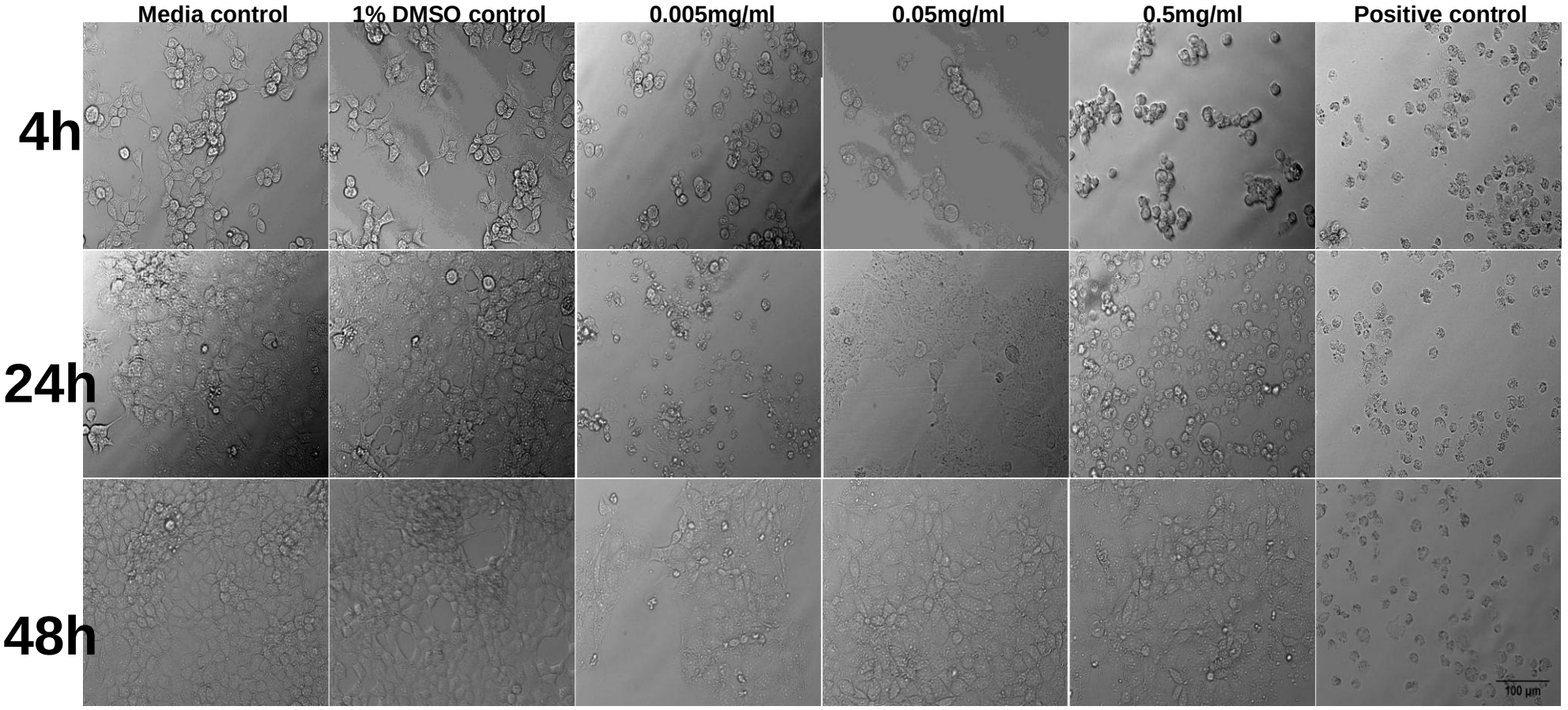


Figure 5.8: Morphological changes in the JAr choriocarcinoma cell line after treatment with fraction one
Morphological changes in the JAr choriocarcinoma cell line after treatment with the semi-purified *D. orbita* fraction one without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

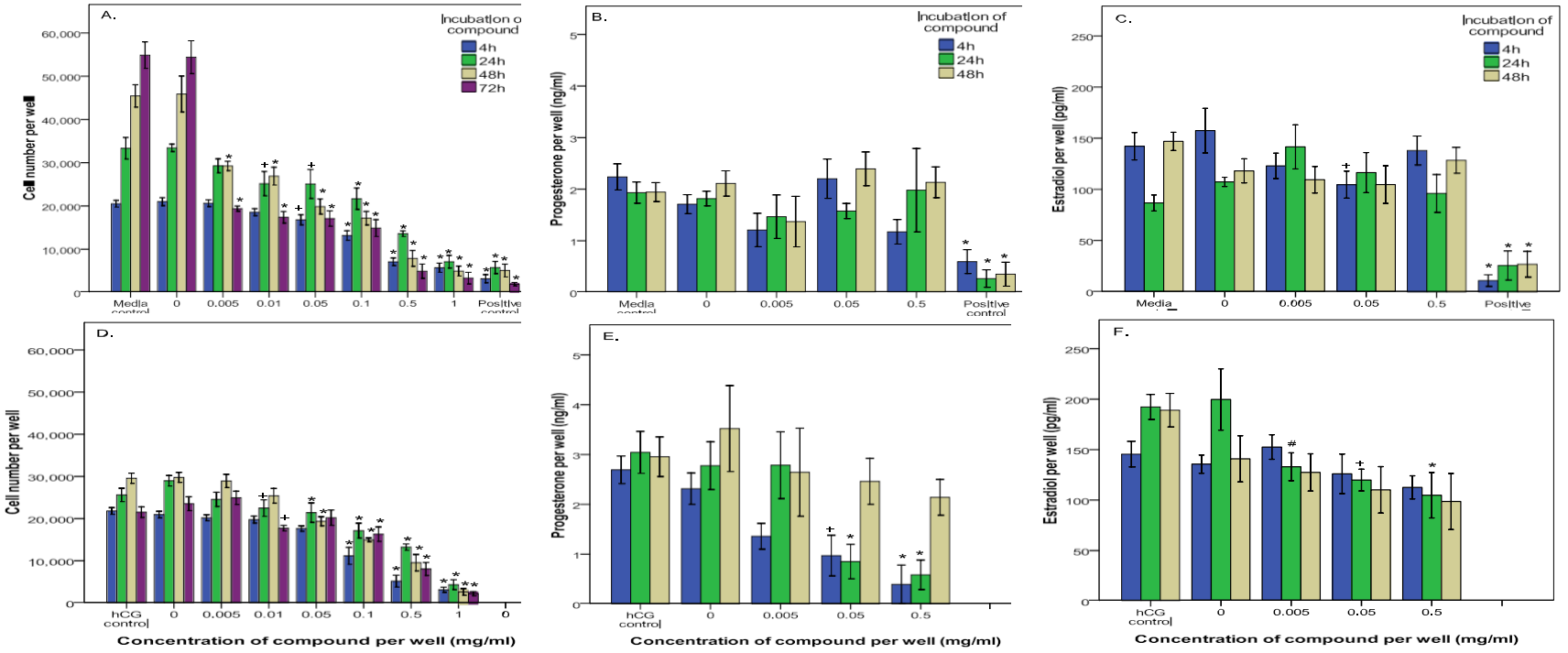


Figure 5.9: **The effect of fraction one on OVCAR-3 cells**

The effect of the semi-purified *D. orbita* fraction one on OVCAR-3 cell viability, progesterone synthesis and estradiol synthesis in the absence (A. B, C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period OVCAR-3 cells (20,000cells/well) were treated with fraction one ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

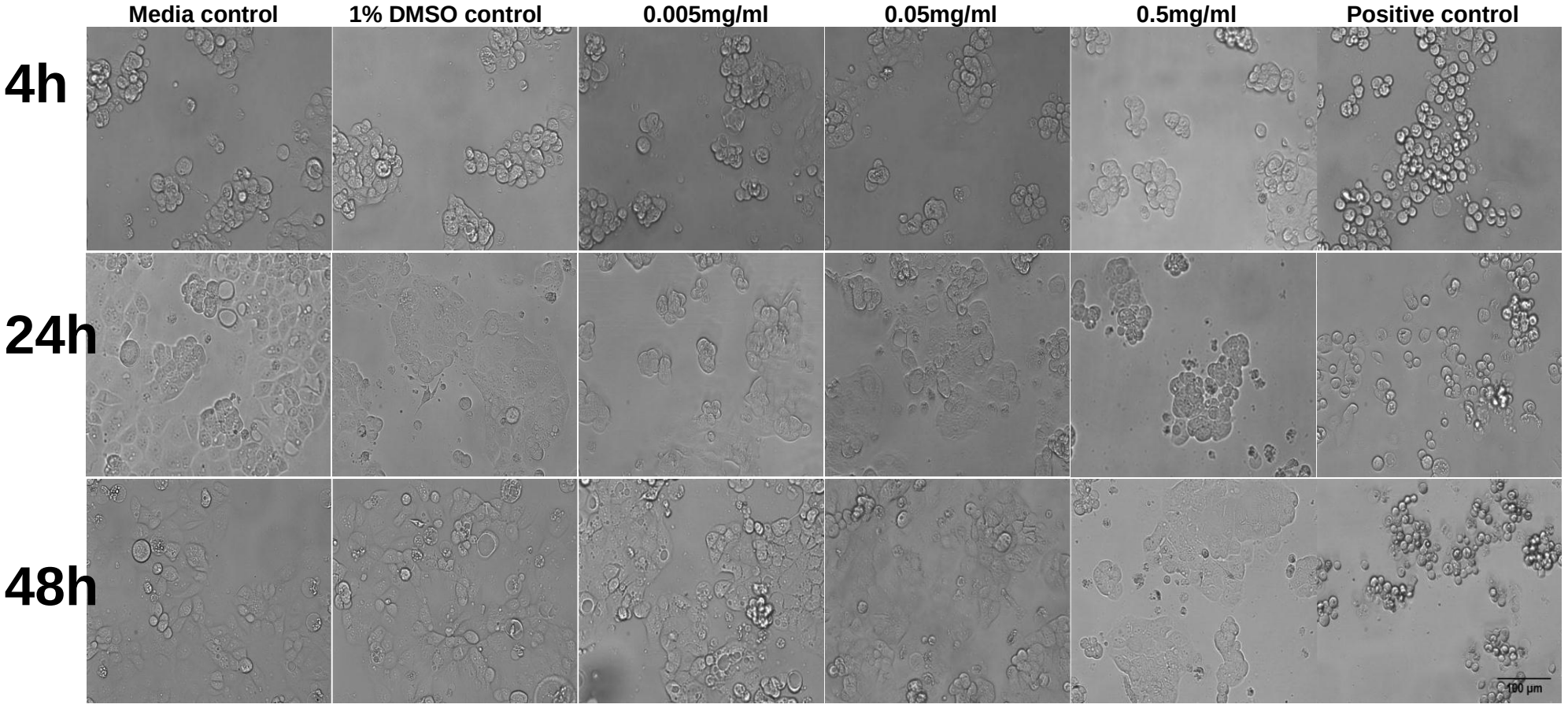


Figure 5.10: Morphological changes in the OVCAR-3 ovarian cancer cell line after treatment with fraction one
Morphological changes in the OVCAR-3 ovarian cancer cell line after treatment with the semi-purified *D. orbita* fraction one without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

5.3.4.2 Effects of Fraction Two on Cell Viability & Hormone Synthesis

Of the three semi-purified fractions tested, fraction two containing predominately the bioactive compound, tyrindoleninone (Figure 5.2B) was the most cytotoxic towards the reproductive cancer cell lines (Tables 5.3, 5.4 & 5.5) than the primary granulosa cells (Table 5.4). In fact, the LC_{50} range, were at least 10-fold lower with the KGN, JAr and OVCAR-3 cell lines, in comparison to the primary granulosa cells after all testing periods. Fraction two had the greatest effect on the KGN granulosa cancer cell line with LC_{50}'s of 0.01, 0.02, 0.005 and < 0.005mg/mL for 4, 24, 48 and 72h respectively, more than 10-fold less than the primary granulosa cells. In general though, fraction two in the presence of hCG and db-cAMP was not as cytotoxic to all three cancer cell lines (Table 5.4). Generally, progesterone synthesis by all cells was inhibited by fraction two ± hCG or db-cAMP. Fraction two however at the low concentration in the absence of hCG or db-cAMP often increased estrogen synthesis by all the cells.

Table 5.4: Results of Fraction two on Reproductive cells

Effects of Fraction two (F2) ± hCG (primary-derived human granulosa, JAr and OVCAR-3 cells) or db-cAMP (KGN cells) on cell viability, progesterone and estradiol synthesis. Cell viability shown as LC_{50} concentration that had an effect on 50% of the cells, & hormone data shown as concentration that was significantly different to the 1% DMSO control GC = primary-derived human granulosa cells; hCG = human chronic gonadotrophin; cAMP = dibutyryl adenosine 3‘,5‘-cyclic monophosphate; LC_{50} = concentration that had an effect on 50% of the cells; F2= Fraction two; F2 + hCG/db-cAMP = Fraction tow in the presence of hCG or db-cAMP; (+) concentration that stimulated hormone synthesis; (-) concentration that inhibited hormone synthesis; N/T = not tested; n/e = no effect.

	Time (h)	**LC_{50} (mg/mL)**		**Effect on hormone synthesis (mg/mL**			
				Progesterone		Estradiol	
Conc. 0005, 0.01, 0.05, 0.1, 0.5, 1.0mg/mL		F2	F2 + hCG/db-cAMP	F2	F2 + hCG/db-cAMP	F2	F2 + hCG/db-cAMP
GC	1	N/T	N/T	N/T	N/T	N/T	N/T
KGN		N/T	N/T	N/T	N/T	N/T	N/T
JAr		0.08	0.05	N/T	N/T	N/T	N/T
OVCAR-3		N/T	N/T	N/T	N/T	N/T	N/T
GC	4	0.9	>1	n/e	n/e	0.5 (-)	n/e
KGN		0.01	0.05	≤0.5 (-)	≤0.5 (-)	0.05(+)	0.005 (-)
JAr		0.01	0.02	≤0.5 (-)	0.05 (-)	0.005 (+)	0.5 (-)
OVCAR-3		0.04	0.08	≤0.5 (-)	0.05 (-)	n/e	0.05 (+)
GC	24	0.4	0.5	0.5 (-)	n/e	0.005, 0.05 (+)	0.005, 0.5 (-)
KGN		0.02	0.05	≤0.5 (-)	≤0.5 (-)	n/e	0.5 (+)
JAr		0.03	0.04	0.5 (-)	≤0.5 (-)	0.005, 0.05 (+)	0.005(+) 0.5 (-)
OVCAR-3		0.06	0.1	≤0.05 (-)	0.005 (-)	≤0.05 (+)	0.005 (-)
GC	48	0.4	0.4	n/e	0.5 (-)	0.005 (+), 0.5 (-)	0.05, 0.5 (-)
KGN		0.005	0.02	≤0.5 (-)	≤0.5 (-)	n/e	0.005 (-)
JAr		0.02	0.06	0.005, 0.5 (-)	≤0.5 (-)	n/e	0.05 (+)
OVCAR-3		0.008	0.07	n/e	n/e	n/e	n/e
GC	72	0.4	0.5	N/T	N/T	N/T	N/T
KGN		<0.005	0.01	N/T	N/T	N/T	N/T
JAr		N/T	N/T	N/T	N/T	N/T	N/T
OVCAR-3		<0.005	0.02	N/T	N/T	N/T	N/T

5.3.4.2.1 Primary-Derived Human Granulosa Cells

Primary granulosa cells were only significantly reduced at 0.05mg/mL ± hCG after 4h treatment (reduced to 88% of control; $p < 0.05$; Figure 5.11A). At longer exposure times (48h), 0.01mg/mL of fraction two in the absence of hCG was found to be more toxic to primary granulosa cells than in the presence of hCG (reduced to 86% of the control without hCG; $p < 0.01$; and to 96% of control with hCG; Figure 5.11A and D). Minor changes in primary granulosa cell morphology were only noted with less cell coverage and cell shrinkage at 0.5mg/mL of fraction two (Figure 5.12).

Fraction two only significantly inhibited progesterone synthesis by primary granulosa at 0.5mg/mL after 24h treatment without hCG and after 48h treatment with hCG (Figure 5.11B and E). In comparison, estradiol synthesis by primary granulosa cells was increased significantly after 24h at 0.005 and 0.5mg/mL and 48h at 0.005mg/mL with fraction two in the absence of hCG (Figure 5.11C.) However, at the higher concentration of 0.5mg/mL at 4 and 48h, there was a notable decrease in estradiol secretion by primary granulosa cells with fraction two in the absence of hCG (Figure 5.11C). In comparison, when primary granulosa cells were treated with fraction two in the presence of hCG there was a decrease in estradiol synthesis after 24h at the concentrations of 0.005 and 0.5mg/mL ($p < 0.01$; Figure 5.11F). Furthermore after 48h at ≥ 0.05mg/mL of fraction two with hCG, estradiol synthesis by primary granulosa cells was significantly decreased ($p < 0.001$; Figure 5.11F).

5.3.4.2.2 KGN Cell Line

Treatment of the KGN cell line (the corresponding granulosa tumour cell line) with fraction two in the absence of db-cAMP significantly reduced cell numbers at all concentrations tested (≥ 0.005mg/mL) and at ≥ 4h of treatment ($p < 0.001$; LC_{50} 0.01; Figure 5.13A). In comparison to primary granulosa cells KGN cells were

reduced to 67% of the control after 4h at the lowest concentration tested (0.005mg/mL; $p < 0.001$) without db-cAMP (Figure 5.13A), whereas primary granulosa cells were only reduced to 93% of control (Figure 5.13A) under the same conditions. Furthermore, at 0.5mg/mL of fraction two without db-cAMP, KGN cells were reduced to 9% of control (Figure 5.13A) and primary granulosa cells to 58% of control (Figure 5.13A) after 4h treatment. Changes in KGN cell morphology by cell shrinkage after treatment with fraction two without db-cAMP was evident at the lowest concentration tested, 0.005mg/mL (Figure 5.14). With prolonged culture (48h) cells were more notably detached and shrivelled (Figure 5.14). However, db-cAMP afforded some protection to the KGN cells because the same cytotoxic effects from fraction two were only noted at $\geq$ 0.01mg/mL and $\geq$ 4h of treatment in the presence of db-cAMP ($p < 0.001$; Figure 5.13D). After 48 and 72h there was a significant reduction in KGN cells at 0.005mg/mL with fraction two and db-cAMP ($p < 0.01$; Figure 5.13D).

Fraction two ± db-cAMP reduced progesterone synthesis by the KGN cells lines at all concentrations tested ($\leq$ 0.5mg/mL) and all incubation periods (Figure 5.13B and E). In comparison, estradiol synthesis by KGN cells increased after 4h of treatment with fraction two in the absence of db-cAMP at 0.05mg/mL (Figure 5.13C). However, in the presence of db-cAMP estradiol synthesis by KGN cells was inhibited at 0.005mg/mL after 4 and 48h with fraction two (Figure 5.13F). After 24h of treatment with fraction two (0.5mg/mL) in the presence of db-cAMP there was a significant increase in estradiol synthesis ($p < 0.01$; Figure 5.13F).

5.3.4.2.3 JAr Cell Line

Fraction two both in the presence and absence of hCG was also found to be cytotoxic to the JAr cell line and after only 1h of treatment (LC_{50} 0.04mg/mL without hCG and 0.05mg/mL with hCG; Figure 5.15A and D). Stronger activity was observed after 4h treatment (± hCG), with fraction two significantly reducing JAr cells at ≥ 0.005mg/mL ($p < 0.001$; Figure 5.15A and D). At 0.005mg/mL of fraction two without hCG, JAr cells were reduced to 63% of the control after 4h (Figure 5.15A). After 4h of treatment and at higher concentrations of fraction two (0.5mg/mL) without hCG JAr cells were reduced to 8% of control (Figure 5.15A). In the presence of hCG a significant reduction in JAr cell viability was only noted after 48h treated with 0.05mg/mL fraction two as JAr cells were reduced to 41% of the control ($p<0.001$; Figure 5.15D). Cell damage and shrinkage after JAr cells were treated with fraction two in the absence of hCG was noted even at all concentrations ≥ 0.005mg/mL and ≤ 48h treatment (Figure 5.16). Furthermore, JAr cells were much less confluent with more notable detached cells in comparison to the DMSO control (Figure 5.16).

In the absence of hCG fraction two inhibited progesterone synthesis by JAr cells at concentrations ≥ 0.005mg/mL after 4h, but only at 0.5mg/mL after 24 and 0.05mg/L at 48h (Figure 5.15B). Whereas progesterone synthesis by JAr cells was significantly inhibited after 24 and 48 h at ≥ 0.005mg/mL with fraction two in the presence of hCG ($p < 0.001$; Figure 5.15E). In comparison, estradiol synthesis by JAr cells was only inhibited in the presence of hCG at 0.5mg/mL after 4 and 24h (Figure 5.15F). In fact, an increase in estradiol synthesis was noted when JAr cells were treated with

fraction two ± hCG at 0.005mg/mL after 24h and 0.05mg/mL after 24 and 48h (Figure 5.15C and F).

5.3.4.2.4 OVCAR-3 Cell Line

The OVCAR-3 cell line was also significantly affected by fraction two even after 4h treatment ± hCG (LC_{50} 0.04mg/mL without hCG and 08mg/mL with hCG). However, at the lowest concentration tested, 0.005mg/mL (without hCG), OVCAR-3 cells were only significantly reduced after 24h of treatment with fraction two (to 85% of control; Figure 5.17A). However, at higher concentrations of fraction two (0.5mg/mL) without hCG OVCAR-3 cell viability was reduced to 18% of control after 4h (Figure 5.17A). In the presence of hCG, fraction two was only noted to be cytotoxic to OVCAR-3 cells at ≥ 0.01mg/mL and ≥ 4h treatment (Figure 5.17D). Morphological changes to the OVCAR-3 cells were evident with clear signs of cell shrinkage, less cell confluence and detachment from wells at all concentrations of fraction two without hCG (Figure 5.18).

Progesterone synthesis by OVCAR-3 cells when treated with fraction two without hCG was significantly inhibited after 4h at all concentrations tested (Figure 5.17B). However, this decrease in progesterone synthesis was only noted after 24h treatment at concentrations of 0.005 and 0.5mg/mL fraction two without hCG, and no significant effect was noted after 48h (Fraction 5.17B). Furthermore, when OVCAR-3 cells were treated with fraction two in the presence of hCG the only significant inhibition of progesterone synthesis was noted after 4h of treatment and at concentrations of 0.005 and 0.5mg/mL (Figure 5.17E).

Estradiol synthesis by OVCAR-3 cells was seen to increase after 24h treatment with fraction two (without hCG) at 0.05 and 0.005mg/mL (Figure 5.17C). This increase in estradiol was also noted in the presence of hCG after 4h treatment with fraction two at 0.05mg/mL (Figure 5.17F). However, after 24h at the lowest concentration tested 0.005mg/mL, there was a significant decrease in estradiol secretion by OVCAR-3 cells treated with fraction two in the presence of hCG ($p < 0.01$; Figure 5.17F).

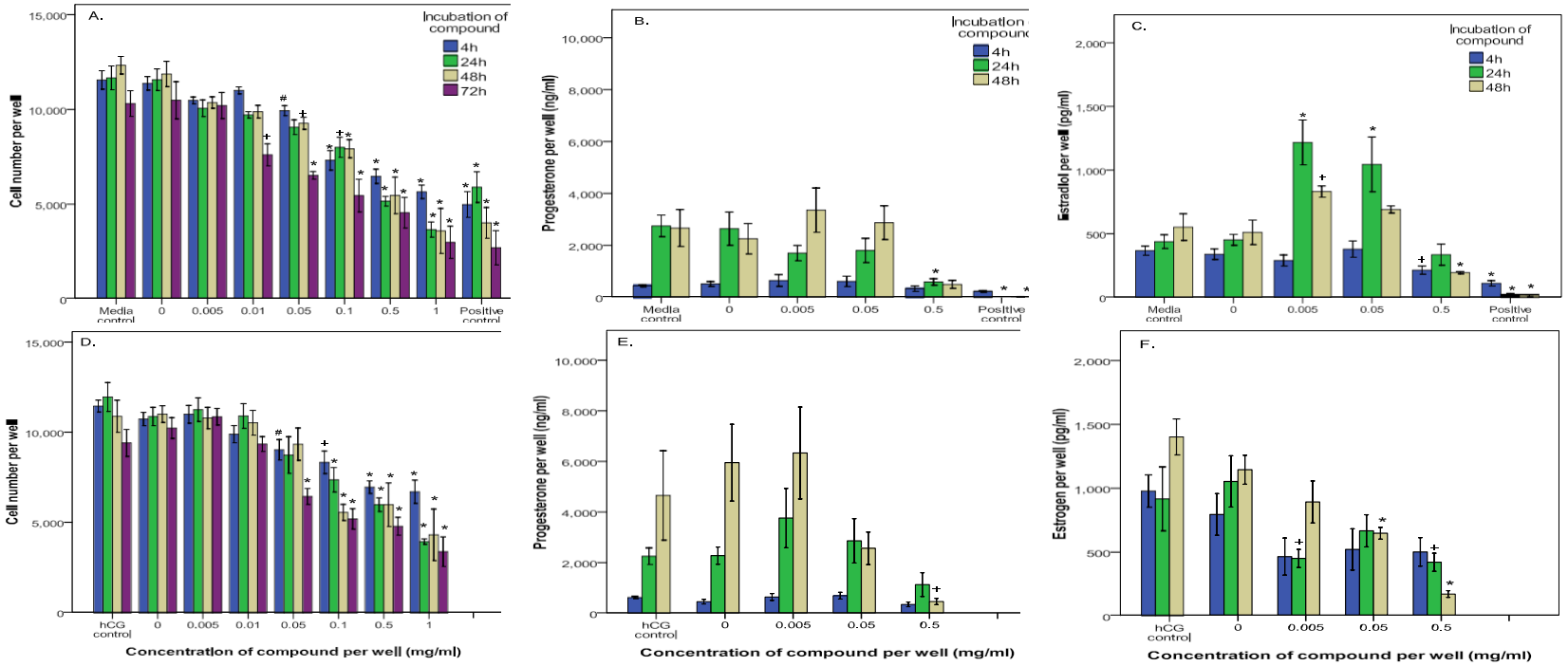


Figure 5.11: The effect of fraction two on primary-derived human granulosa cells

The effect of the semi-purified *D. orbita* fraction two on primary-granulosa cell viability, progesterone synthesis, estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction two ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

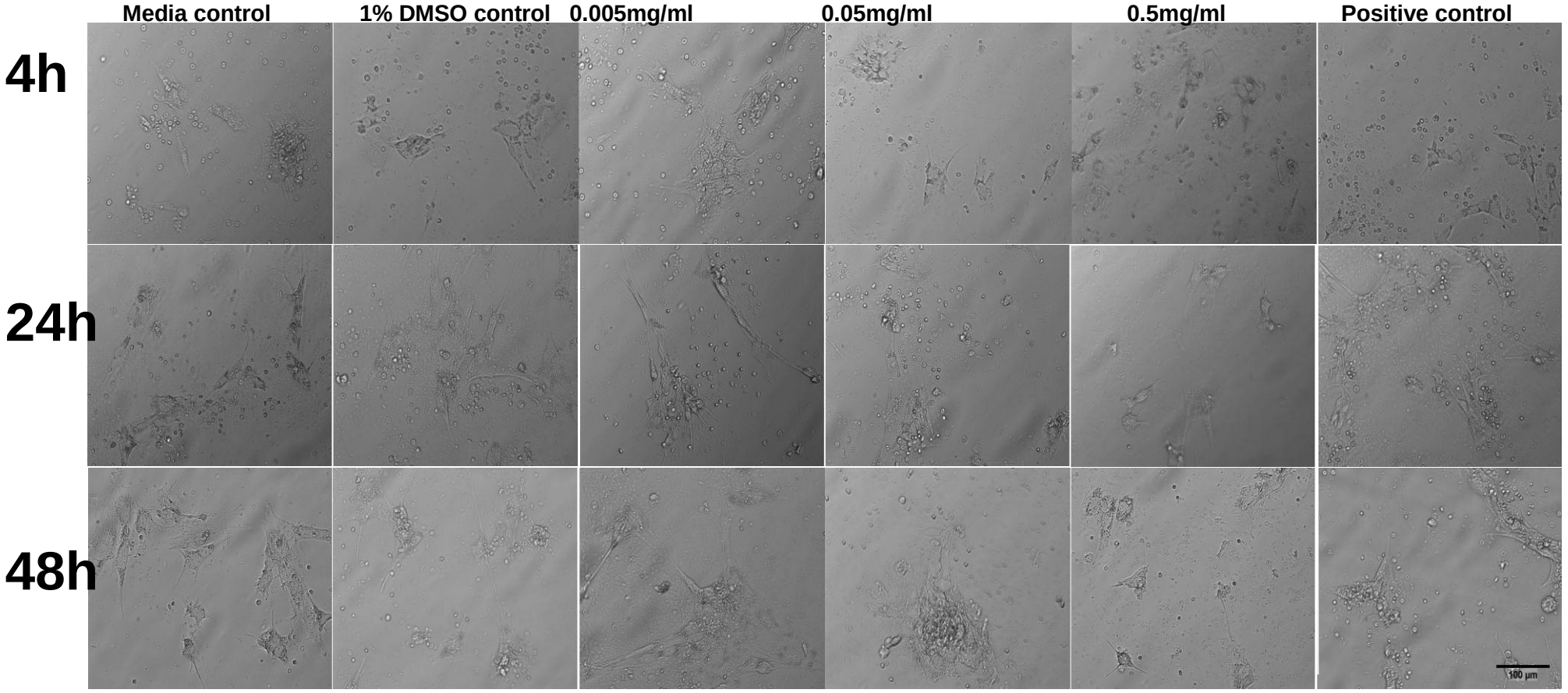


Figure 5.12: Morphological changes in the primary-derived human granulosa cells after treatment with fraction two
Morphological changes in the primary-derived human granulosa cells after treatment with the semi-purified *D. orbita* fraction two without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

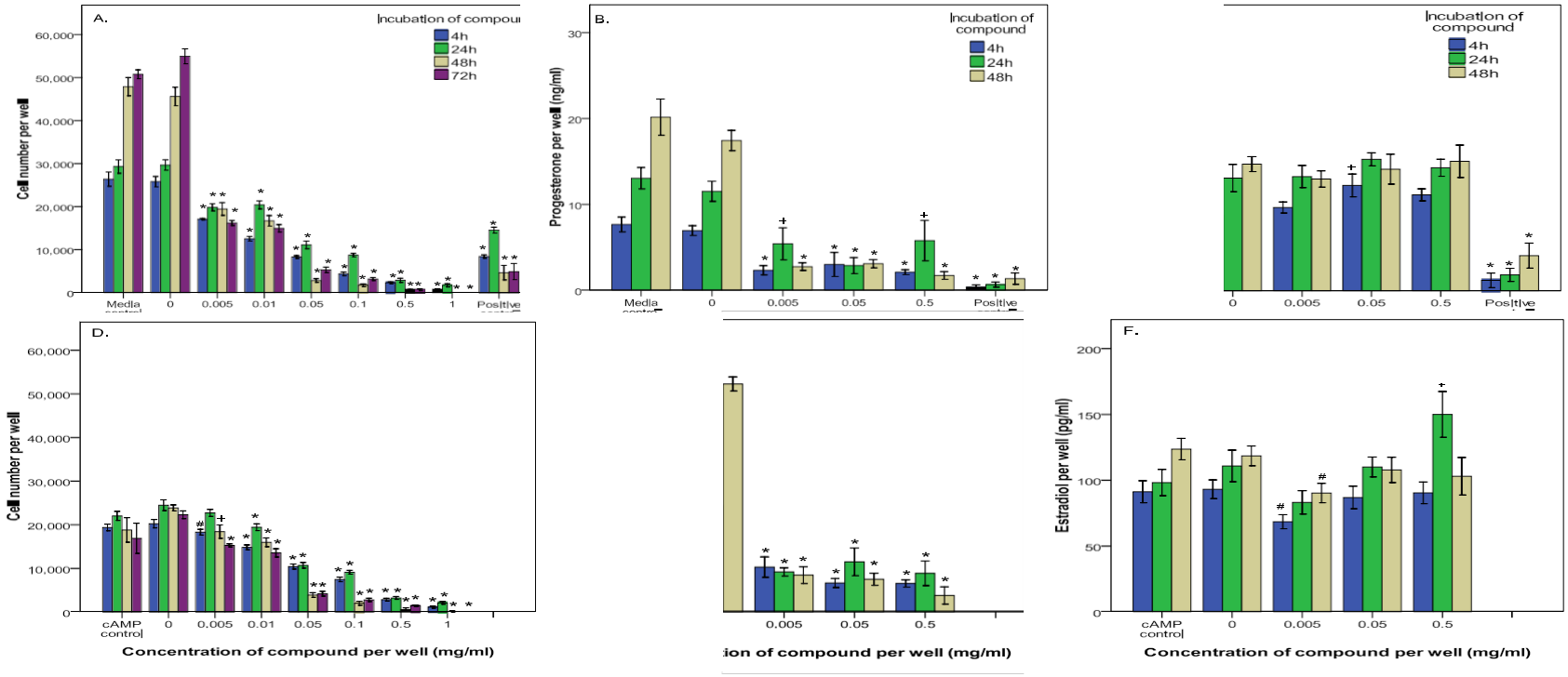


Figure 5.13: The effect of fraction two on KGN cells

The effect of the semi-purified *D. orbita* compound fraction two on KGN cell viability, progesterone synthesis, estradiol synthesis in the absence (A, B, C) and in the presence of db-cAMP (D, E, F). After an initial 24h cell attachment period KGN cells (20,000cells/well) were treated with fraction two ± db-cAMP for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

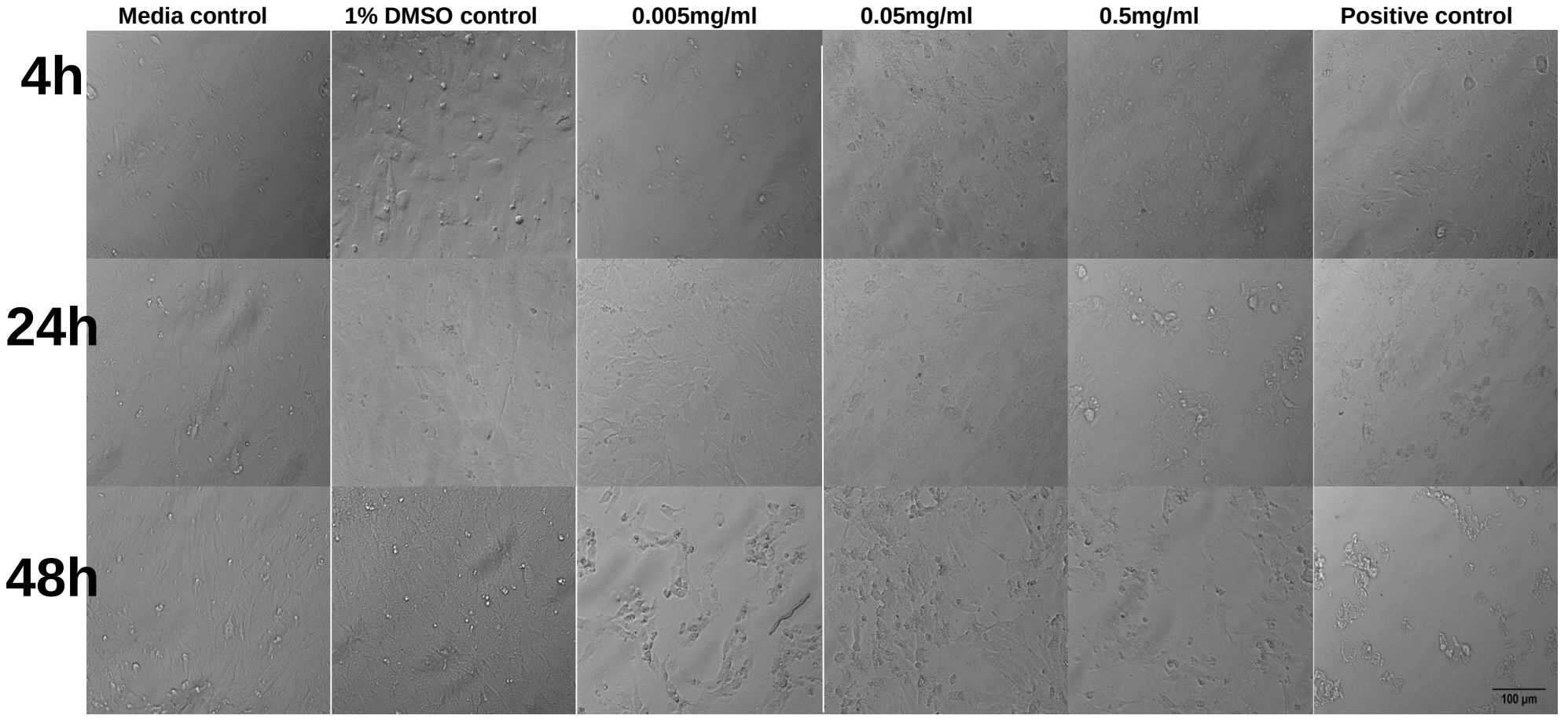


Figure 5.14: Morphological changes in the KGN granulosa cancer cell line after treatment with fraction two
Morphological changes in the KGN granulosa cancer cell line after treatment with the semi-purified *D. orbita* fraction two without db-cAMP after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

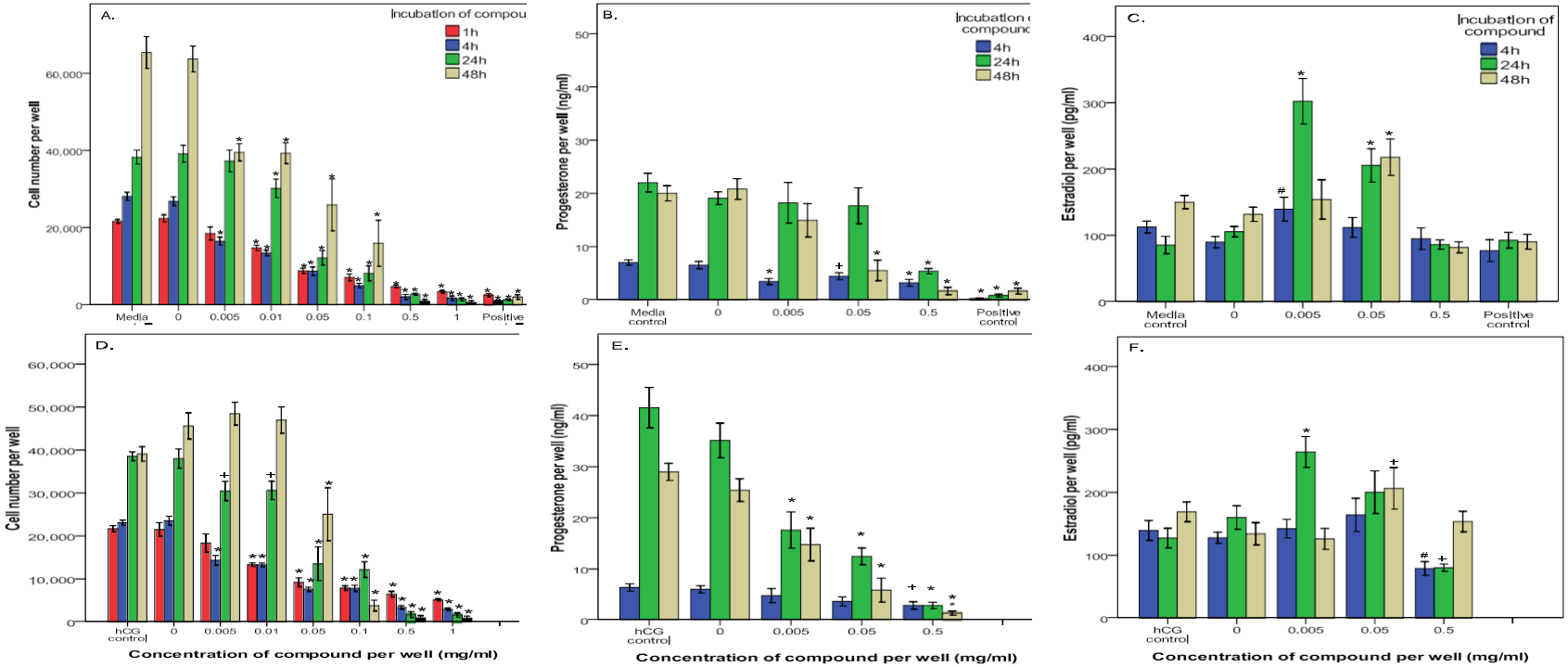


Figure 5.15: The effect of fraction two on JAr cells

The effect of the semi-purified *D. orbita* fraction two on JAr cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 2h cell attachment period JAr cells (20,000cells/well) were treated with fraction two ± hCG for 1, 4, 24 and 48h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

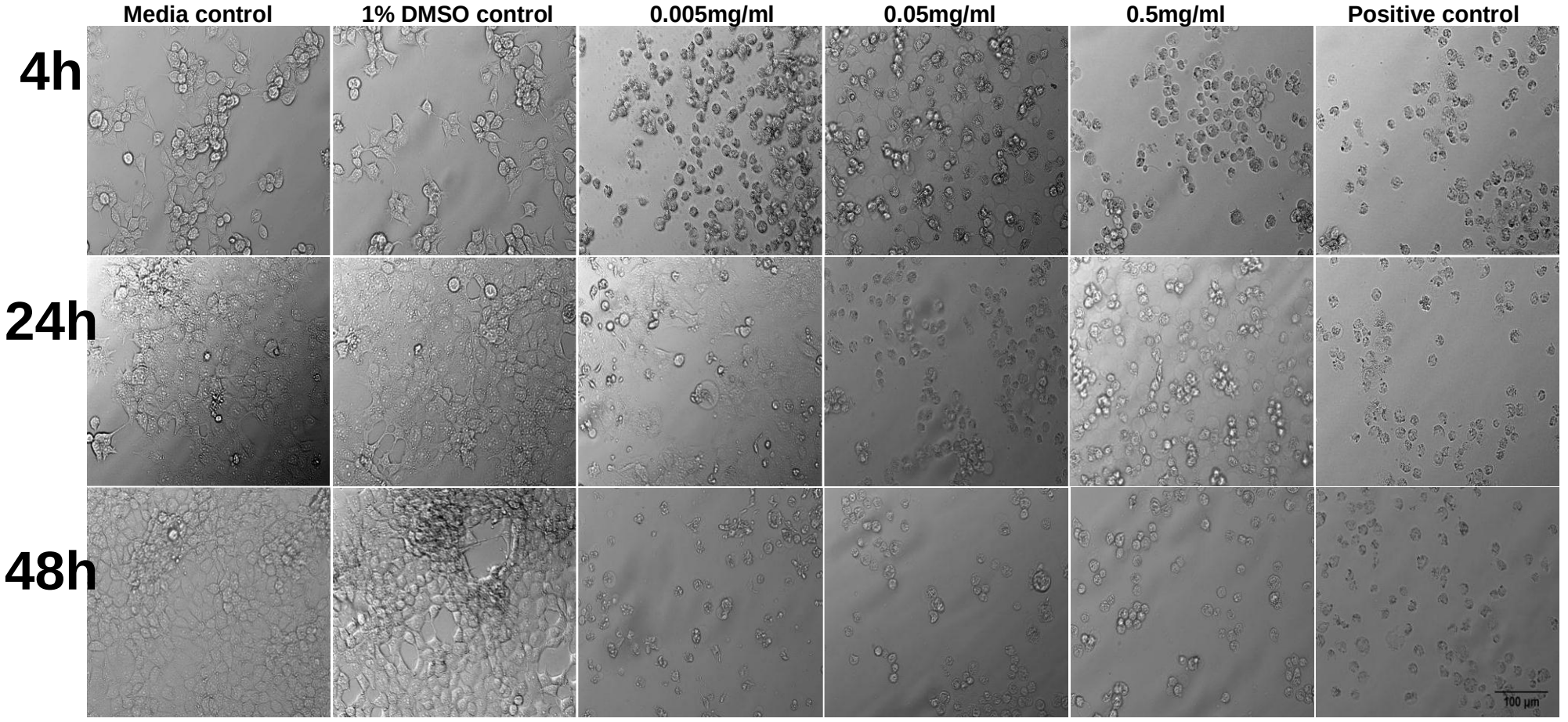


Figure 5.16: Morphological changes in the JAr choriocarcinoma cell line after treatment with fraction two
Morphological changes in the JAr choriocarcinoma cell line after treatment with the semi-purified *D. orbita* fraction two without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

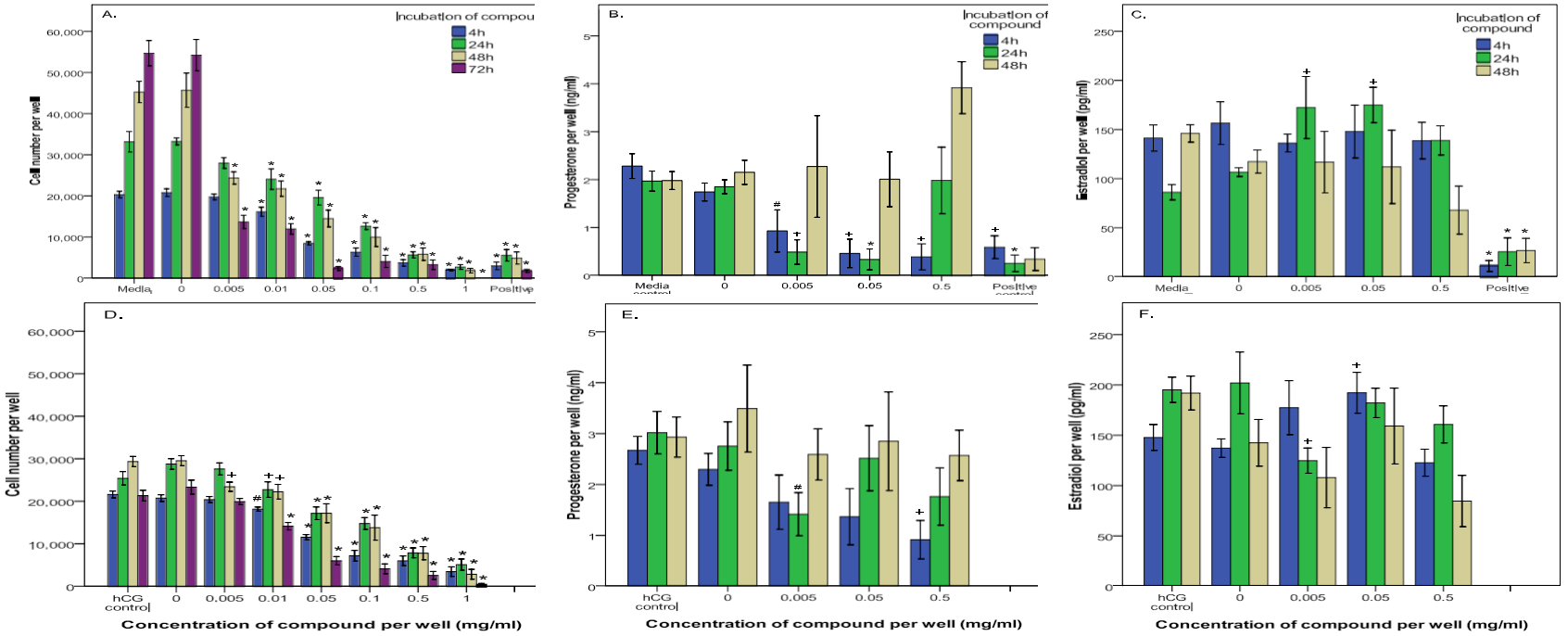


Figure 5.17: The effect of fraction two on OVCAR-3 cells

The effect of the semi-purified *D. orbita* compound fraction two on OVCAR-3 cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period OVCAR-3 cells (20,000cells/well) were treated with fraction two ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

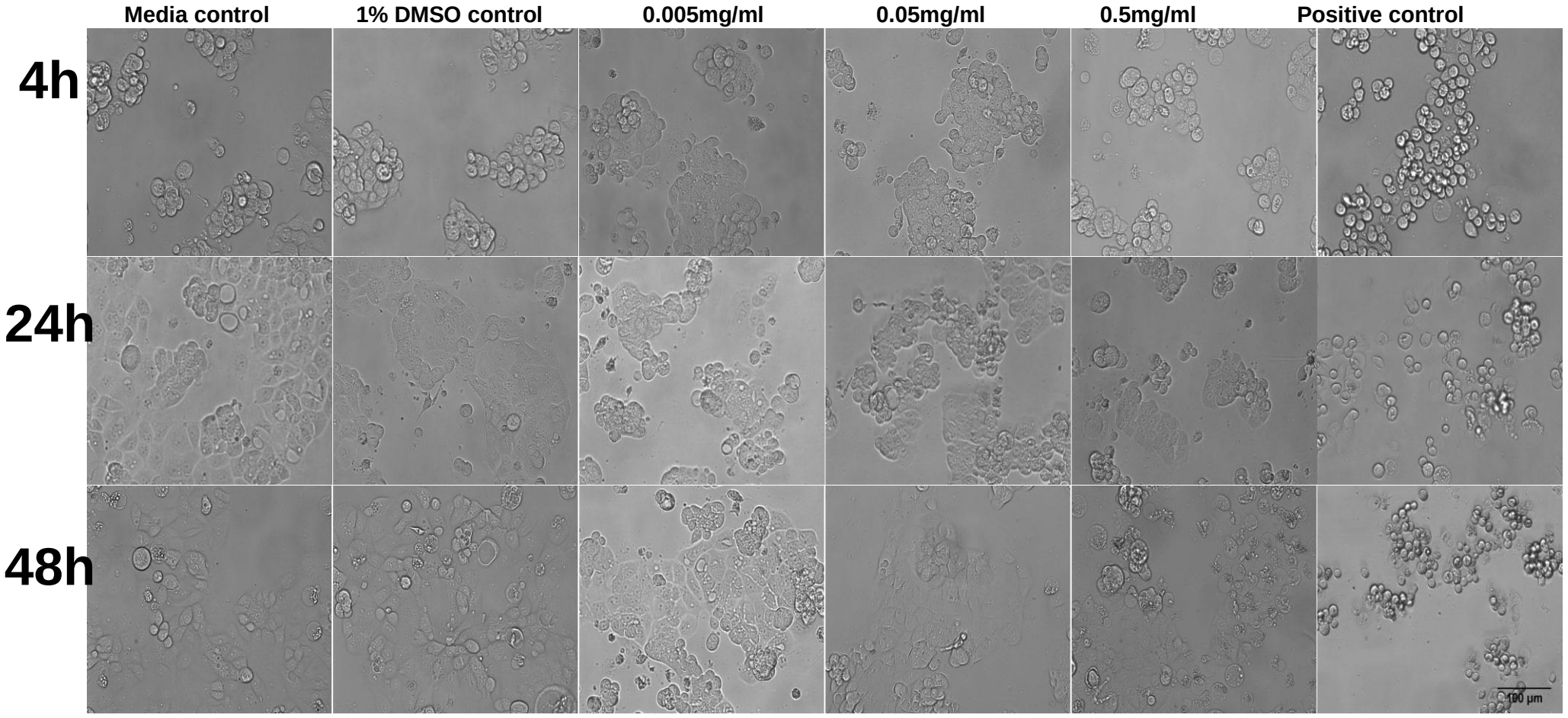


Figure 5.18: Morphological changes in the OVCAR-3 ovarian cancer cell line after treatment with fraction two
Morphological changes in the OVCAR-3 ovarian cancer cell line after treatment with the semi-purified *D. orbita* fraction two without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

5.3.4.3 Effects of Fraction Three on Cell Viability & Hormone Synthesis

Fraction three containing the bioactive compound 6-bromoisatin (Figure 5.2C) demonstrated greater activity towards the three reproductive cancer cell lines in comparison to the primary granulosa cells; with a magnitude of 10-fold or more, as shown by the LC_{50}'s (Table 5.5). However, in the presence of hCG or db-cAMP fraction three wasn't as cytotoxic towards the three cancer cell lines (Table 5.5). Like fraction two (Table 5.4), fraction three was generally more cytotoxic than fraction one (Table 5.3) to the reproductive cancer cell lines, although not as toxic as fraction two. Progesterone synthesis by all cell lines was inhibited when treated with fraction three ± hCG or db-cAMP (Table 5.5). In comparison estrogen synthesis by KGN and JAr cells was stimulated with fraction one without db-cAMP or hCG but inhibited in the presence of db-cAMP or hCG.

Table 5.5: Results of Fraction three on Reproductive Cells

Effects of Fraction three (F3) ± hCG (primary-derived human granulosa, JAr and OVCAR-3 cells) or db-cAMP (KGN cells) on cell viability, progesterone and estradiol synthesis. Cell viability shown as LC_{50} concentration that had an effect on 50% of the cells, & hormone data shown as concentration that was significantly different to the 1% DMSO control GC = primary-derived human granulosa cells; hCG = human chronic gonadotrophin; db-cAMP = dibutyryl adenosine 3‘,5‘-cyclic monophosphate; LC_{50} = concentration that had an effect on 50% of the cells; F3= Fraction three; F3 + hCG/db-cAMP = Fraction three in the presence of hCG or db-cAMP; (+)concentration that stimulated hormone synthesis; (-) concentration that inhibited hormone synthesis; N/T = not tested; n/e = no effect

	Time (h)	**LC_{50} (mg/mL)**		**Effect on hormone synthesis (mg/mL**			
				Progesterone		Estradiol	
Conc. 0005, 0.01, 0.05, 0.1, 0.5, 1.0mg/mL		F3	F3 + hCG/db-cAMP	F3	F3 + hCG/db-cAMP	F3	F3 + hCG/db-cAMP
GC	1	N/T	N/T	N/T	N/T	N/T	N/T
KGN		N/T	N/T	N/T	N/T	N/T	N/T
JAr		0.5	0.1	N/T	N/T	N/T	N/T
OVCAR-3		N/T	N/T	N/T	N/T	N/T	N/T
GC	4	>1	>1	0.005 (-)	n/e	0.005, 0.5 (-)	≤0.05 (-)
KGN		0.04	0.1	≤0.5 (-)	≤0.5 (-)	0.005, 0.5 (+)	n/e
JAr		0.05	0.06	≤0.05 (-)	0.005 (-)	0.5 (+)	≤0.5 (-)
OVCAR-3		0.09	0.1-0.05	0.05 (-)	0.05 (-)	n/e	n/e
GC	24	>1	>1	n/e	n/e	n/e	n/e
KGN		0.1	0.3	≤0.5 (-)	≤0.5 (-)	0.05, 0.5 (+)	0.05 (-)
JAr		0.1	0.1	n/e	≤0.5 (-)	0.05, 0.5 (+)	n/e
OVCAR-3		0.1	0.1-0.05	n/e	n/e	n/e	<0.5 (-)
GC	48	0.4	0.8	n/e	n/e	n/e	<0.5 (-)
KGN		0.005	0.04	≤0.5 (-)	≤0.5 (-)	n/e	n/e
JAr		0.1	0.4	0.005, 0.5 (-)	≤0.5 (-)	n/e	n/e
OVCAR-3		0.02	0.1-0.5	n/e	0.05, 0.5 (-)	n/e	0.005 (-)
GC	72	0.5	0.1	N/T	N/T	N/T	N/T
KGN		<0.005	0.02	N/T	N/T	N/T	N/T
JAr		N/T	N/T	N/T	N/T	N/T	N/T
OVCAR-3		<0.005	0.2	N/T	N/T	N/T	N/T

5.3.4.3.1 Primary-Derived Human Granulosa Cells

Fraction three (in the absence of hCG) after 4h treatment, was not cytotoxic to primary granulosa cells until the concentration reached 0.05mg/mL and cells were reduced to 83% of control (Figure 5.19A). However, as the incubation period increased to 72h fraction three (without hCG) significantly reduced primary granulosa cells at the lowest concentration tested, 0.005mg/mL (to 66% of control; $p < 0.001$; Figure 5.19A). In the presence of hCG after 72h, fraction three was only found to be significantly cytotoxic to primary granulosa cells after exposure to 0.01mg/mL (65% of control; $p < 0.001$; Figure 5.19D). There was no effect on primary-granulosa cell morphology with fraction three (Figure 5.20).

Fraction three only inhibited progesterone synthesis by primary granulosa cells after 4h at 0.005mg/mL and in the absence of hCG ($p < 0.01$; Figure 5.19B). However, estradiol synthesis by primary granulosa cells was inhibited at 0.005 and 0.5mg/mL after 4h treatment of fraction three in the absence of hCG, although at longer incubation period (24 and 72h) no significant inhibition of estradiol synthesis was noted (Figure 5.19C). In contrast when primary granulosa cells were exposed to fraction three in the presence of hCG estradiol production was significantly inhibited after 4 and 48h treatment and at all concentrations tested (Figure 5.19F).

5.3.4.3.2 KGN Cell Line

In comparison to the primary granulosa cells the KGN cells were significantly reduced at all concentrations of fraction three and at all incubation periods in the absence of db-cAMP (LC_{50} 0.04mg/mL at 4h; Figure 5.21A). After 4h of 0.005mg/mL fraction three without db-cAMP KGN cells were reduced to 71% of control (Figure 5.21A), whereas under the same conditions primary granulosa cells were only reduced to 98% of control (Figure 5.21A). After longer exposure times

(48h) with fraction three and at the lowest concentration 0.005mg/mL tested without db-cAMP, KGN cell viability was reduced to 48% of control (Figure 5.21A). In comparison primary granulosa cells treated under the same conditions were only reduced to 96% of the control (Figure 5.11A). However, in the presence of db-cAMP, fraction three only significantly inhibited the mitochondrial enzymes of the KGN cells at the lowest concentration tested (0.005mg/mL) after 48h of treatment ($p < 0.01$; Figure 5.21A). Furthermore, after 72h of treatment with fraction three in the presence of db-cAMP cell viability was only significantly inhibited at 0.1mg/mL ($p < 0.001$; Figure 5.21B). Cell rounding, shrinkage and detachment indicated cell damage after the KGN cells were treated fraction three (Figure 5.22).

Fraction three ± db-cAMP significantly inhibited progesterone synthesis by the KGN cells at all concentrations and all incubation periods (Figure 5.21B and E). In comparison, estradiol synthesis by KGN cells was significantly increased at 0.005 and 0.5mg/mL of fraction three (without db-cAMP) after 4h treatment (Figure 5.21C). This increase in estradiol synthesis was also noted after 24h treatment at 0.05 and 0.5mg/mL with fraction three and in the absence of db-cAMP (Figure 5.21C). In the presence of db-cAMP the only observe effect on estradiol synthesis by the KGN cell line was noted after 24h treatment with fraction three at the concentration of 0.05mg/mL where estradiol synthesis was slightly inhibited (Figure 5.21F).

5.3.4.3.3 JAr Cell Line

Fraction three (without hCG) showed an anti-proliferative effect on the JAr cell line after the short incubation periods of 1, and 4h at all concentrations tested (LC_{50} 0.04mg/mL at 1h and 0.05mg/mL at 4h; Figure 5.23A). After 4h at 0.005mg/mL of fraction three without hCG, JAr cells were reduced to 70% of control (Figure 5.23A).

After 24h there was a decrease in JAr cell numbers when treated with fraction three (0.005 and ≥ 0.05mg/mL) in the absence of hCG (Figure 5.23A). However, by 48h of treatment with fraction three (without hCG) there was a significant decrease in JAr cells at all concentrations tested, with cells reduced to 60% of control at 0.005mg/mL fraction three ($p < 0.001$; Figure 5.23A). In comparison, in the presence of hCG fraction three only significantly reduced JAr cell numbers after 4h ≥ 0.005mg/mL and not after 1h (Figure 5.23D). However, less activity was observed after 24 and 48h when JAr cells were treated with fraction three in the presence of hCG (Figure 5.23D). After 48h, a significant effect on JAr cell viability was only noted at 0.1mg/mL of fraction three in the presence of hCG with cells reduced to 73% of control (Figure 5.23A). Cell shrinkage, damage and detachment from wells was noted at the lowest concentrations of fraction three (0.005mg/mL) at 4 and 24h and at 0.05mg/mL after 48h (Figure 5.24).

A notable reduction in progesterone synthesis by the JAr cells was seen after 4h treatment of fraction three without hCG at the concentrations of 0.005 and 0.05mg/mL (Figure 5.23B). After 48h progesterone synthesis was also inhibited at the lowest concentration of fraction thre (0.005mg/mL in the absence of hCG (Figure 5.23B). However, in the presence of hCG, fraction three significantly inhibited progesterone synthesis after 24 and 48h at all concentrations tested (Figure 5.23E). In comparison, estradiol secretion by the JAr cells was seen to increase after 24h at 0.05 and 0.5mg/mL of fraction three without hCG and after 4h at 0.5mg/mL (Figure 5.23C). Estradiol synthesis by JAr cells treated with fraction three in the presence of hCG was significantly inhibited after 4h at ≥ 0.005mg/mL (Figure 5.23F).

5.3.4.3.4 OVCAR-3 Cell Line

Fraction three had a greater anti-proliferative effect on OVCAR-3 cells in the absence of hCG (LC_{50} 0.09mg/mL at 4h; Figure 5.25A). At the lowest concentration tested (0.005mg/mL) fraction three in the absence of hCG significantly reduced OVCAR-3 cells to 60% of control after 48h ($p < 0.001$; Figure 5.25A). After 4h treatment there was a significant effect on OVCAR-3 cell viability at ≥ 0.05mg/mL of fraction three ± hCG and at ≤ 48h ($p < 0.001$; Figure 5.25A and D). Changes to OVCAR-3 cell morphology indicated cell damage and detachment from wells after treatment with fraction three in the absence of hCG (Figure 5.26).

Progesterone synthesis by the OVCAR-3 cell line was significantly inhibited in the presence of fraction three ± hCG at 0.05mg/mL after 4h treatment ($p < 0.01$ without hCG and $p < 0.5$ with hCG; Figure 5.25B and E). However, fraction three in the presence of hCG inhibited OVCAR-3 progesterone synthesis after 48h at 0.05 and 0.5mg/mL ($p < 0.05$; Figure 5.25E). Estradiol synthesis by the OVCAR-3 cell line was only inhibited by fraction three in the presence of hCG and after 24h at ≤ 0.5mg/mL and after 48h at 0.005mg/mL (Figure 5.25F).

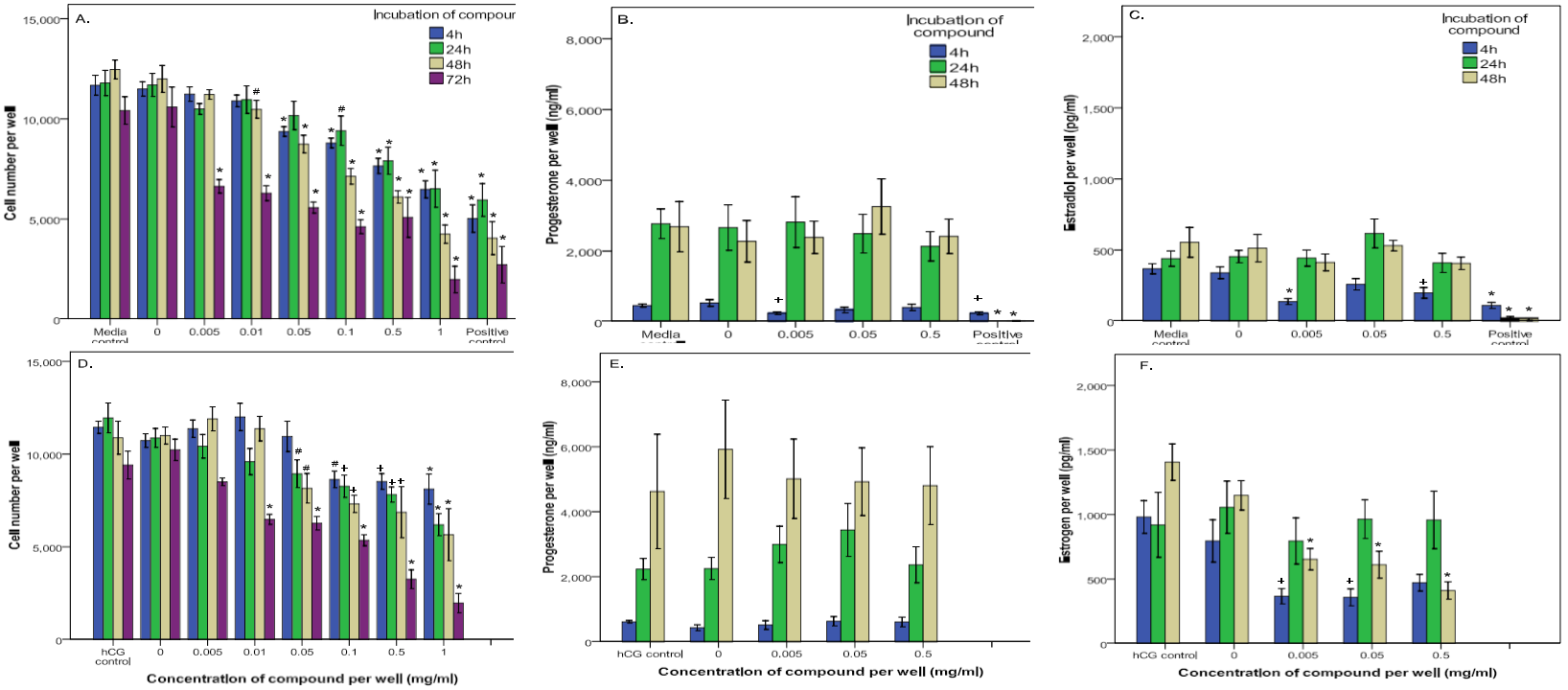


Figure 5.19: The effect of fraction three on primary-derived human granulosa cells

The effect of the semi-purified *D. orbita* compound fraction three on primary-granulosa cell viability, progesterone synthesis, estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction three ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*) and the alpha value was adjusted to $p < 0.01$ (**).

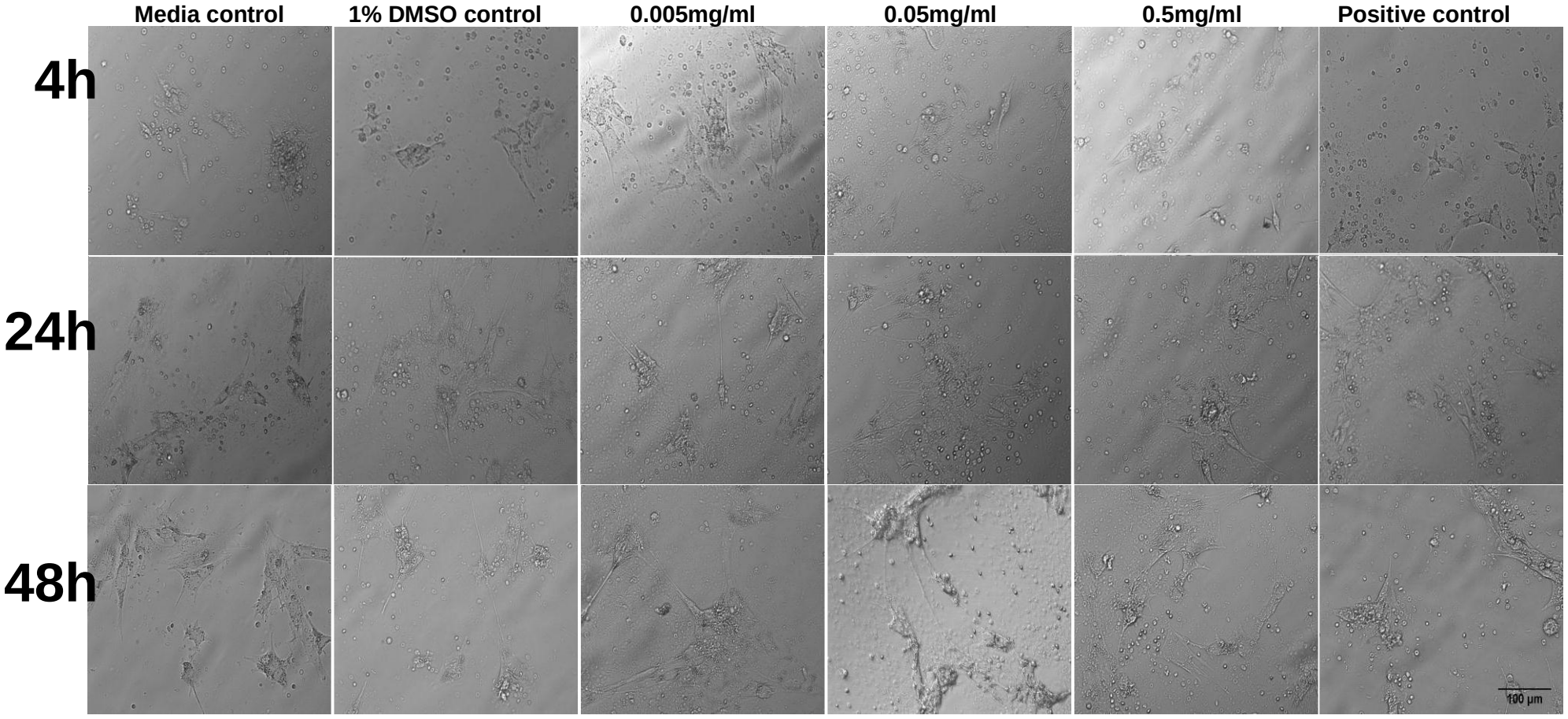


Figure 5.20: Morphological changes in the primary-derived granulosa cells after treatment with fraction three
Morphological changes in the primary-derived granulosa cells after treatment with the semi-purified *D. orbita* fraction three without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

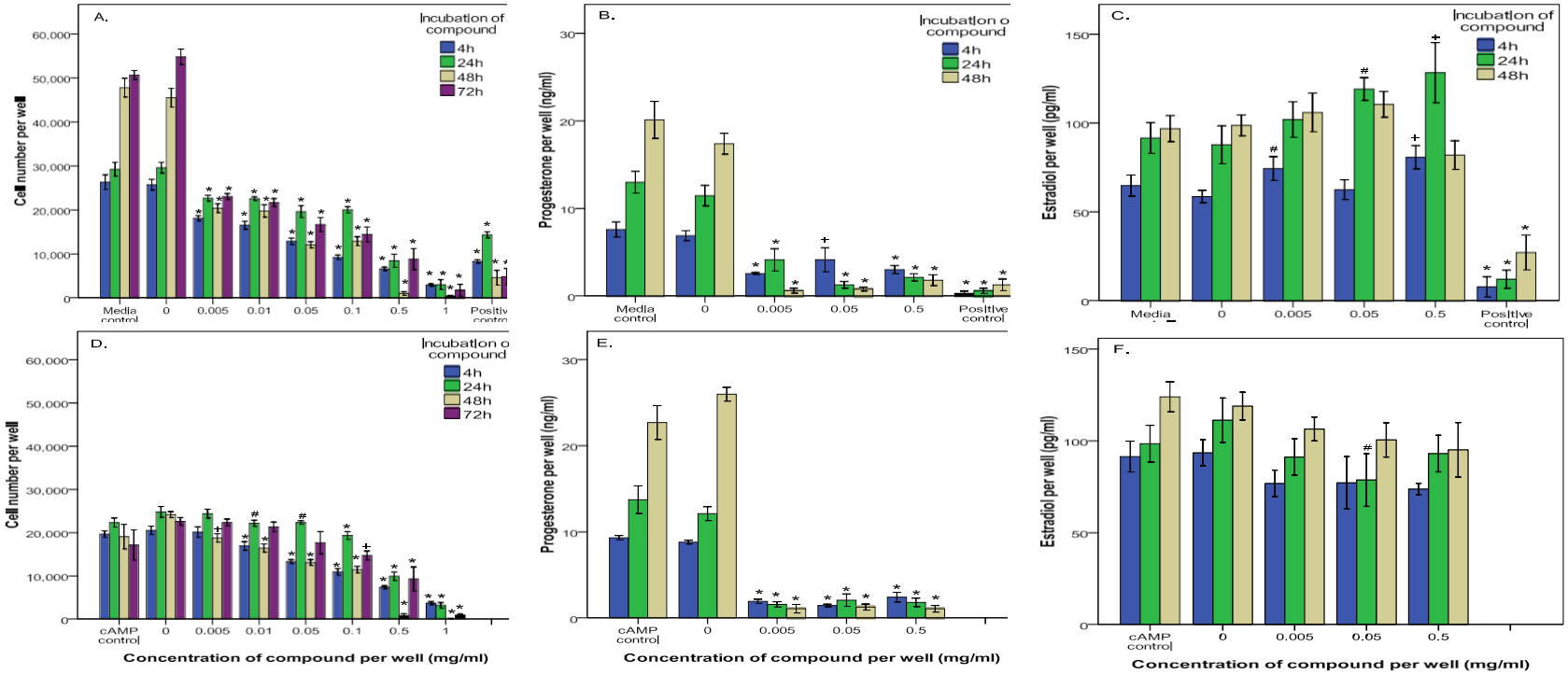


Figure 5.21: The effect of fraction three on KGN cells

The effect of the semi-purified *D. orbita* compound fraction three on KGN cell viability, progesterone synthesis, estradiol synthesis in the absence (A, B, C) and in the presence of db-cAMP (D, E, F). After an initial 24h cell attachment period KGN cells (20,000cells/well) were treated with fraction three ± db-cAMP for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

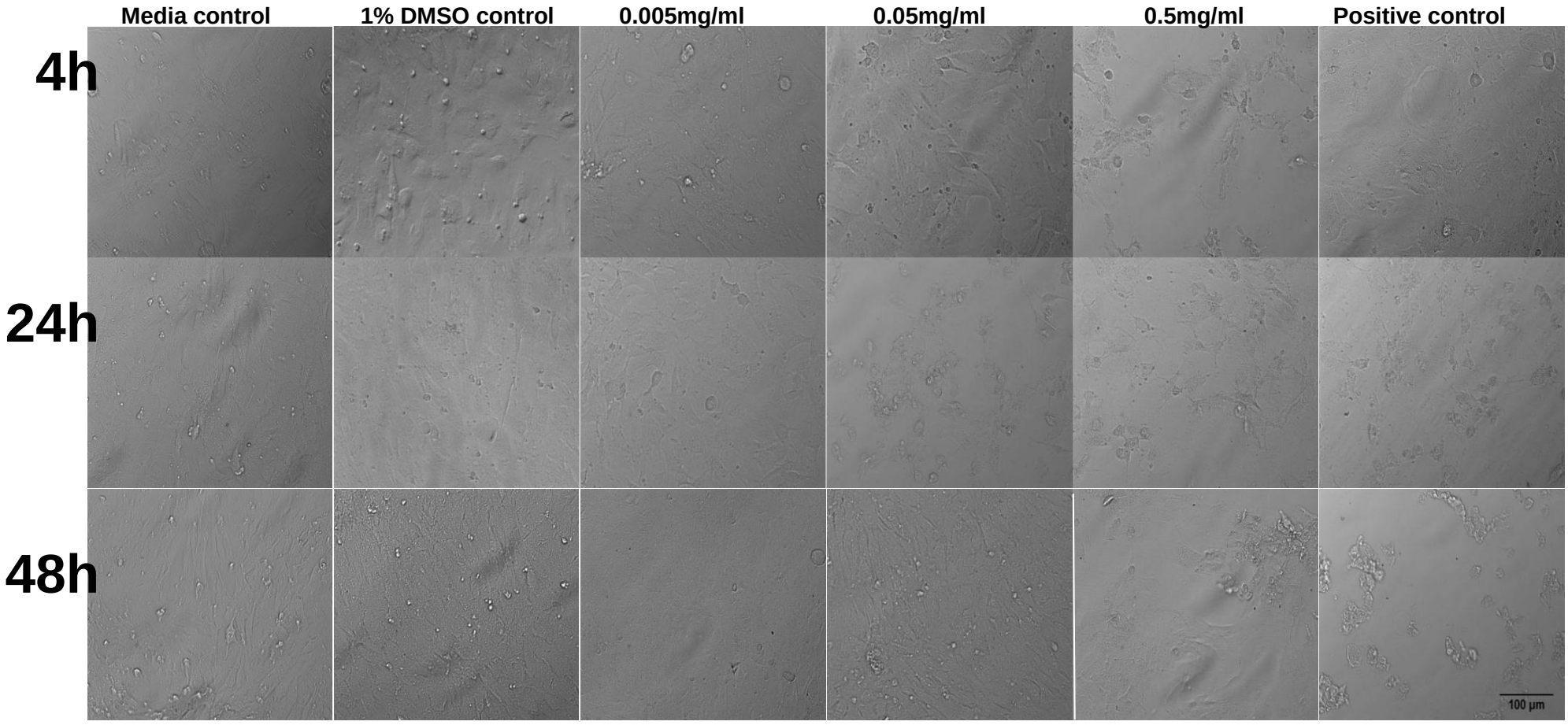


Figure 5.22: Morphological changes in the KGN granulosa cancer cell line after treatment with fraction three
Morphological changes in the KGN granulosa cancer cell line after treatment with the semi-purified *D. orbita* fraction three without db-cAMP after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

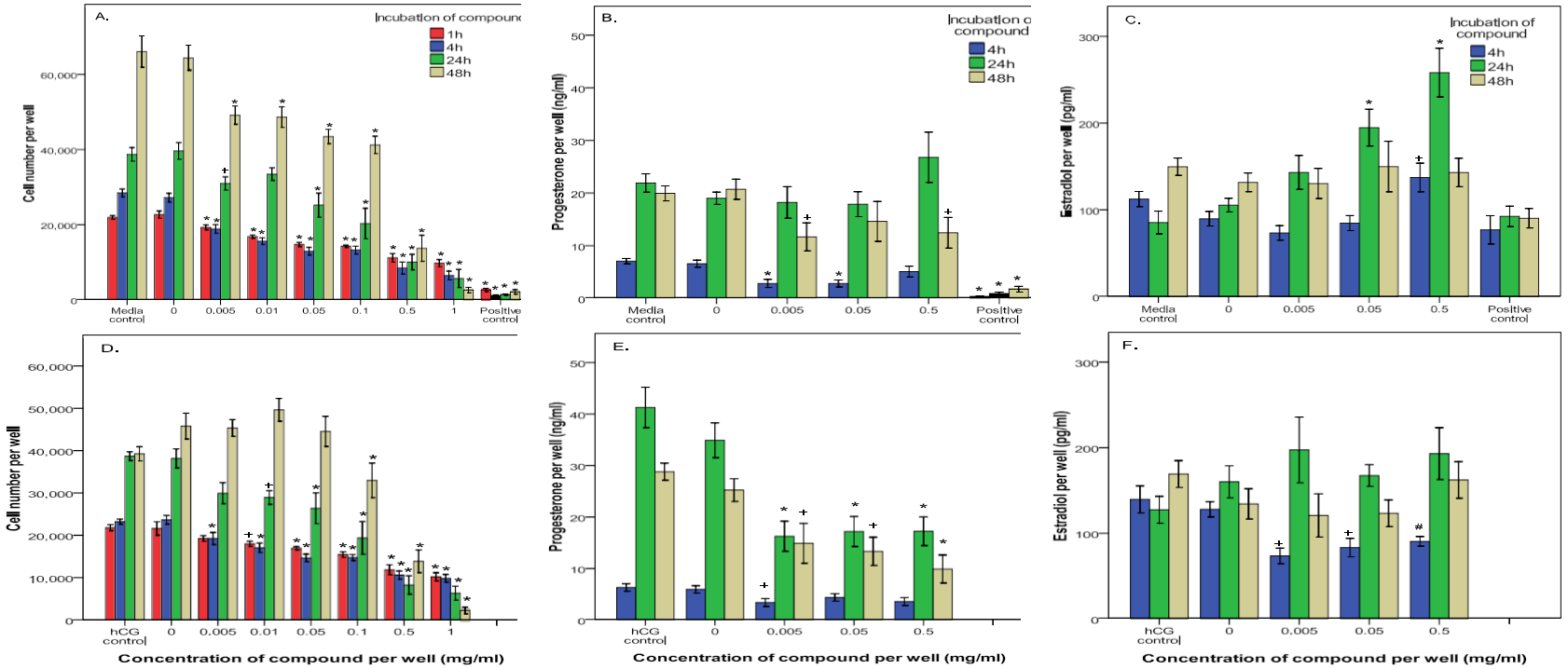


Figure 5.23: The effect of fraction three on JAr cells

The effect of the semi-purified *D. orbita* fraction three on JAr cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) and in the presence of hCG (D, E, F). After an initial 2h cell attachment period JAr cells (20,000cells/well) were treated with fraction three ± hCG for 1, 4, 24 and 48h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

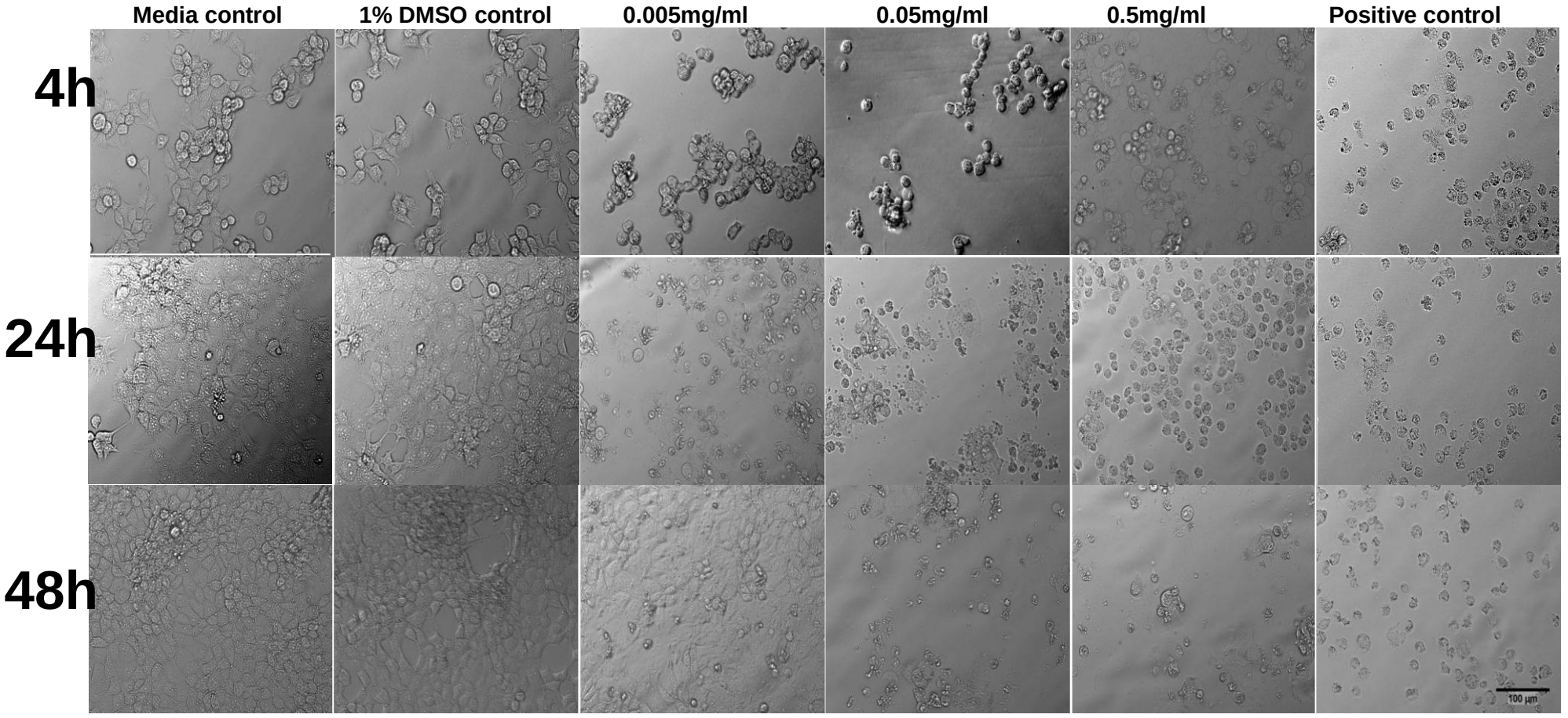


Figure 5.24: Morphological changes in the choriocarcinoma JAr cell line after treatment with fraction three
Morphological changes in the choriocarcinoma JAr cell line after treatment with the semi-purified *D. orbita* fraction three without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

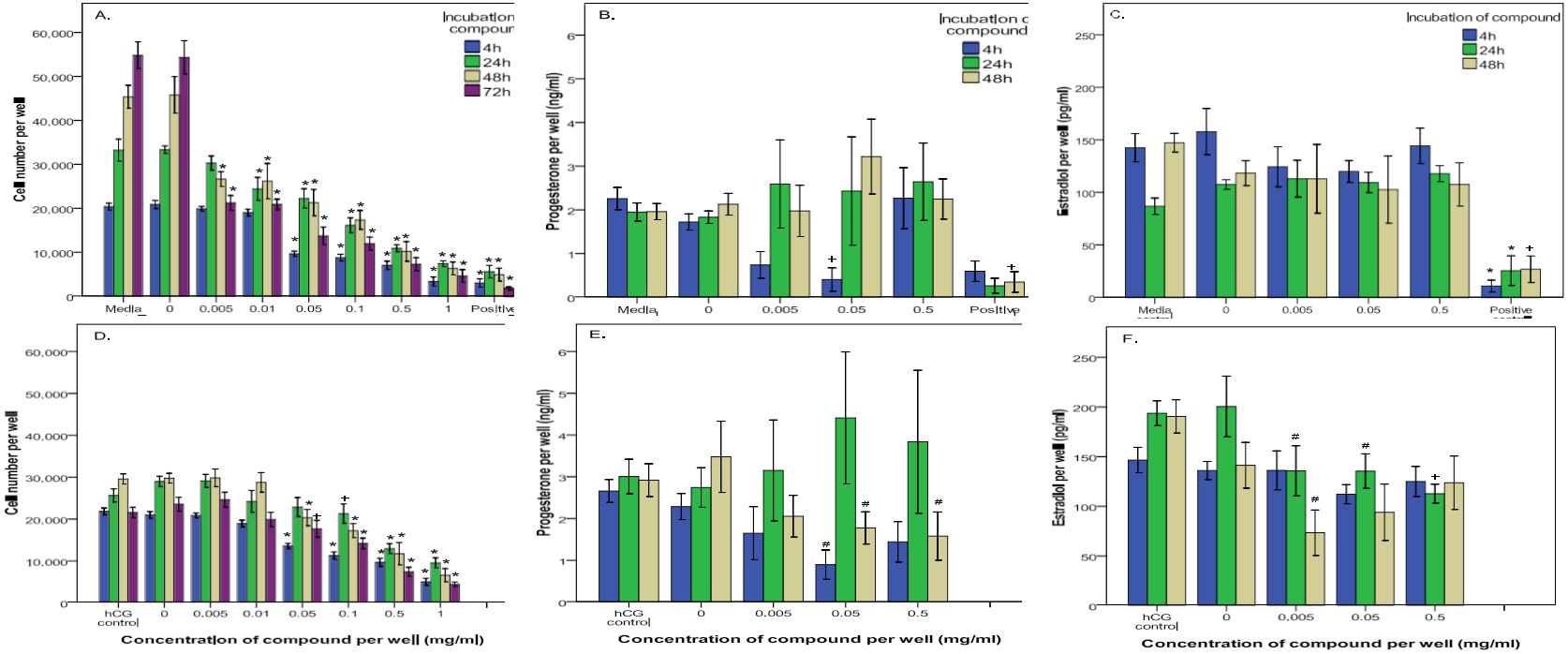


Figure 5.25: The effect of fraction three on OVCAR-3 cells

The effect of the semi-purified *D. orbita* fraction three on OVCAR-3 cell viability, progesterone synthesis and estradiol synthesis in the absence (A, B, C) in the presence of hCG (D. E, F). After an initial 24h cell attachment period OVCAR-3 cells (20,000cells/well) were treated with fraction three ± hCG for 4, 24, 48 and 72h. Cell viability was determined by the MTT assay at 570nm with reference absorbance 630nm and hormone synthesis by RIA. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

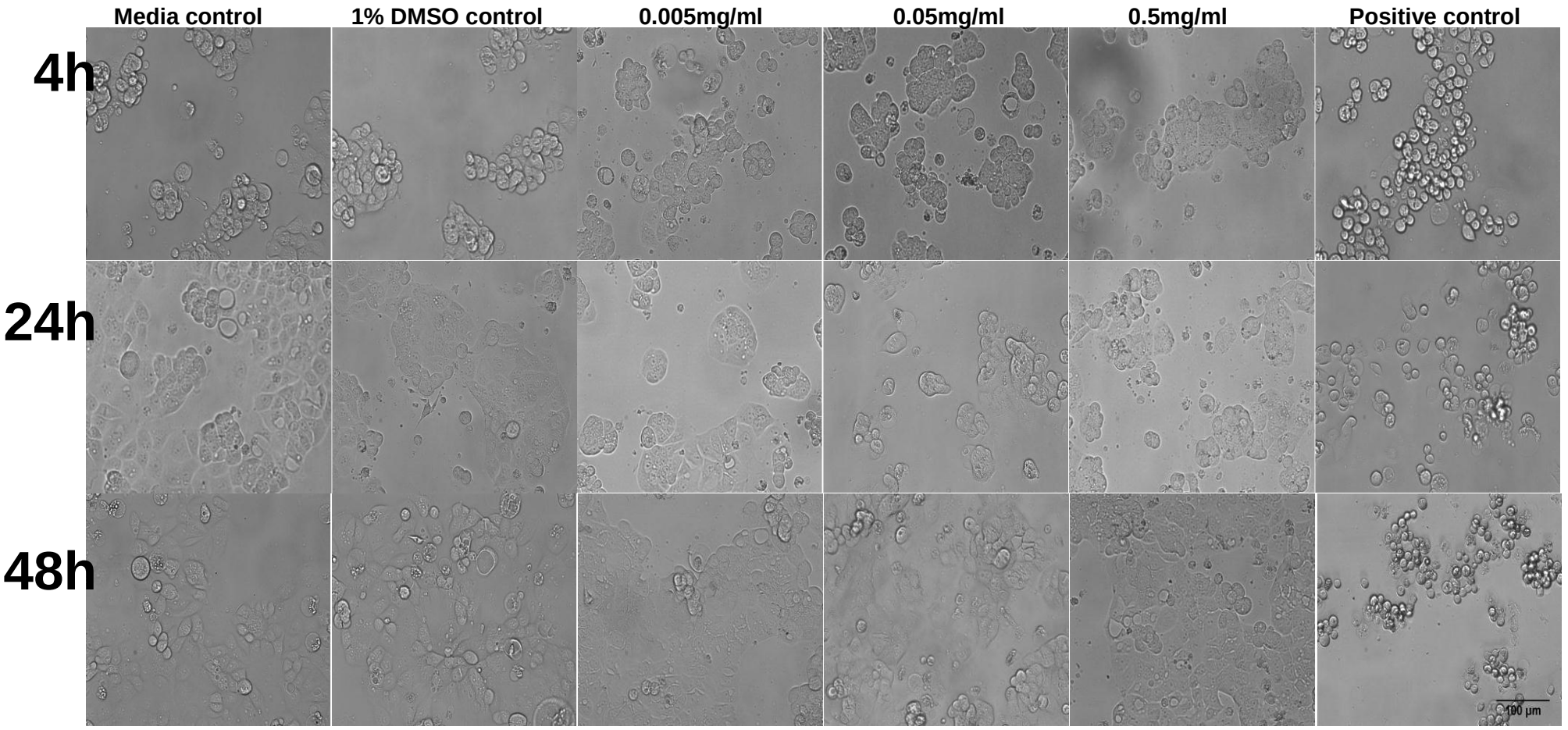


Figure 5.26: Morphological changes in the OVCAR-3 ovarian cancer cell after treatment with fraction three
Morphological changes in the OVCAR-3 ovarian cancer cell after treatment with the semi-purified *D. orbita* fraction three without hCG after 4, 24 and 48h compared to the DMSO control. Photographed at 200x magnification. Scale bar = 100µM

5.4 Discussion

After extracting a crude sample from the hypobranchial glands of specimens of *D. orbita,* semi-purification by flash silica chromatography was successful in concentrating the known bioactive compounds, tyrindoleninone, tyrindolinone and 6-bromoisatin, as has been previously reported (Benkendorff, *et al.*, 2011). Cell culture analysis of the semi-purified *D. orbita* fractions confirmed the hypothesis that these compounds demonstrated greater specificity towards the human reproductive cell lines, KGN, JAr and OVCAR-3, in comparison to the primary-derived human granulosa cells. This effect was more significant in the absence of hCG or db-cAMP for all three cancer cell lines and with all three fractions tested. Hence, the presence of hCG or db-cAMP in the culture medium afforded some protection to the cancer cell lines from the cytotoxic effects of the semi-purified *D. orbita* compounds. Previous research has shown that when hCG has been added to cell culture in the presence of known cytotoxic drugs, cell death in the choriocarcinoma, and ovarian cancer cell line is inhibited (Kuroda, *et al*., 1998; Lei, et al., 1999). This may explain why the *D. orbita* fractions had less cytotoxic effects on the reproductive cancer cell lines in the presence of hCG.

An interesting finding from this study, which contradicts other research (Kraemer, *et* al., 2001; Hamada, *et al*., 2005) was that hCG (1,000mIU/mL) and db-cAMP (1mM) inhibited cell proliferation in the KGN, JAr and OVCAR-3 cell lines in the absence of the *D. orbita* compounds. For example, Kramer *et al*., (2001) have shown that the addition of exogenous hCG or hCG together with FSH, increases estradiol (E_2) secretion by OVCAR-3 cells, which in turn increases cell proliferation, thereby promoting ovarian cancer growth. In contrast, other research has shown, *in vitro*, that

hCG (200mIU/mL), at a level produced during early pregnancy *in vivo*, decreases proliferation in ovarian epithelial cancer cell lines (Tourgeman, *et al*., 2002). Tourgeman *et al*., (2002) have further shown that, hCG (200mIU/mL) continues to inhibit cell proliferation in ovarian epithelial carcinomas in the presence of FSH (40mIU/mL). Other research has shown that hCG can inhibit apoptosis in cells. For example Kuroda *et al*., (1998) have demonstrated that hCG (0.1µg/mL) in the presence of cisplatin, a drug specifically designed to treat ovarian cancer, inhibits cisplatin-induced apoptosis in ovarian cancer cells. The same study did however demonstrate that OVCAR-3 cells do not proliferate in the presence of hCG alone; using the BrdU assay (Kuroda, *et al*., 1998).

Further studies have shown that hCG (300-1,000mIU/mL) added to JAr cells in culture, along with the large amount of hCG secreted by the cells, greatly up-regulates the LH/hCG receptors resulting in an increase in cell proliferation (Hamada, *et al*., 2005). Contrary to this, Lei *et al*., (1999), have demonstrated that hCG does not actually increase JAr cell proliferation. Instead, hCG prevents JAr cell death by inhibiting apoptosis of the cells (Lei, *et al*., 1999); which seems to support our findings herein. The authors of the same study hypothesized that choriocarcinoma cells rely on high serum levels of hCG for the tumour survival (Lei, *et al.*, 1999). There are contradictions in the literature regarding the effect of hCG on cell proliferation, however the only logical explanation we could interpret from our findings of hCG and db-cAMP inhibiting cell proliferation was that, in the presence of hCG or db-cAMP may be cellular energy is being redirected to other processes in the cell such as, hormone synthesis or differentiated functions, rather than cell growth and proliferation.

Despite this, in the presence or absence of hCG or db-cAMP all three semi-purified *D. orbita* fractions greatly inhibited the mitochondrial enzyme activity in the reproductive cancer cell lines, as opposed to the primary granulosa cells. This selectivity towards reproductive cancer cell lines over primary-derived human reproductive cells emphasizes the potential benefits of these naturally derived marine compounds in reproductive anticancer therapy. Fraction one, containing a mixture of brominated indole and isatin compounds was not as active towards the reproductive cancer cell lines in comparison to the more purified fractions, two and three; similar to previous studies (Benkendorff, *et al.,* 2011). However, cell viability was reduced considerably more in the human reproductive cancer cells, in comparison to previous studies with other tumour cell lines, including colorectal carcinomas (HT29 and CaCo2) and the MCF-7, breast cancer cell line (Benkendorff, *et al.*, 2011).

Fraction two containing tyrindoleninone and tyrindolinone, was the most active of all the three semi-purified fractions tested. Cell numbers were significantly reduced by fraction two after just 4h exposure, in all three reproductive cancer cell lines and at low concentrations (LC_{50} 0.01mg/mL KGN and JAr and 0.04mg/mL OVCAR-3), without significantly affecting primary granulosa cells (LC_{50} 0.9mg/mL). Previous studies have also shown that fractions containing tyrindoleninone are more selective towards cancer cells (lymphoma cell line U937) in comparison to primary cells (human mononuclear cells; MNC) *in vitro* (Benkendorff, *et al*., 2011). Furthermore, fraction two induced morphological changes associated predominately with apoptosis rather than necrosis in the KGN, JAr and OVCAR-3 cells; which is also consistent with previous findings *in vitro* studies (Benkendorff, *et al.*, 2011).

Compounds that interrupt the cell cycle and thus, inhibit the proliferation of cancer cells are increasing being investigated as potential new anticancer agents (Bacher, *et al*., 2001; Wang and Mencher, 2006). The mode of action of these compounds at present is unknown however this selective cytotoxic effect towards the reproductive cancer cell lines in the present study warrants further investigations for the use of this fraction in the treatment of granulosa cell tumours. Other research has shown that similar compounds, indolinones inhibit proliferation of cells by interfering with microtubule in cell division (Bacher, *et al.*, 2001; Chen, *et al.*, 2005c; Matesic, *et al.*, 2008). Chen *et a*l., (2005) have demonstrated that the novel indolinone A-432411 disrupts mitosis of proliferating cells by de-stabilizing microtubules and inhibiting spindle formation at low concentrations. The synthetic indole compound D-24851 has also been shown to destabilise microtubules in tumour cells by selectively blocking the cell cycle at the metaphase (Bacher, *et al*., 2001). These studies of structurally similar indole compounds, along with our findings of fraction two, containing the bioactive compound tyrindoleninone, highlight the potential of these brominated indole compounds in the developed of natural products for cancer therapy.

The relatively pure fraction three, containing 6-bromoisatin also demonstrated very promising results. 6-bromoisatin was less potent towards the primary-granulosa cells than the three reproductive cancer cell lines, KGN, JAr and OVCAR-3. In fact, exposure of KGN, JAr and OVCAR-3 cells to 6-bromoisatin for 24h, LC_{50} was 0.1mg/mL, in comparison to primary granulosa cells (LC_{50} > 1mg/mL) in the absence of hCG or db-cAMP. Studies have also shown that a range of substituted isatins including 5 and 7-dibromoisatin inhibit the mitochondrial enzymes and

therefore, decreases cell proliferation in a series of tumour cell lines including the lymphoma cell lines, U937 and Jurkat (Vine, *et al*., 2007a). Further research by Vine *et al*., (2007b) have shown that the novel 5,7-dibromo-N-(p-methylbenzyl)isatin is actually 22 times more active against U937 and Jurkat, then 5 and 7-dibromoisatin compounds. Furthermore, the 5,7-dibromo-N-(p-trifluoro-methylbenzyl)isatin compound was found to be at least 5 times more active against human cancer cell lines in comparison to human peripheral blood lymphocytes (Vine, *et al*., 2007b). Vine *et al.*, (2007b) along with similar studies, (Li, *et al*., 2005; Matesic, *et al*., 2008) have demonstrated that isatin compounds inhibit proliferation of cancer cells by activating caspase-3 and -7 effector enzymes and inhibiting microtubular formation thereby, inducing apoptosis. Further examination of cell changes in the reproductive cancer cell lines indicated that the cells predominately underwent apoptosis as opposed to necrosis in the presence of fraction three. In comparison, other research has shown 6-bromoisatin can induce both apoptosis or necrosis depending on the cell line tested (Vine, *et al.*, 2007a; Westley, *et al.*, 2010; Benkendorff, *et al.*, 2011). However, it should be noted that, the *in vivo* study by Westley *et al*., (2010), involved a crude mixture of the muricid compounds, not a semi-purified fraction containing, 6-bromoisatin. Therefore, it would be of interest to conduct studies to further investigate the mode of action of these brominated indole compounds on the reproductive cancer cell lines, including apoptosis and necrosis assays.

The potential beneficial effects of these semi-purified *D. orbita* fractions on reproductive cancer cell lines were promising, however the effects of these compounds on hormone synthesis were more cell and dose specific. Fraction one had no inhibitory effect on progesterone or estradiol synthesis by primary granulosa cells

at any concentration and any time point tested in the absence of hCG. In fact, basal estradiol synthesis by primary granulosa cells actually increased after 24 and 48h treatment (at 0.5mg/mL) with fraction one in the absence of hCG. Estrogen secretion is not only essential for reproductive functions in women, but also plays major role in maintaining healthy bones, heart and blood vessels (WHO, 1981). During menopause, the natural estrogen levels become depleted and as a result, hormone replacement therapy is often recommended (WHO, 1981). However, research indicates that women would rather take herbs or naturally derived therapies rather than drugs for their condition (Tesch, 2003). Therefore, this increase in estrogen synthesis by primary reproductive cells in the presence of fraction one could potentially be beneficial for women during menopause when the bodies' natural estrogen levels become depleted. However, at present, the concentration of the muricid fraction and time points that are generating these effects, are inconsistent and therefore would require further investigations before any solid conclusions could be made.

Synthesis of hormones by primary granulosa cells when treated with fraction two was also inconsistent as no clear patterns were evident. In the presence of fraction two, basal progesterone secretion by primary granulosa cells was either significantly inhibited (24), or not affected (4 and 48h). In comparison, basal estradiol synthesis by granulosa cells was inhibited at high concentrations of fraction two (0.5mg/mL) at 4 and 48h but stimulated at 24h at low concentrations. Furthermore, in the presence of hCG estradiol synthesis was often inhibited by fraction two however, it again was cell and dose specific. For example in the presence of db-cAMP of hCG, at 24h

treatment, fraction two inhibited estrogen synthesis by the cancer cell lines OVCAR-3 and KGN cell lines at the lowest concentration tested (0.005mg/mL).

Fraction three was also found to inhibit progesterone synthesis in all cell lines tested. In comparison basal estrogen was often stimulated by KGN and JAr cells but inhibited in the presence of hCG or db-cAMP. High endogenous estrogen is known to be associated with breast cancer in women (Dı´az-Cruz, *et al*., 2005). In fact, 60% of all breast cancers, are hormone-dependent cancers (Dı´az-Cruz, *et al*., 2005), because they specifically have receptors for estrogen and require estrogen for tumour growth (Dı´az-Cruz, *et al*., 2005; Kleinsmith, *et al*., 2006). As a result the development of estrogen blocking drugs (agonists) such as, Tamoxifen have been developed to block estrogen binding to its receptor thereby preventing proliferation of tumour growth (Kleinsmith, *et al*., 2006). Structurally similar compounds such as the indole-3-carbinol (I3C) compound, derived from cruciferous vegetables, has been reported to inhibit tumour growth in estrogen-sensitive cancers both *in vitro* and *in vivo*, including breast (Bradlow, *et al*., 1991), endometrical (Kojima, et al., 1994) and cervical cancer (Jin, *et al.*, 1999), and also in humans in clinical trials (Michnovicz and Bradlow, 1990; Michnovicz and Bradlow, 1991). Research has shown that I3C is only biologically active after being metabolised under highly acidic conditions such as, the acidic levels in the stomach, whereby I3C is metabolised into an active dimer, diindolylmethane (DIM) (Gao, *et al*., 2002; Auborn, *et al*., 2003). I3C/DIM once metabolised can then cause hydroxylation of estradiol at the C-2 position, to 2-hydroxyestrone, which is essentially nonestrogenic (Michnovicz and Bradlow, 1990). Further research has shown that the active dimer of I3C, diindolylmethane (DIM), acts by interfering with and blocking estrogen binding to its estrogen alpha receptor

(ERα), thereby interfering with the estrogen signalling pathway (Auborn, *et al.*, 2003). As both fraction two and three inhibited both growth and estrogen synthesis by the three cancer cells, these bioactive compounds could potentially be developed as a natural therapy for regulating endogenous estrogen thereby, assist in the prevention of hormone dependent cancers.

In conclusion, a major issue of developing new anti-cancer drugs is identifying candidates that can selectively inhibit cancer cell growth while having minimal or no effects on primary cells. The results of the *D. orbita* bioactive compounds assessed in this experiment demonstrate that the cytotoxicity of these indolinone and isatins compounds were amplified with the reproductive cell lines KGN, JAr and OVCAR-3 in comparison to the primary-granulosa cells. The hormone results varied between cell line and fraction tested, however the inhibitory effects on estrogen is worth investigation for estrogen-responsive cancers. Furthermore, the stimulatory effect of basal estrogen by primary granulosa cells also warrants investigation into a possible natural therapy for increasing estrogen in menopausal women.

Chapter 6 Characterisation of Cell Death in Reproductive Cells Induced by *D. orbita* Compounds

6.1 Introduction

Apoptosis or programmed cell death, although a highly complex signalling pathway (Chapter 1 Figure 1.10), is a normal physiological occurrence in eukaryotic cells (Elmore, 2007). Cell death by apoptosis involves death promoter proteins and a family of cysteine proteases called 'caspases' (Chapter 1 section 1.23; Elmore, 2007). This process involves intracellular biochemical events that produce morphological changes, including chromatin and nuclear condensation, DNA fragmentation, membrane blebbing and microspikes on the cell surface and eventually, the formation of apoptotic bodies (Figure 6.1; Gavrieli, *et al*., 1992; Kroemer, *et al*., 2005; Elmore, 2007; Kroemer, *et al*., 2009). The DNA of apoptotic cells is cleaved into 180-200bp fragments at the 3'OH end by the endonuclease, DNase (Wieder, 2008). Cells undergoing apoptosis shrink, and subtle changes to the cell membrane (blebbing) allows recognition by neighbouring cells, which phagocytise the apoptotic cell before the membrane breaks up and releases its contents; therefore preventing an inflammatory response (Kroemer, *et al*., 2005).

Necrosis, as opposed to apoptosis is usually thought of as the direct result of acute injury to the cell, which predominately occurs to the cell surface (Figure 6.1; Collins, *et al*., 1997). However, accumulating evidence now suggests that, like apoptosis, necrosis maybe highly regulated (Kroemer, *et al.*, 2009). Prior to these findings, necrosis was thought of as a process of cell death independent of the cells adenosine triphosphate (ATP) energy levels (Alonso-Pozos, *et al.*, 2003), although research now suggests that a necrotic cell death signal transduction pathway may exist in cells

undergoing necrosis (Elmore, 2007; Kroemer, *et al.*, 2009). Cells undergoing necrosis exhibit morphological signs of cell swelling, blistering and membrane damage (Alonso-Pozos, *et al*., 2003), resulting in the cell bursting and spilling its contents over neighbouring cells; inducing an inflammatory response (Figure 6.1; Collins, *et al*., 1997). Research also indicates that cells undergoing apoptosis can convert to the necrosis pathway or in other words undergo secondary necrosis (Pozhilenkova, *et al.*, 2008).

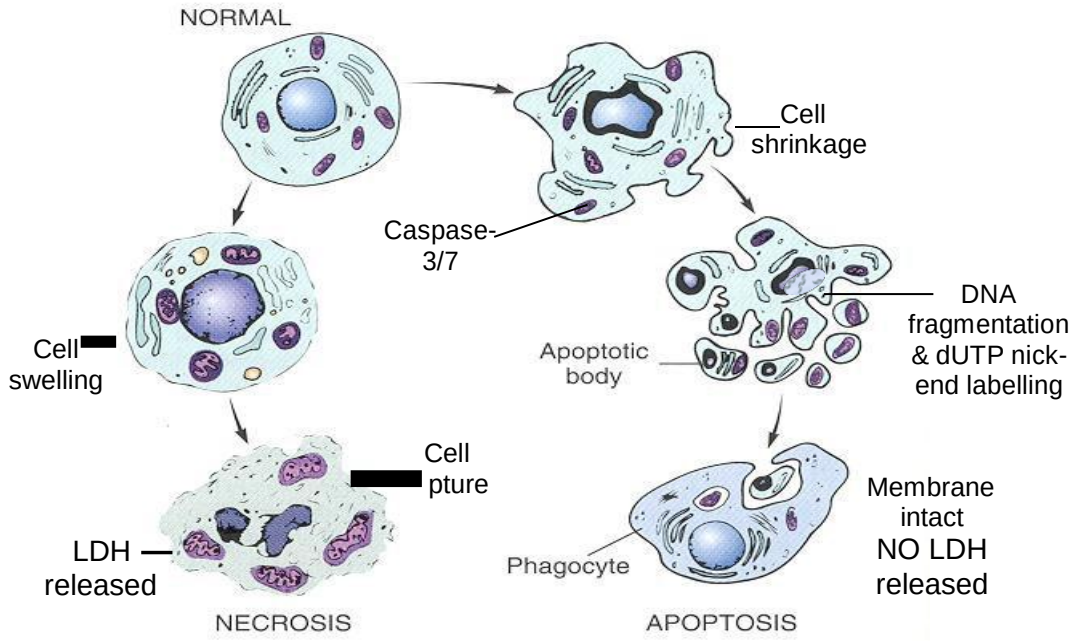


Figure 6.1: Cell death induced by apoptosis and necrosis
Cell death by apoptosis is a highly regulated sequential process of programmed cell death. Morphological changes during apoptosis include nuclear and chromatin condensation, cell shrinkage, the activation of effector caspases such as the executioner caspase-3, followed by DNA fragmentation and the formation of apoptotic bodies. In comparison, cell death by necrosis is generally a disorderly event of cell death and often occurs as a result of injury to the cell. Cells undergoing necrosis lose membrane integrity, swell and eventually burst causing an inflammatory response (Dolan, 2009).

In the ovary, apoptosis is a highly regulated mechanism that is responsible for granulosa cell death during follicle degradation and corpus luteum regression (Tilly, 1996; Vaskivuo and Tapanainen, 2003). A number of mammalian genes and enzymes have been implicated in granulosa cell apoptosis which has been well

documented (Quirk, *et al*., 1995; Jääskeläinen, *et al*., 2009). However, the entire mechanistic pathway of apoptosis in granulosa cells is not complete. Both the extrinsic and intrinsic pathways have been implicated in apoptosis in granulosa cells involving death domain ligands such as FADD, Fas/TNF and TRAIL (Manabe, *et al.*, 2004; Ren, *et al.*, 2004; Jääskeläinen, *et al.*, 2009). Activation of the death domain on the surface of granulosa cells results in cytochrome C release from the mitochondria which interacts with pro-caspase 9 causing a conformational change, thereby activating downstream effector caspases including caspase 3; resulting in the activation of the enzyme, caspase activated DNase (CAD) (Manabe, *et al.*, 2004).

The Fas- tumor necrosis factor (TNF) signalling pathway has been shown to induce apoptosis in placenta cells (Saulsbury, *et al.*, 2008). When the human choriocarcinoma JAr cell line was treated with chlorpyifos, an organphosphate pesticide, TNFα and Fas mRNA were both up-regulated and the anti-apoptotic Bcl-2 protein was down-regulated, leading to nuclear condensation and DNA fragmentation (Saulsbury, *et al.*, 2008). Other research has shown the JAr cells highly express both caspase 3 and 9 when treated with C93 and anti-tumour inhibitor of fatty acid synthase, a tumour associated marker (Ueda, *et al.*, 2009). TNFα has also been shown to induce apoptosis in the ovarian carcinoma cell line, OVCAR-3 (Powell, *et al.*, 1996). Recent studies have shown that apoptosis is activated in the OVCAR-3 cell line when exposed to fluoxetine (FLX), an antidepressant, by the up-regulation of Bax in the mitochondria, leading to cytochrome C release and subsequent caspase-3 activation (Lee, *et al.*, 2009).

There are a number of different techniques that can be implemented in the detection of both necrosis and apoptosis. The CytoTox ONE™ (Promega) assay is a measurement of lactate dehydrogenase (LDH) release from cells with damaged membranes, indicating cell death by necrosis (Promega, 2009b). This assay is based on the conversion of the substrate resazurin into the fluorescent product resorufin, catalyzed by the enzymatic conversion of lactate to pyruvate (Figure 6.2; Niles, *et al.*, 2007).

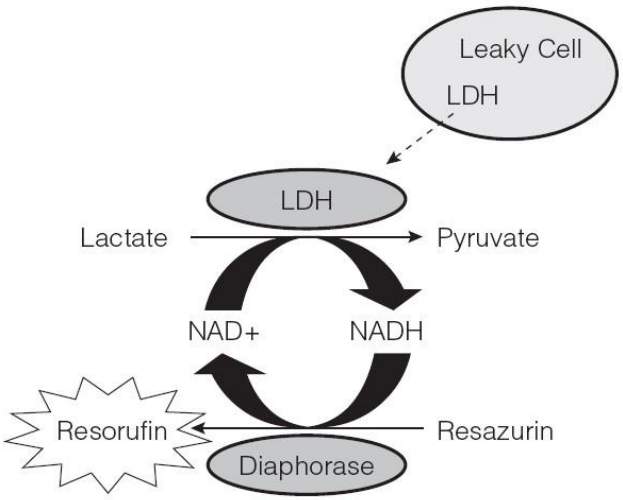


Figure 6.2: Overview of the Membrane Integrity Assay.
Damaged to the cell membrane through necrosis releases LDH, this can be measured by adding lactate, NAD^+ and resazurin as substrates in the presence of diaphorase. The resulting fluorescent product resorufin is proportional to the amount of LDH release, indicating cell death by necrosis (Promega, 2009b).

The Caspase-Glo 3/7® (Promega) assay measures the direct amount of the executioner caspase-3 and -7 in a cell culture assay. This is a direct measurement of apoptosis, as once caspase-3 is activated the cell is irreversibly directed towards cell death (Elmore, 2007). A pro-luminescent caspase 3/7 substrate containing the tetrapeptide DEVD sequence (Asp-Glu-Val-Asp; which is a caspase-3 and 7-recognition site), along with a reagent to lyse the cells, is added to the cell culture (Promega, 2009a). After the cells are lysed, any caspase-3 and -7 in solution cleaves

the luminogenic substrate, this in turn generates a luminescence signal which is directly proportional to the amount of caspase 3/7 activity (Figure 6.3; Niles, *et al*., 2007).

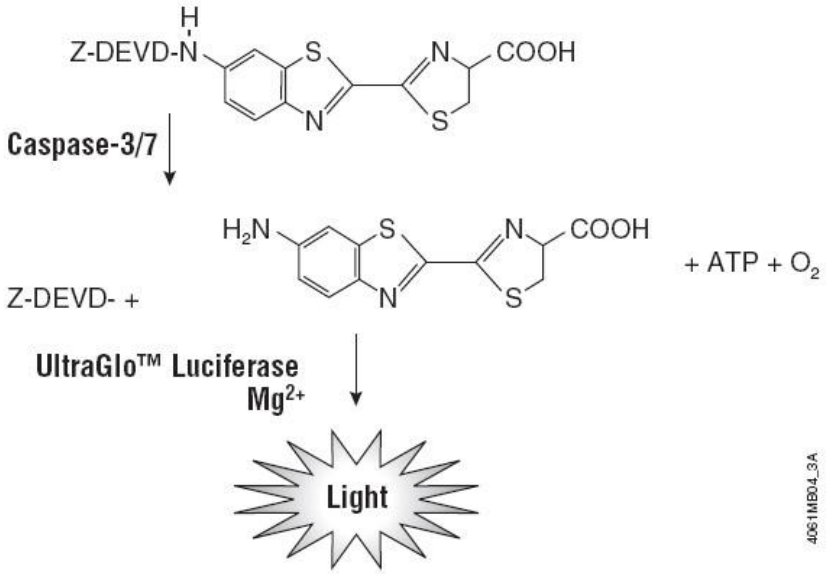


Figure 6.3: Overview of the Caspase-Glo 3/7 Assay. Caspase 3/7 cleavage of the luminogenic substrate containing the DEVD sequence results in the release of the recombinant luciferase generating a luminescence signal, which is proportional to the amount of caspase 3/7 activity, indicating cells are undergoing apoptosis (Promega, 2009a).

The Terminal dUTP Nick End-Labelling (TUNEL) technique is an alternative method for detecting apoptosis (Promega, 2009c). The DNA of cells that are undergoing apoptosis is cleaved into 180-200bp at the 3'OH end by the caspases activated DNase (CAD) enzyme, generally activated by caspase-3. The 3' end can be labelled with fluorescent dUTP, using a recombinant Terminal Deoxynucleotidyl Transferase enzyme, (rTdT; Promega, 2009c; Gavrieli, *et al*., 1992). The labelled DNA can then be visualised and scored under a fluorescent microscope (Gavrieli, et al., 1992).

Changes in cell morphology during apoptosis including DNA fragmentation, nuclear shrinkage and nuclear fragmentation, can also be selectively identified by staining the DNA undergoing apoptosis with fluorescent dyes such as 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Benachour and Seralini, 2009; Peluso, *et al.*, 2009). The condensed or fragmented nuclei of cells undergoing apoptosis fluoresce intensely with the DAPI stain, which can then be visualised using a fluorescent microscope (Travert, *et al.*, 2006; Benachour and Seralini, 2009).

The bioactive compounds extracted from *D. orbita* whelks are not only cytotoxic but can also induce both apoptosis and/or necrosis in different cancer cell lines (Benkendorff, *et al.*, 2011). For example, both apoptosis and necrosis were activated in the T-cell carcinoma, Jurkat cell line, whereas only necrosis was observed in the colon cancer cell line, HT29 (Benkendorff, *et al.*, 2011). Animal studies have also shown that crude extracts from the muricid, *D. orbita* are pro-apoptotic in cells of the distal colon in response to administration of the genotoxic agent, azoxymethane (AOM; Westley, *et al*., 2010).

Synthetic isatin and indole compounds structurally similar to that of the natural *D. orbita* compounds, have also been shown to activate apoptosis and inhibit mitosis in cancer cell lines. Vine *et al*., (2007a) have shown that isatins including 5 and 7-dibromoisatin selectively promote apoptosis in the lymphoma cell lines U937 and Jurkat, by the activation of effector caspases-3 and -7. Further research has shown that indoles and isatins can disrupt cell division in cancer cell lines by targeting spindle formation in microtubules (Bacher, *et al.*, 2001; Chen, *et al.*, 2005c; Vine, *et al.*, 2007b; Matesic, *et al.*, 2008). Vine, *et al* (2007b) established that the 5, 7,

dibromo-N-(p-methylbenzyl)isatin was 22 times more active than 5 and 7-dibromoisatin, against a series of cancer cell lines. Furthermore, mitosis in U937 cells was arrested at the G2/M phase of the cell cycle by inhibiting tubulin polymerization when cells were exposed to 5,7,dibromo-N-(p-methylbenzyl)isatin, (Vine, *et al.*, 2007b).

The experiments in chapter 5, not only demonstrated that indole and isatin based muricid compounds have selective anti-proliferative effects in reproductive human cancer cell lines, but further microscopic examination of cells, after exposure to the semi-purified *D. orbita* compounds revealed morphological changes associated with apoptosis, such as cell shrinkage and signs of cell surface blebbing (Collins, *et al*., 1997; Nguyen and Wells, 2003)

A natural extension of this research therefore was to further investigate the mode of cell death induced by these bioactive compounds in female human reproductive cancer cell lines in comparison with female human reproductive primary cells using assays to detect apoptosis and necrosis. Since apoptosis involves the activation of specific caspases, and necrosis involves membrane damage resulting in the release of LDH, a combined apoptosis and necrosis assay can be performed. However, caution should be taken when selecting specific caspase assays, as only the activation of executioner caspases, such as caspase-3 and -7 leads to cell death. For example, caspases can be activated in differentiation pathways (Kroemer, *et al.*, 2009). Therefore, the effector caspases, or executioner caspases, 3 and -7 are the most common caspases selected for apoptosis assays (Elmore, 2007). The effector caspases-3 enzyme mediates the apoptotic pathway and is considered a key regulator

of apoptosis and hence, is often used as a positive marker for apoptosis (Glamoclija, *et al.*, 2005).

The main aim of this experiment was to determine if the semi-purified *D. orbita* fractions caused cell death by apoptosis or necrosis in the primary-derived human granulosa cells and the human reproductive cancer cell lines. A Caspase-Glo 3-7 assay was multiplexed with a LDH cell membrane integrity assay to determine if cell death by the *D. orbita* fractions is induced by apoptosis or necrosis (Niles, *et al.*, 2007; Promega, 2009a; Promega, 2009b). Cell cytotoxicity in the presence of these semi-purified fractions was also determined using the crystal violet assay to compare with the results obtained previously (Chapter 5) from the MTT assay (Tosetti, *et al.*, 2003). To confirm the mode of cell death by apoptosis induced by the semi-purified fractions, a DNA fragmentation assay (TUNEL) was also performed to detect apoptosis in the treated cells (Gavrieli, *et al.*, 1992; Promega, 2009c). Finally, DNA condensation induced by apoptosis was also detected by staining the nuclei with the fluorescent dye, DAPI (Travert, *et al.*, 2006; Benachour and Seralini, 2009; Peluso, *et al.*, 2009). More specifically the aims were as follows and as summarised in Table 6.1:

1. To isolate, semi-purify and identify the bioactive compounds from the egg masses of the muricid, *D. orbita*
2. To simultaneously detect caspase 3/7 activity and determine loss of membrane integrity by LDH release, using a multiplex assay, as a measurement of apoptosis and necrosis respectively, in primary-derived human granulosa cells and the corresponding KGN granulosa cancer cell line.

3. To confirm the results of the MTT cell viability assay in chapter 5 in all the reproductive cancer cell lines, KGN, JAr and OVCAR-3, along with the primary-derived human granulosa cells by using the crystal violet assay.
4. To determine if the semi-purified *D. orbita* fractions induce apoptosis in the primary-derived human granulosa cells and the corresponding cancer cell line KGN by measurement of apoptotic DNA fragmentation using the TUNEL assay.
5. To determine if the *D. orbita* fractions induce necrosis in the JAr and OVCAR-3 reproductive cancer cell lines using the LDH membrane integrity assay.
6. To confirm whether the KGN cancer cell line were undergoing apoptosis by staining of DNA condensation with DAPI.

Since the *D. orbita* fractions (Chapter 5) altered the normal morphology of the reproductive cancer cell lines, KGN, JAr and OVCAR-3, indicating apoptosis, the hypothesis in this study was that after treatment with two semi-purified *D. orbita* fractions, the effector caspase-3 and -7 enzymes would be upregulated. Furthermore, DNA fragmentation would also be identified in cells undergoing apoptosis by fluorescent labelling of dUTP and DAPI staining.

Table 6.1: Experimental plan
Experimental plan outlining the cell viability, apoptosis and necrosis assays performed on primary-derived human reproductive cells and three reproductive cancer cell lines.

Cell Type	Assay Type				
	Crystal violet	Caspase-3/7	LDH membrane integrity	TUNEL	DAPI
Primary GC	√	√	√	√	
KGN	√	√	√	√	√
JAr	√		√		
OVCAR-3	√		√		

6.2 Methods

6.2.1 Extraction and Isolation of Bioactive compounds from *D. orbita*

The bioactive compounds were extracted from egg capsules (285g) as previously described (section 2.2.4.1.1). Briefly, the egg capsules were cut open and soaked in chloroform and methanol (v/v) for 24h, during which time the solvent was replaced twice (section 2.2.4.1.1). The chloroform layer was then separated from the aqueous layer, filtered under water pressure and then through glass wool to remove tissue residue and evaporated to dryness, yielding a brown/red oil of 3.726g. Chemical analysis was performed by LC/MS (sections 2.2.4.4.2). The brown/red oil was stored at -20^{o}C under N_2 gas in amber vials until semi-purified.

To facilitate the separation of the bioactive compounds tyrindoleninone, tyrindolinone and 6-bromoisatin, the crude samples extracted from the egg masses were semi-purified using flash silica chromatography under N_2 pressure using a solvent system with increasing polarity of DCM, 5% methanol in DCM and 10% methanol in DCM (section 2.2.4.2). Two separate silica columns were run using the crude extracts collected from the egg masses and from these, two main fractions were collected and pooled from subsequent columns (Fraction one: 0.120g eluted

from the column with DCM and Fraction two: 0.105g eluted from the column with 5% DCM). The two fractions were stored in aliquots at -20^{o}C under N_2 until required for apoptosis, necrosis and crystal violet cytotoxicity assays. LC/MS chemical analysis was used to identify the bioactive compounds in collected semi-purified fractions (section 2.2.9). The collected fractions were dried on a rotary evaporator and stored under N_2 at -20^{o}C in amber vials prior to cell culture assays.

6.2.1.1 Compound Preparation for Cell Culture Assays

For cell culture assays, aliquots of each sample fraction were thawed fresh on the day of each experiment and prepared in DMSO (100%; section 5.2.1.1.3). Concentrations of 0.005, 0.01, 0.05, 0.1 and 0.5mg/mL were prepared in complete cell culture medium (sections 2.1.3.1.3 and 2.1.3.6.4) fresh on the day of each experiment (section 5.2.1.1.3).

6.2.2 Combined Caspase 3/7, Membrane Integrity & Cell Viability Assays

6.2.2.1 Preparation of Cells

Primary-derived human granulosa cells were isolated from the follicular aspirates donated by informed consenting women undergoing assisted reproductive technology (ART; section 2.2.1.1) who were considered to be fertile women, based on the criteria previously specified (section 2.2.1.1 Appendix II). For the following experiment, aspirates were collected from three separate women (cases 39, 40 and 41; Appendix II). The collected granulosa cells were re-suspended in DMEM/F12 + 10% FBS complete cell medium (section 2.1.3.1.3). The KGN, JAr and OVCAR-3 cell lines were detached from flasks at 80% confluence (section 2.2.1.2) and re-suspended in DMEM/F12 + 10% FBS complete cell medium (section 2.1.3.1.3) for KGN cells and RPMI + 10 and 20% complete cell medium (section 2.1.3.6.4) for JAr and OVCAR-3 cell lines, respectively. Primary-derived granulosa, KGN, JAr and

OVCAR-3 viable cell numbers were determined using the trypan blue exclusion assay (section 2.2.2.3).

6.2.2.2 Cell Treatment with D. orbita *Semi-purified Fractions*

The primary-derived granulosa cells, KGN, JAr and OVCAR-3 cell lines (10,000 cells per well) were then plated into sterile white (opaque) 96-well plates (Interpath) and clear sterile 96-well plates (Interpath) in 0.1mL per well of complete cell culture medium (section 2.1.3.1.3 and 2.1.3.6.4) for 24h (granulosa, KGN and OVCAR-3 cells) and 2h (for JAr cells) to allow cell adherence to plates. All opaque treatment plates contained six wells of cell culture medium only, and outside wells of all plates contained 0.1mL of sterile PBS only (section 2.1.3.5), to prevent evaporation of the inside treatment wells of the plates. Standard curve plates of 0-40,000 cells per well (primary granulosa cells) and 0-80,000 cells per well (for KGN, JAr and OVCAR-3 cells) in six replicate wells, plated into clear 96-well plates, were setup alongside the test plates.

After the initial cell adherence period, the spent medium was removed from all treatment wells and primary-derived granulosa, KGN, JAr and OVCAR-3 cells were treated with two semi-purified *D. orbita* fractions (section 6.2.1.1.4) at concentrations of 0.005, 0.01, 0.05, 0.1, and 0.5mg/mL in a final volume of 0.1mL per well, for 4 and 24h at 37^{o}C + 5% CO_2. 1h prior to the end of each incubation period two positive controls were added to the opaque plates in triplicate wells each; one for apoptosis, and the second for necrosis. For apoptosis in the primary granulosa and KGN cell plates, the media was removed from three replicate wells and 1µg/mL DNase I in DNase I buffer (section 2.1.8.2) was added, and the plates incubated at 37^{o}C + 5% CO_2 for a further 1h. For the necrosis positive control, 2µL per well of a

cell lysis buffer (supplied by the manufacturer; section 2.1.4) was added to three replicate wells of all opaque plates and the plates were incubated at 37^{o}C + 5% CO_2 for a further 1h.

6.2.2.3 LDH Membrane Integrity Assay and Caspase 3/7 Assay

After 4 and 24h exposure to the *D. orbita* fractions, the combined caspase 3/7 and membrane integrity assay was performed on the primary granulosa cells and KGN cells in the opaque plates. The membrane integrity assay was performed only on the JAr and OVCAR-3 cells. After the designated incubation period, the opaque plates were equilibrated to 22^{o}C for 30 minutes. The equilibrated opaque plates had 0.05mL of the LDH substrate (section 2.1.4) added to all treatment wells, giving a final volume of 0.15mL per well, and the plates were incubated for 10 minutes with shaking at 22^{o}C, protected from light. After this, the fluorescence in the plates was immediately read using a fluorescence plate reader and Multimode detection Software at $535_{EX}/590_{EM}$. The no-cell control signals in medium only wells were subtracted from the signals produced by the treated cells; to account from any signal generated from cell culture medium alone.

The primary-derived human granulosa cell and KGN cell plates containing 0.15mL per well had 0.1mL of solution removed from all treatment wells (which was discarded). Then 0.05mL of the Caspase 3/7 reagent (section 2.1.2) was added to all wells, giving a final volume per well of 0.1mL. The plates were incubated in the dark for 1h to achieve luminescence. The opaque plates were read on an automatic spectrophotometer and Multimode detection Software, on full light to capture total luminescence. After 4 and 24h exposure to the *D. orbita* fractions, the combined caspase 3/7 and membrane integrity assay was performed on the primary granulosa

cells and KGN cells in the opaque plates. Only the membrane integrity assay was performed on the JAr and OVCAR-3 cells. After the designated incubation period, the opaque plates were equilibrated to 22^{o}C for 30 minutes. The equilibrated opaque plates had 0.05mL of the LDH substrate (section 2.1.4) added to all treatment wells, giving a final volume of 0.15mL per well, and the plates were incubated for 10 minutes with shaking at 22^{o}C, protected from light. After this, the fluorescence in the plates was immediately read using a fluorescence plate reader and Multimode detection Software at $535_{EX}/590_{EM}$. The no-cell control signals in medium only wells were subtracted from the signals produced by the treated cells, to account for any signals generated from cell culture medium alone. The assay was completed on three separate occasions and the results are the mean of three repeats ± 1 SEM.

6.2.2.4 Crystal Violet Cell Viability Assay

After the initial cell adherence period in the standard curve plates, cell viability in the plates was determined by the crystal violet assay as previously described (0.5%; section 2.1.2.1). At the end of the treatment periods with the *D. orbita* fractions, the medium was removed from the clear 96-well plates. The wells were then rinsed with sterile 1x PBS (section 2.1.19) and the crystal violet assay was performed as previously described (0.5%; section 2.1.2.1). The absorbance in the wells of the crystal violet plates was read on an automatic spectrophotometer at 570nm, with reference absorbance 630nm, with the assistance of KC Junior Software.

6.2.2.5 Statistical Analysis

Two-way analyses of variance (ANOVA) using the sensitive contrast K Matrix analysis tests (Pallant, 2002) were conducted to examine the effects of the semi-purified *D. orbita* fractions on caspase 3/7 activity, LDH release and cell viability using SPSS software package version 17. Homogeneity of variance was determined

using the Levine's Test of Equality of Error and the alpha value adjusted to < 0.01 where homogeneity of variance was violated (Appendix VIII).

6.2.3 Detection of Apoptotic Cells by TUNEL

6.2.3.1 Preparation of Cells

Primary-derived human granulosa cells were isolated from the follicular aspirates collected from women undergoing ART (cases 42, 43, and 44; Appendix II). These women were considered fertile under the criteria specified in section 2.2.1.1 and Appendix II. The isolated granulosa cells were re-suspended in DMEM/F12 + 10% FBS complete cell culture medium (section 2.1.3.1.3). The corresponding granulosa tumour cell line, KGN, was detached from flasks (at 80% confluence; section 2.1.3.1.3) and re-suspended in DMEM/F12 + 10% FBS complete cell culture medium (section 2.2.1.2). Cell viability was determined using the trypan blue exclusion assay on a haemocytometer for primary granulosa and KGN cells (section 2.2.2.3).

Cells were plated into Nunc Lab-Tek II CC2 Chamber Slides at 30,000 cells per chamber well in a final volume of 0.3mL of DMEM/F12 + 10% FBS complete cell culture medium (section 2.1.3.1.3) and incubated for 24h at 37^{o}C + 5% CO_2 to allow cell attachment to slides.

6.2.3.2 Treatment of Cells with Semi-Purified D. orbita *Fractions*

After the 24h cell adherence period the spent medium was removed from the wells and two semi-purified *D. orbita* fractions (section 6.2.1.1.2) were added at concentrations of 0.005, 0.01 and 0.05mg/mL in a final volume of 0.3mL of DMEM/F12 + 10% complete cell culture medium (section 2.1.3.1.3) per chamber well. A media only and 1% DMSO control were also added to chamber wells and the slides were then incubated for 4 and 24h at 37^{o}C + 5% CO_2.

After the 4 and 24h incubation period the supernatant was removed and the chamber wells were rinsed with 1x sterile PBS (section 2.1.3.5). The cells were then fixed to the slides with 4% paraformaldehyde solution (section 2.1.2.6.2) for 25 minutes at room temperature. The slides were then rinsed 2x with sterile PBS (section 2.2.3.5) for 5 minutes each and the chambers were removed.

6.2.3.3 TUNEL assay to Detect Apoptotic Cells

The TUNEL assay was performed as previously described (section 2.2.3.4) and the nuclei were counter stained with propidium iodide (section 2.1.2.6.3). Positive control slides of 1µg/mL DNase I and negative control slides consisting of the TUNEL incubation buffer without the rTdT enzyme (section 2.2.3.4) were run in conjunction with the assay. The cells were examined and photographed at x 200 magnification using a Fluorescence Olympus BX50 Microscope (section 2.2.3.4). Photomicrographs of four random microscope fields were taken for each fraction concentration in each repeat of the assay and the number of TUNEL positive nuclei, stained bright green, were calculated as a fraction of the total number of propidium iodide stained nuclei, stained bright red, in each image. The average of the four fields was then calculated and the results are the mean ± 1 SEM of three separate repeated assays.

6.2.3.4 Statistical Analysis

A two-way analysis of variance (ANOVA) using the sensitive contrast K Matrix analysis test (Pallant, 2002) was conducted to determine the number of apoptotic cells induced by the semi-purified *D. orbita* fractions using SPSS software package version 17. The alpha value was adjusted when necessary to significant if $p < 0.01$ where homogeneity of variance was violated using the Levine's Test of Equality of Error (Appendix VIII).

6.2.4 Detection of Apoptotic Cells by DAPI Staining

6.2.4.1 Cell Preparation

KGN cells were detached from flasks at 80% confluence, and re-suspended in DMEM/F12 + 10% FBS complete cell culture medium (section 2.1.3.1.3). Viable cells were determined using the trypan blue exclusion assay on a haemocytometer (section 2.2.2.3). Viable cells were plated into Nunc Lab-Tek II CC2 Chamber slides at 30,000 cells per well in a final volume of 0.3mL of DMEM/F12 + 10% FBS complete cell culture medium (section 2.1.3.1.3) and incubated for 24h at 37^{o}C + 5% CO_2 to allow cell attachment to chamber slides.

6.2.4.2 Treatment of KGN Cells with Semi-purified D. orbita *Fractions*

After the 24h attachment period the medium was removed from the wells and two semi-purified *D. orbita* fractions (section 6.2.1.1.2) were added at concentrations of 0.005, 0.01 and 0.05mg/mL in a final volume of 0.3mL DMEM/F12 + 10% FBS cell culture medium (section 2.1.3.1.3) per chamber well. A media only and 1% DMSO control were also added to separate chamber wells and the slides were then incubated for 4 and 24h at 37^{o}C + 5% CO_2.

6.2.4.3 Detection of Apoptotic Cells by DAPI Staining of DNA

After the 4 and 24h incubation period the supernatant was removed from all the wells and the wells were rinsed with 1x sterile PBS (section 2.1.3.5). The cells were then fixed to the slides with an ethanol, chloroform and acetic acid solution v/v/v (section 2.1.2.3.1) overnight at -20^{o}C. The chambers were then removed and the slides were rinsed twice with sterile PBS (section 2.2.3.5) for 5 minutes each. The DAPI stain (section 2.1.2.3.2) at 1µg/mL was then added to the slides in the dark for 30 minutes (section 2.2.3.2). The assay was repeated on three separate occasions and the results are the mean ± 1 SEM of the three separate repeated assays. Four to eight random photo micrographs were taken of each treatment well for each repeat of the assay at x

200 and x 400 oil magnification using a Fluorescence Olympus AX70 microscope (section 2.2.3.2).

6.3 Results

6.3.1 Chemical Analysis

Chemical analysis by LC/MS of the crude sample extracted from the egg masses identified four major compounds shown on the diode array chromatogram (Figure 6.4A). 6-Bromoisatin (*m/z* 224, 226) at t_R 6.43 minutes was the main component of the crude sample, followed by tyrindolinone (*m/z* 302, 304) at t_R 9.60 minutes, tyrindoleninone (*m/z* 255, 257) at t_R 11.32 minutes and a smaller peak, was confirmed to be tyriverdin (*m/z* 463, 465, 467) at t_R 12.97 minutes.

Chemical analysis of the two semi-purified fractions by LC/MS identified one major compound in fraction one at (t_R) 11.32 minutes, which corresponded to the molecular mass of tyrindoleninone (ESIMS insert *m/z* 255, 257; Figure 6.4B). A minor product, tyrindolinone was also detected at t_R 9.8 minutes. Tyrindoleninone and tyrindolinone can interconvert during chromatography however the equilibrium lies towards tyrindoleninone as it is the more stable product; tyrindolinone readily loses a methane thiol group to form tyrindoleninone (Cooksey, 2001). The second fraction consisted of one major HPLC peak at t_R 6.45 minutes on the diode array chromatogram was confirmed to be 6-bromoisatin by the molecular mass on the ESI/MS (insert; *m/z* 224, 226; Figure 6.4C).

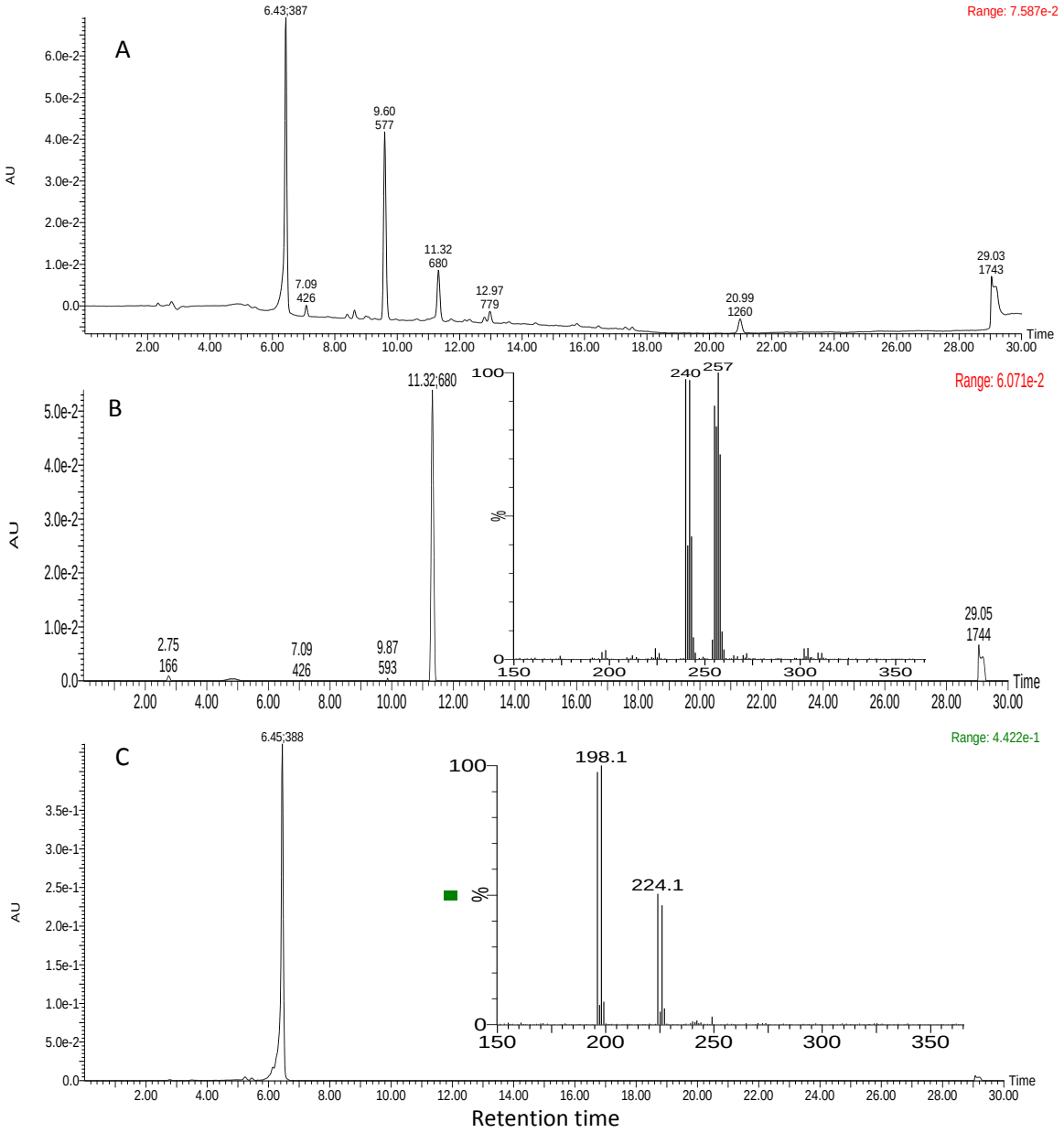


Figure 6.4: LC/MS of a crude and semi-purified *D. orbita* egg extract
Liquid chromatography mass spectrometry analysis of the crude sample an two semi-purified fractions extracted from the egg capsules of *D. orbita* and the two semi-purified fractions. (A.) The chromatogram from the Diode array at 300 + 600nm shows the retention times (t_R) and absorbance units (AU) of the main brominated indole compounds in the crude sample. The major peak at 6.43 minutes corresponds to the molecular mass of 6-bromoisatin (*m/z* 224, 226). Another compound at t_R 9.60 minutes was confirmed to be tyrindolinone (*m/z* 302, 304). Smaller peaks at t_R 11.32 and 12.97 minutes correspond to the molecular mass of tyrindoleninone (*m/z* 255, 257) and tyriverdin (*m/z* 463, 465, 467) respectively; (B) The first fraction collected from semi-purification of the egg capsule crude sample which shows the retention times (t_R) of the major compound at t_R 11.32 minutes. This fraction was confirmed to be primarily tyrindoleninone by the molecular mass (ESI/MS insert; *m/z* 255, 257); (C) The second semi-purified fraction corresponded to that of 6-bromoisatin, as confirmed by its molecular weight (ESI/MS insert; *m/z* 224, 226).

6.3.2 Combined Caspase 3/7, Membrane Integrity & Cell Viability assay

6.3.2.1 Results of Fraction One Tyrindoleninone

Table 6.2: Results of Tyrindoleninone on the Reproductive Cells
The concentration of Fraction one (Tyrindoleninone) that caused 50% death on primary-derived human granulosa (GC), KGN, JAr and OVCAR-3 cells, along with the concentration that induced caspase 3/7 up-regulation and LDH release, after exposure for 4 and 24h. Cell viability was determined by the crystal violet assay (CV) and is shown as LC_{50} concentration that killed 50% of the cells. Caspase 3/7 and LDH activity is shown as the lowest concentration that was significantly different to the 1% DMSO control. N/T = not tested.

Cell Type	Time (h)	CV LC_{50}	Caspase 3/7 (mg/mL)	LDH (mg/mL)
Primary GC	4	>0.5	0.1	0.5
KGN		0.04	0.005	0.5
JAr		0.06	N/T	0.05
OVCAR-3		0.08	N/T	0.1
Primary GC	24	>0.5	0.1	0.5
KGN		0.04	0.005	0.05
JAr		0.05	N/T	0.05
OVCAR-3		0.09	N/T	0.1

6.3.2.1.1 Primary-Derived Human Granulosa cells

After 4h exposure to tyrindoleninone, a statistically significant increase in caspase 3/7 was noted in primary granulosa cells at 0.1mg/mL ($p < 0.01$; Figure 6.5A) while LDH release from the cells was only detected at 0.5mg/mL ($p < 0001$; Figure 6.5A). The crystal violet assay detected cell death at a lower concentration (0.05mg/mL) than either the caspase and LDH assays at 4h ($p < 0.05$; Figure 6.5B), although the LC_{50} was higher (>0.5mg/mL, Table 6.2).

After 24h exposure to tyrindoleninone, caspase 3/7 activity was significantly increased in primary granulosa cells at 0.05mg/mL ($p < 0.001$; Figure 6.5A). Again LDH associated fluorescence was significantly increased after 24h exposure to the higher 0.5mg/mL concentration of tyrindoleninone (Figure 6.5A) whereas cell death

detected by the crystal violet cell death was detected at the lower concentration of 0.1mg/mL ($p < 0.01$; Figure 6.4B).

6.3.2.1.2 KGN Cell Line

In comparison, when the cancer granulosa KGN cell line were treated with tyrindoleninone for 4h significant caspase 3/7 activity was detected at the lower concentrations of 0.005, 0.01 and 0.05mg/mL ($p < 0.001$; Figure 6.6A), but at the higher concentrations of 0.1 and 0.5mg/mL caspase activity was not significantly different to the DMSO control. LDH release only significantly increased at 0.5mg/mL compared to the DMSO control ($p < 0.001$; Figure 6.6A), and cell viability measured by the crystal violet assay was statistically different to the DMSO control at all concentrations tested, $\leq$ 0.5mg/mL (LC_{50} 0.04mg/mL; Figure 6.6B).

After 24h, tyrindoleninone caused the largest increase in caspase 3/7 activity in KGN cells at the lowest concentration (0.005mg/mL; $p < 0.001$; Figure 6.6A), but then there was a dose dependant decrease in caspase activity as the concentration of tyrindoleninone increased (Figure 6.6A). LDH release was detected at 0.05mg/mL of tyrindoleninone ($p < 0.01$; Figure 6.6A), and cell viability was significantly affected at 0.005 and 0.05mg/mL after 24h ($p < 0.01$ and 0.001; LC_{50} 0.04mg/mL; Figure 6.6B).

6.3.2.1.3 JAr Cell Line

When JAr cells were treated for 4h with 0.05mg/mL of tyrindoleninone, fluorescence significantly increased ($p < 0.01$), indicating LDH release, and cell viability decreased significantly in the crystal violet assay ($p < 0.001$; Figure 6.7A and B). After 24h treatment with tyrindoleninone at 0.05mg/mL, LDH release by JAr cells significantly increased compared to the DMSO control ($p < 0.001$; Figure 6.7A) and

cell viability by the crystal violet assay significantly decreased ($p < 0.001$; LC_{50} 0.05mg/mL; Figure 6.7B).

6.3.2.1.4 OVCAR-3 Cell Line

Both LDH release and cell viability in OVCAR-3 cells under the same treatment conditions with tyrindoleninone for 4h were only statistically different to the DMSO control at 0.1mg/mL ($p < 0.001$; Figure 6.8A and B). Furthermore, LDH release and cell viability in the OVCAR-3 cell line were only significantly different to the DMSO control after 24h treatment at 0.1mg/mL of tyrindoleninone ($p < 0.001$; LC_{50} 0.09mg/mL; Figure 6.8A and B).

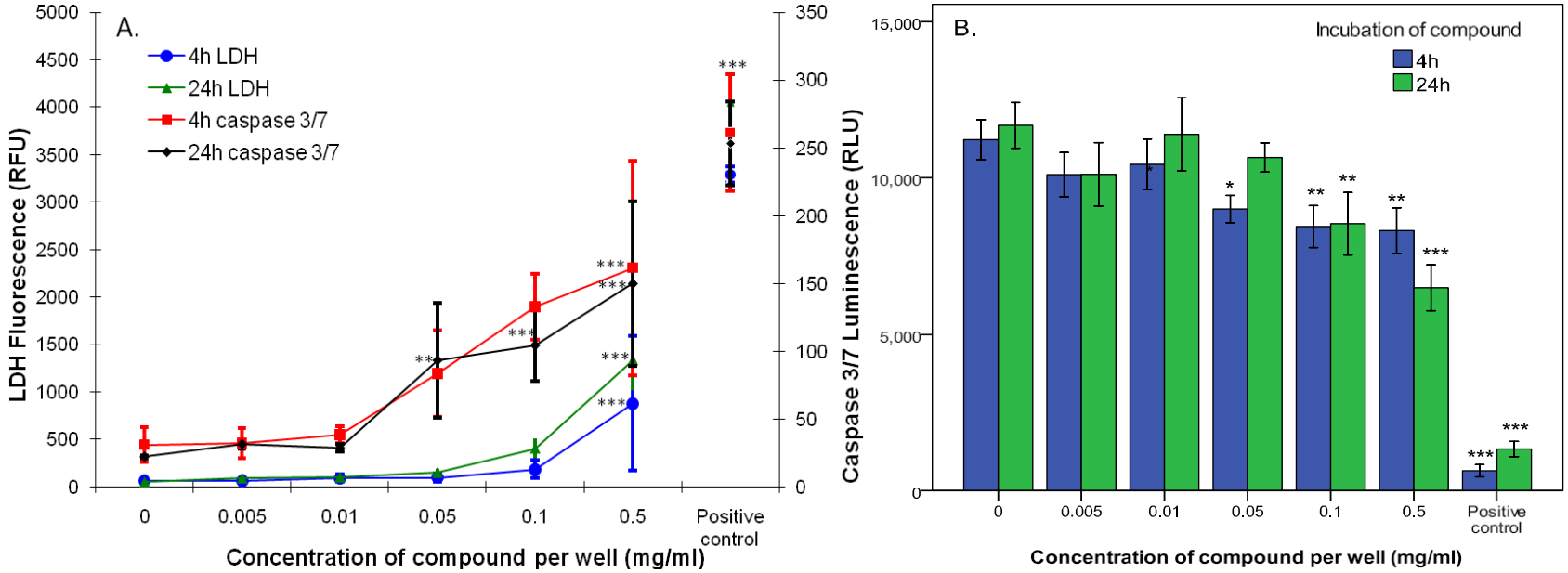


Figure 6.5: Caspase 3/7, LDH and cell viability results of primary-derived human granulosa cells after treatment with tyrindoleninone
Activity of Caspase 3/7 and LDH release (A) and cell viability (B) in primary-derived human granulosa cells after incubation with the semi-purified *D. orbita* fraction one containing tyrindoleninone. After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with tyrindoleninone for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and Caspase 3/7 activity was measured at full light on an automatic plate reader. Cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The results are the mean of three separate repeat assays (n = 3; ± 1 SEM). The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer), for the Caspase 3/7 assay 1µg/mL DNase and for the crystal violet assay no FBS serum. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on caspase 3/7 activity, LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h shown as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

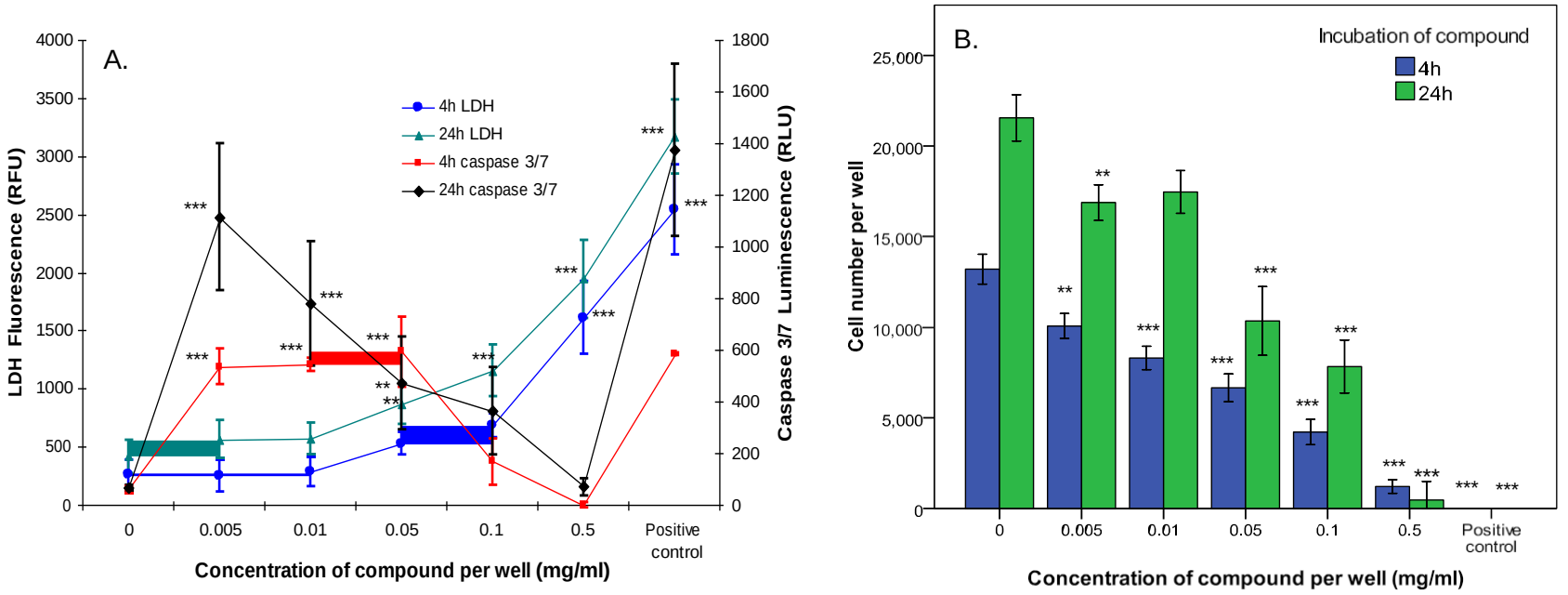


Figure 6.6: Caspase 3/7, LDH and cell viability results of KGN cells after treatment with tyrindoleninone

Activity of Caspase 3/7 and LDH release (A) and cell viability (B) in the KGN cells after incubation with the semi-purified *D. orbita* compound tyrindoleninone. After an initial 24h cell attachment period KGN cells (10,000cells/well) were treated with tyrindoleninone for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and Caspase 3/7 activity was measured at full light on an automatic plate reader. Cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The results are the mean of three separate repeat assays (n = 3; ± 1 SEM). The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer), for the Caspase 3/7 assay 1µg/mL DNase and for the crystal violet assay no FBS serum. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on caspase 3/7 activity, LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h are shown as $p < 0.01$ (**) and $p < 0.001$ (***).

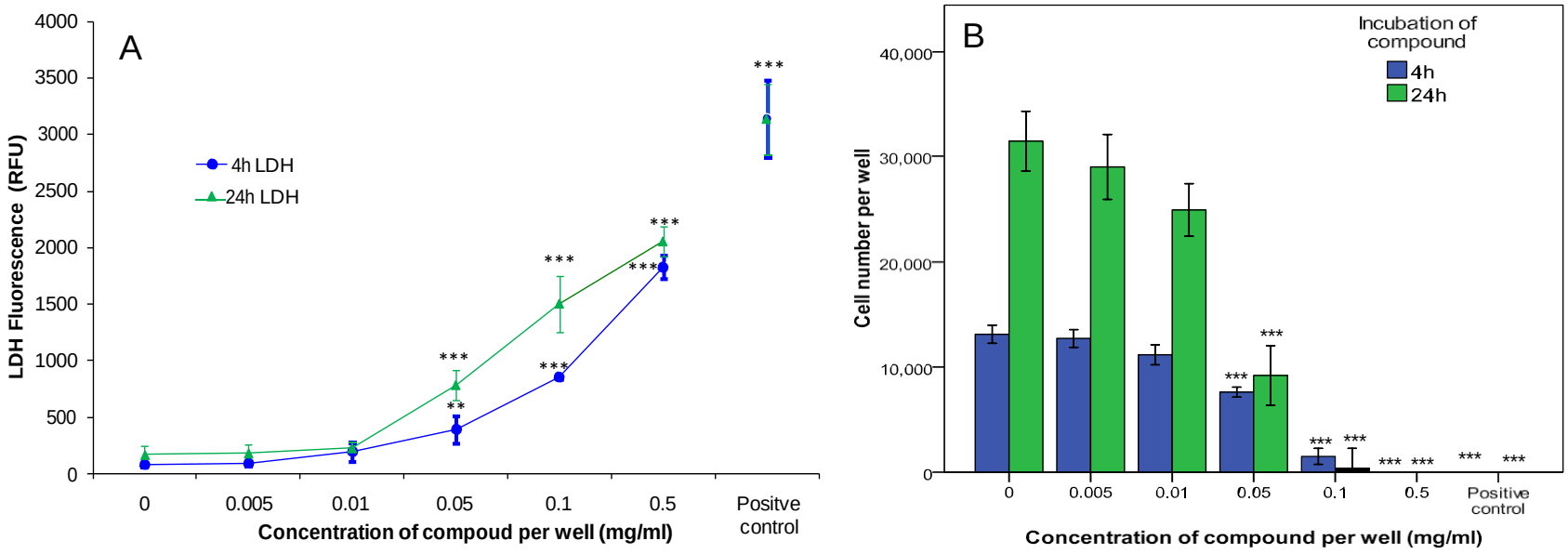


Figure 6.7: LDH and cell viability results of JAr cells after treatment with tyrindoleninone
LDH release (A) and cell viability (B) in the JAr cells after incubation with the semi-purified *D. orbita* fraction one containing tyrindoleninone. After an initial 2h cell attachment period JAr cells (10,000cells/well) were treated with tyrindoleninone for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The positive controls were as follows, for the LDH assay a lysis solution (supplied by the manufacturer) and for the crystal violet assay H_2O_2 (1,000µg/mL). The results are the mean for three separate repeat assays (n = 4; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well and hormone synthesis against the 1% DMSO control at different incubation periods. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h are shown as $p < 0.01$ (**) and $p < 0.001$ (***).

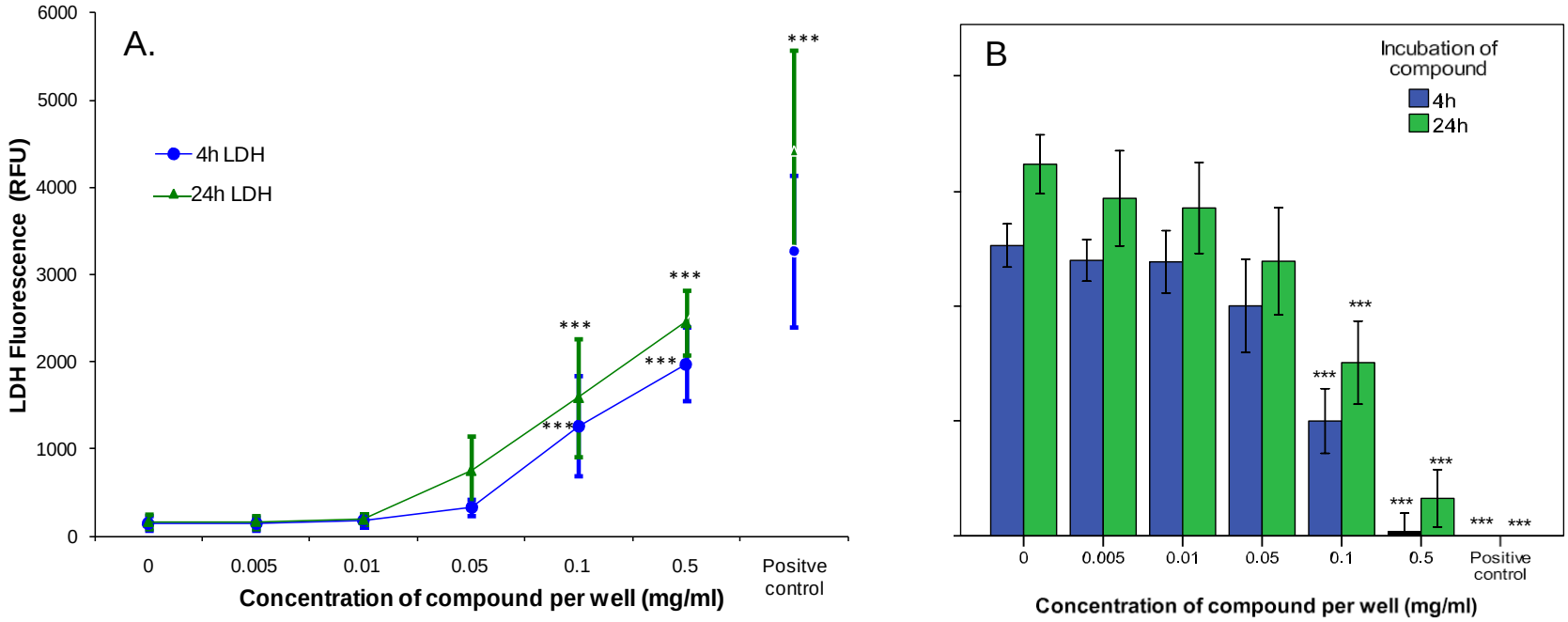


Figure 6.8: LDH and cell viability results of OVCAR-3 cells after treatment with tyrindoleninone

LDH release (A) and cell viability (B) in the OVCAR-3 cells after incubation with the semi-purified *D. orbita* fraction one containing tyrindoleninone. After an initial 2h cell attachment period OVCAR-3 cells (10,000cells/well) were treated with tyrindoleninone for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The positive controls represent the following: for the LDH assay a lysis solution (supplied by the manufacturer) and for the crystal violet assay no FBS serum. The results are the mean for three separate repeat assays (n = 4; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h is shown as $p < 0.01$ (**) and $p < 0.001$ (***).

6.3.2.2 Results of Fraction Two 6-Bromoisatin

Table 6.3: Results of 6-Bromoisatin on the Reproductive Cells
The effective concentration of Fraction two (6-bromoisatin) on primary-derived human granulosa (GC), KGN, JAr and OVCAR caspase 3/7 release, LDH release and cell viability after exposure for 4 and 24h. Cell viability was determined by the crystal violet assay (CV) and is shown as LC_{50}. Caspase 3/7 and LDH activity is shown as the lowest concentration that was significantly different to the 1% DMSO control. N/T = not tested

Cell Type	Time (h)	CV LC_{50}	Caspase 3/7 (mg/mL)	LDH (mg/mL)
Primary GC	4	>0.5	0.1	0.5
KGN		0.2	0.005	0.05
JAr		0.05	N/T	0.1
OVCAR-3		0.1	N/T	0.1
Primary GC	24	>0.5	0.05	0.5
KGN		0.05	0.005	0.5
JAr		0.04	N/T	0.05
OVCAR-3		0.06	N/T	0.1

6.3.2.2.1 Primary-Derived Granulosa Cells

Caspase 3/7 activity was detected in primary granulosa cells after 4h treatment with 6-bromoisatin at 0.1mg/mL ($p < 0.001$; Figure 6.9A) and cell viability decreased at the same concentration (Figure 6.9B). Furthermore, necrosis, as indicated by LDH release was only noted at 0.5mg/mL 6-bromoisatin ($p < 0.001$; Figure 6.9B). After primary granulosa cells were treated treatment with 6-bromoisatin for 24h, caspase 3/7 activity increased at 0.05mg/mL ($p < 0.01$; Figure 6.9A). However, cell viability did not significantly decrease until treatment with 0.5mg/mL of 6-bromoisatin ($p < 0.001$; Figure 6.9B), and at the same time LDH fluorescence was also significantly higher than the DMSO control ($p < 0.001$; Figure 6.9A).

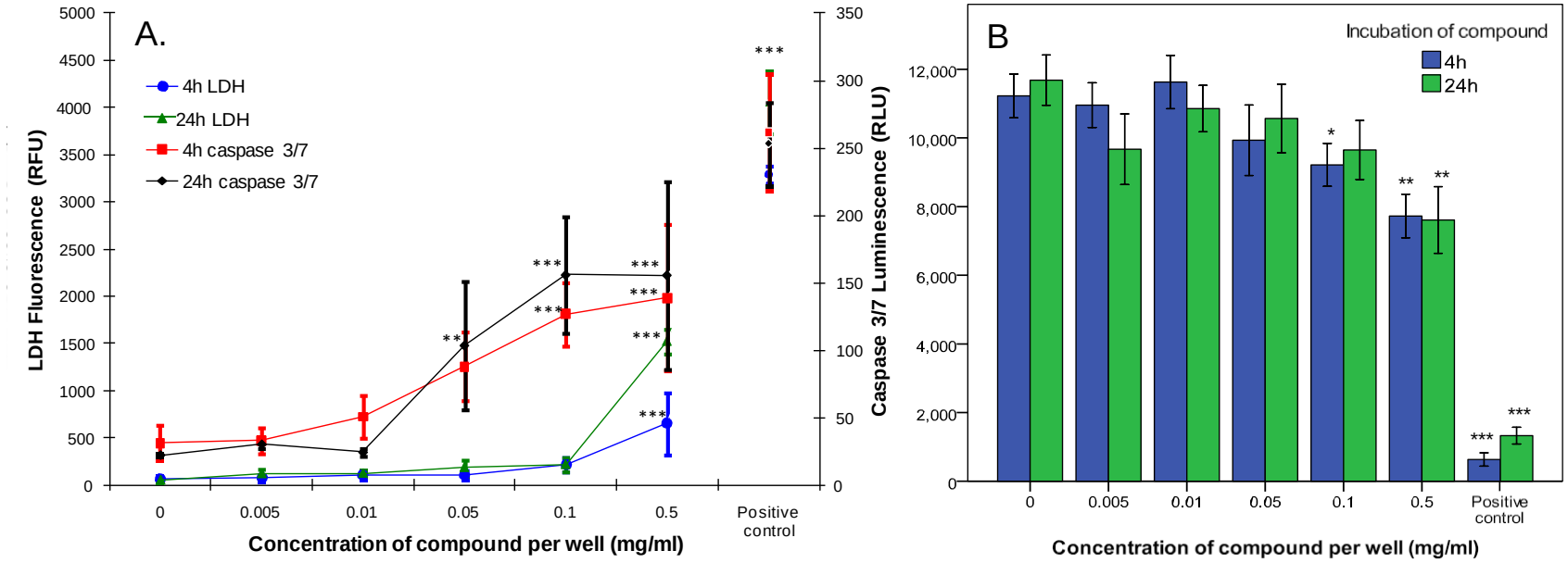


Figure 6.9: Caspase 3/7, LDH and cell viability results of primary-derived human granulosa cells after treatment with 6-bromoisatin
Activity of Caspase 3/7 and LDH release (A) and cell viability (B) in primary-derived human granulosa cells after incubation with the semi-purified *D. orbita* fraction two containing 6-bromoisatin. After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with 6-bromoisatin for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and caspase 3/7 activity was measured at full light on an automatic plate reader. Cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The results are the mean of three separate repeat assays (n = 3; ± 1 SEM). The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer), for the Caspase 3/7 assay 1µg/mL DNase and for the crystal violet assay no FBS serum. Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on caspase 3/7 activity, LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h shown as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

6.3.2.2.2 KGN Cell Line

In comparison to primary-derived granulosa cells, caspase 3/7 activity in KGN cells was significantly higher than DMSO control after 4h treatment with 0.005, 0.01 and 0.05mg/mL of 6-bromoisatin ($p < 0.001$; Figure 6.10A), whereas fluorescence indicating LDH release was only significantly different to the DMSO control at ≥ 0.05mg/mL of 6-bromoisatin. After 4h, KGN cell viability decreased at 0.5mg/mL of 6-bromoisatin in comparison to the DMSO control (LC_{50} 0.2mg/mL; Figure 6.10A and B).

When KGN cells were treated with fraction two for 24h, caspase-3/7 decreased in a dose-dependent manner from 0.005-0.5mg/mL ($p < 0.001$; Figure 6.10A). LDH release did not occur in the KGN cells until the concentration reached 0.5mg/mL after 24h ($p < 0.001$; Figure 6.10A). KGN cell viability, as noted by the crystal violet assay, decreased significantly after 24h when treated with 0.05mg/mL of 6-bromoisatin ($p < 0.001$; LC_{50} 0.05mg/mL; Figure 6.10B).

6.3.2.2.3 JAr Cell Line

Necrosis in the JAr cells was noted at 0.1mg/mL ($p < 0.001$; Figure 6.11A) when treated with 6-bromoisatin for 4h, however, cell viability only significantly decreased at 0.05mg/mL in the crystal violet assay ($p < 0.001$; LC_{50} 0.05mg/mL; Figure 6.11B). After 24h treatment with 6-bromoisatin, cell viability decreased and LDH release increased in the JAr cells at 0.05mg/mL (LC_{50} 0.04mg/mL; Figure 6.11A and B).

6.3.2.2.4 OVCAR-3 Cell Line

In the OVCAR-3 cell line, cell viability decreased and LDH activity increased at 0.1mg/mL of 6-bromoisatin after 4h treatment (LC_{50} 0.1mg/mL; Figure 6.12A and B). OVCAR-3 cell viability also decreased at 0.05mg/mL of 6-bromoisatin after 24h ($p < 0.01$; Figure 6.12B). However, necrosis, as indicated by LDH release from the cells, was only noted at 0.1mg/mL ($p < 0.001$ Figure 6.12A).

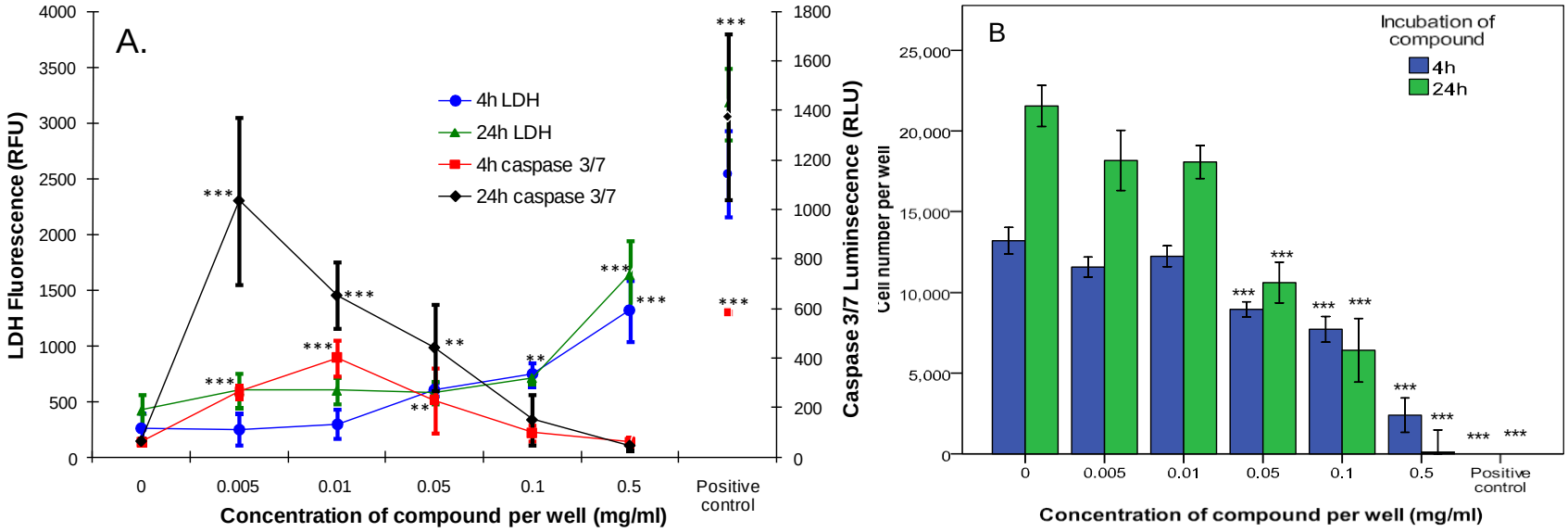


Figure 6.10: Caspase 3/7, LDH and cell viability results of KGN cells after treatment with 6-bromoisatin

Activity of Caspase 3/7 and LDH release (A) and cell viability (B) in the KGN cells after incubation with the semi-purified *D. orbita* fraction two containing 6-bromoisatin. After an initial 24h cell attachment period KGN cells (10,000cells/well) were treated with 6-bromoisatin for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and caspase 3/7 activity was measured at full light on an automatic plate reader. Cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer), for the Caspase 3/7 assay 1µg/mL DNase and for the crystal violet assay no FBS serum. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on caspase 3/7 activity, LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h is shown as $p < 0.01$ (**) and $p < 0.001$ (***).

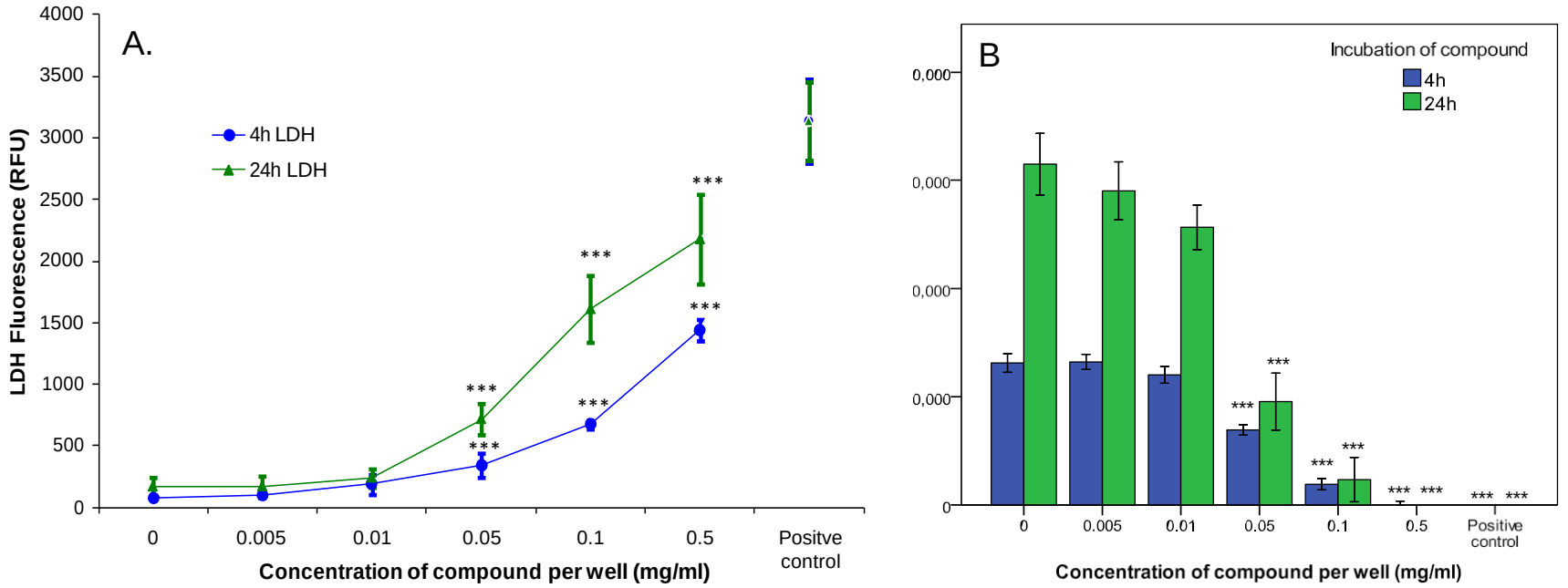


Figure 6.11: LDH and cell viability results of JAr cells after treatment with 6-bromoisatin

LDH release (A) and cell viability (B) in the JAr cells after incubation with the semi-purified *D. orbita* fraction two containing 6-bromoisatin. After an initial 2h cell attachment period JAr cells (10,000cells/well) were treated with 6-bromoisatin for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer) and for the crystal violet assay H_2O_2 (1,000µg/mL). The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h is shown as $p < 0.01$ (**) and $p < 0.001$ (***).

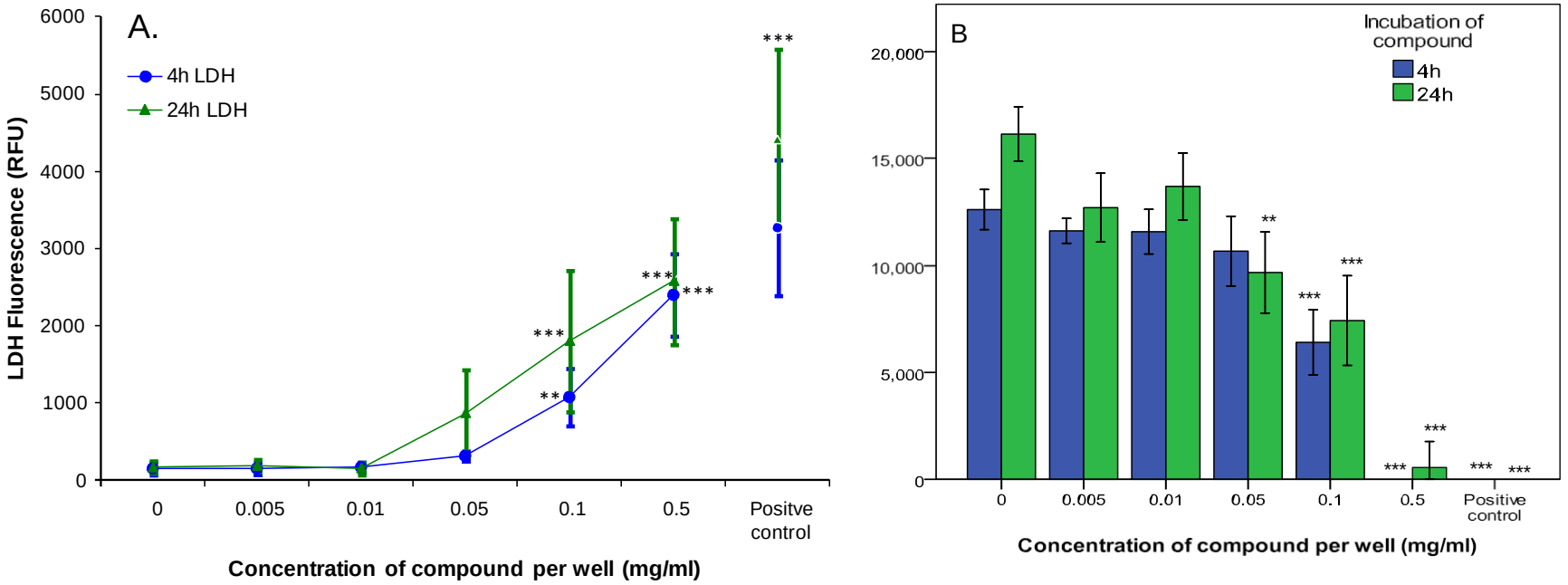


Figure 6.12: LDH and cell viability results of OVCAR-3 cells after treatment with 6-bromoisatin

LDH release (A) and cell viability (B) in the OVCAR-3 cells after incubation with the semi-purified *D. orbita* fraction two containing 6-bromoisatin after 4 and 24h. After an initial 2h cell attachment period OVCAR-3 cells (10,000cells/well) were treated with 6-bromoisatin for 4 and 24h. LDH release was measured by fluorescence at $535_{EX}/590_{EM}$ and cell viability was determined by the crystal violet assay at 570nm with reference absorbance 630nm. The positive controls represent the following: for the LDH assay, a lysis solution (supplied by the manufacturer) and for the crystal violet assay no FBS serum. The results are mean for three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on LDH release and cell viability against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h shown as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

6.3.3 Detection of Apoptotic cells by TUNEL

6.3.3.1 Fraction One Tyrindoleninone

To further assess whether the two semi-purified *D. orbita* fractions containing the bioactive compounds tyrindoleninone and 6-bromoisatin induced cell death by apoptosis in the primary granulosa cells and KGN cells, the TUNEL assay was performed, after exposing the cells to each fraction for 4 and 24h. The DNase treated primary granulosa and KGN cells (positive controls) were 100% TUNEL stained after 4 and 24h (Figures 6.13 and 6.18A and B; Figures 6.14A, 6.15A, 6.16A, 6.17A, 6.19A, 6.20A, 6.21A and 6.22A). The negative control (without rTdT) primary-derived granulosa and KGN cells were TUNEL negative (Figures 6.14B, 6.15B, 6.16B, 6.17B, 16.9B, 6.20B, 6.21B and 6.22B). After 4h incubation, 9% of untreated (DMSO control) primary granulosa, and 10% of KGN cells were TUNEL labelled (Figure 6.13A and B). After 24h, 12% of untreated (control) primary granulosa cells and 8% of KGN cells were TUNEL positive (Figure 6.13A and B).

In the presence of tyrindoleninone at 0.05mg/mL after 4h treatment, 31% of primary granulosa cells (Figure 6.13A & 6.14F) and 66% of KGN cells were TUNEL positive (Figure 6.13B & 6.15F). In comparison after 24h exposure to 0.005mg/mL of tyrindoleninone only 16% of primary granulosa cells were labelled (Figure 6.13A & 6.16D) in comparison with 62% of KGN cells ($P < 0.001$; Figure 6.13B & 6.17D).

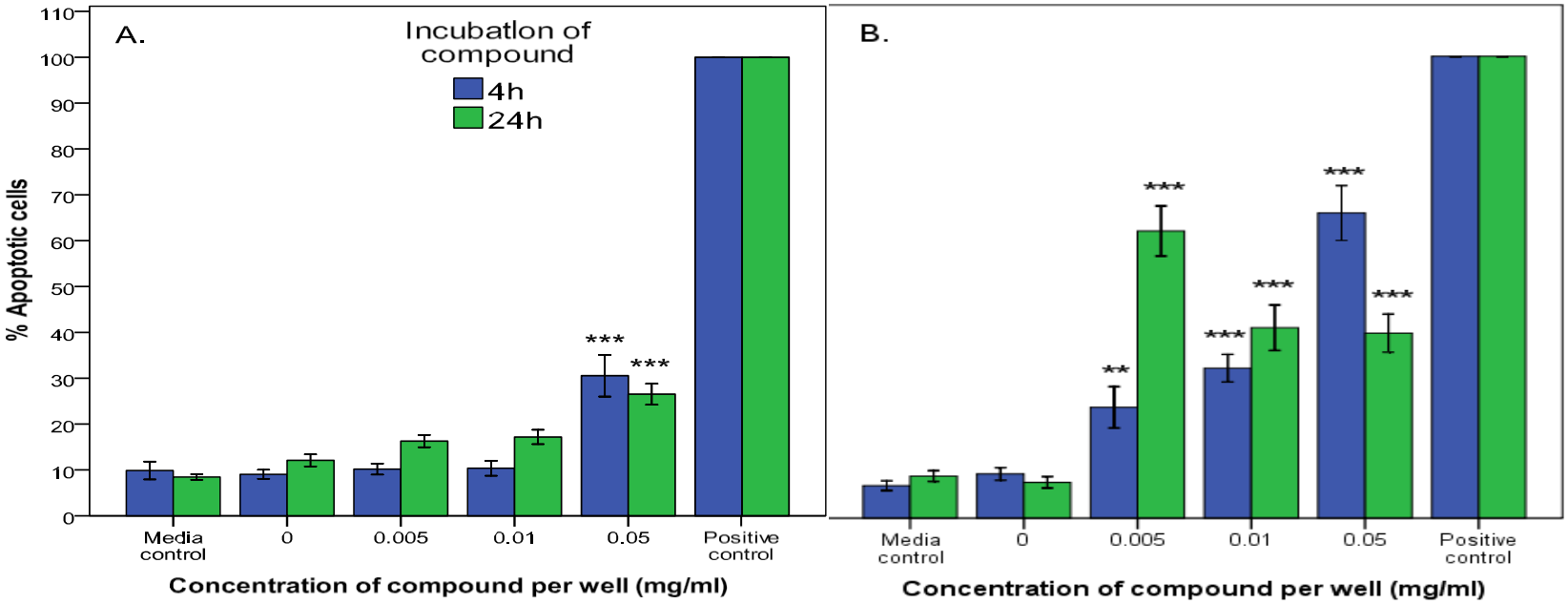


Figure 6.13: TUNEL-stained apoptotic primary-derived human granulosa (A) and KGN (B) cells after treatment with tyrindoleninone
After an initial incubation period of 24h to allow cell adherence, primary granulosa and KGN cells (30,000 cells per well) were treated with tyrindoleninone for 4 and 24h at concentrations of 0.005, 0.1 and 0.5mg/mL at 37^{o}C + 5% CO_2. The positive control represents 1µg/mL DNase I. The cells were then stained for apoptotic bodies using the DeadEnd^{TM} Fluorometric TUNEL System (Promega) and fixed onto slides. Photo micrographs were taken of four random microscope fields, and the number of TUNEL positive nuclei were calculated as a fraction of the total number of Propidium Iodide stained nuclei in each image. The average of the four fields was then calculated and the results are the mean ± 1 SEM of 3 separate repeated assays. Univariate analysis of variance with contrast (K Matrix) were conducted to determine the percentage of apoptotic cells from total cells induced by fraction one against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h shown as $p < 0.01$ (**) and $p < 0.001$ (***).

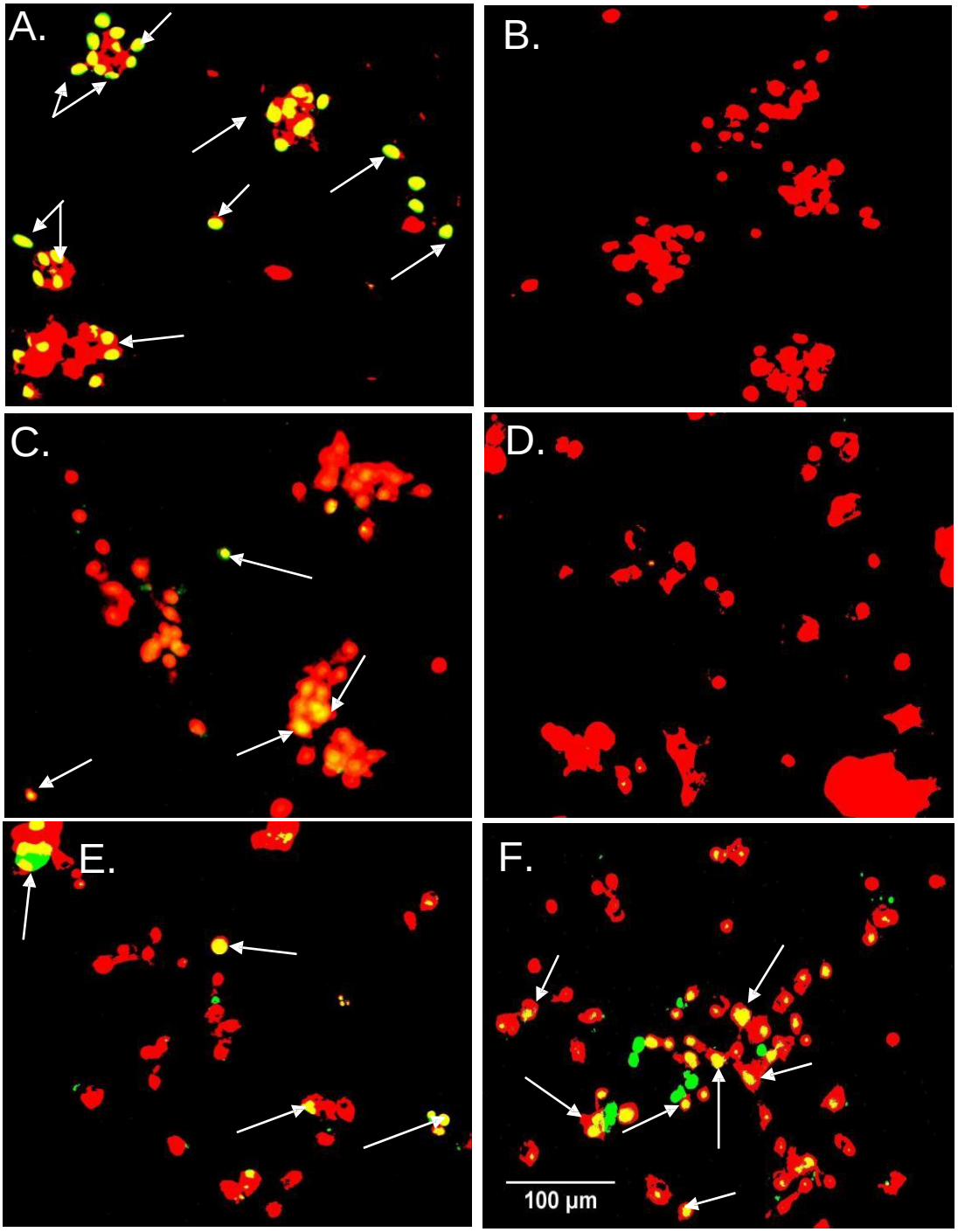


Figure 6.14: TUNEL staining of primary-derived human granulosa cells after treatment for 4h with tyrindoleninone

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), micro photographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction one; (E) after treatment with 0.01mg/mL fraction one; (F) after treatment with 0.05mg/mL fraction one. Scale bar 100µM.

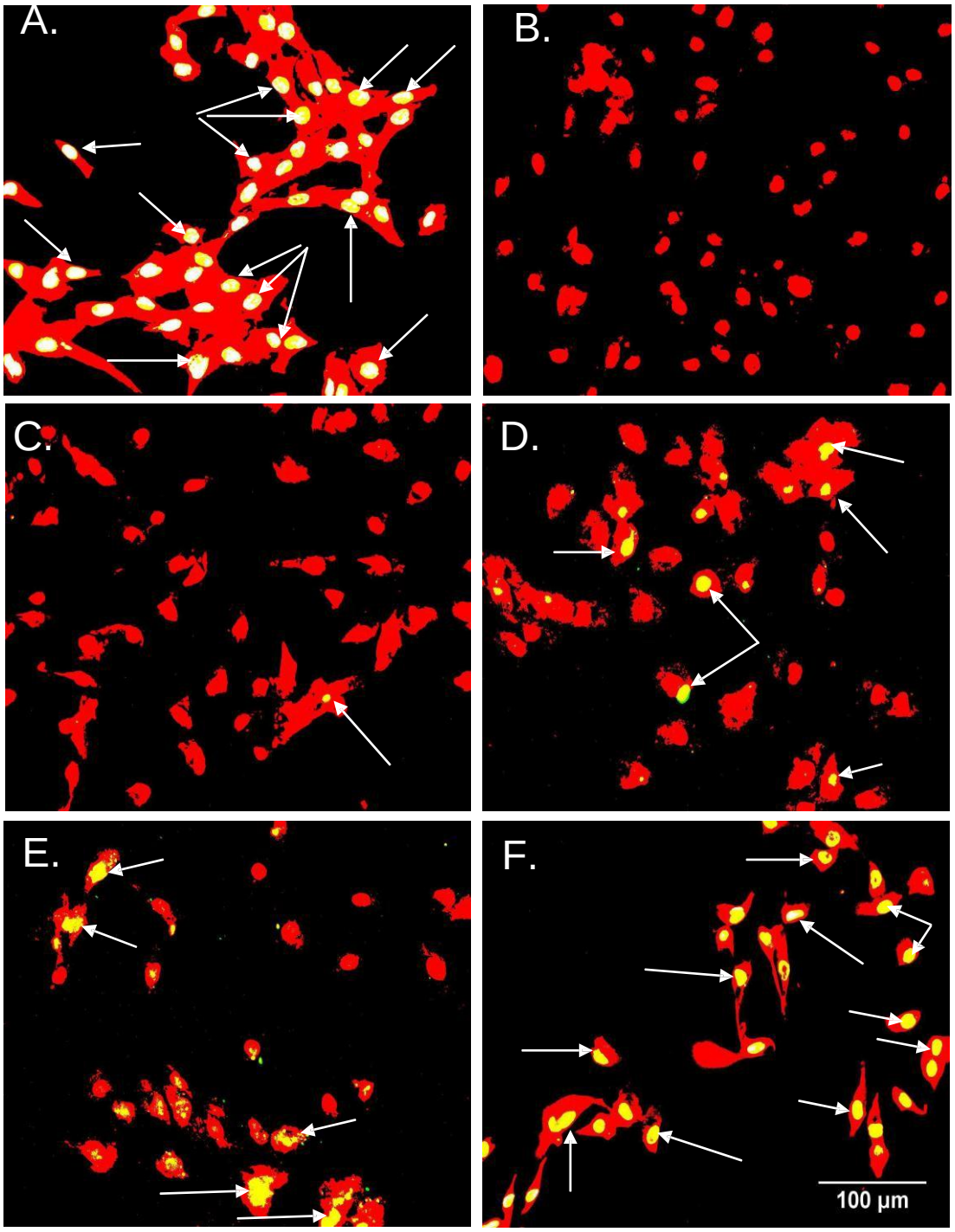


Figure 6.15: TUNEL staining of KGN cells after treatment for 4h with tyrindoleninone

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), micro photographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction one; (E) after treatment with 0.01mg/mL fraction one; (F) after treatment with 0.05mg/mL fraction one. Scale bar 100µM.

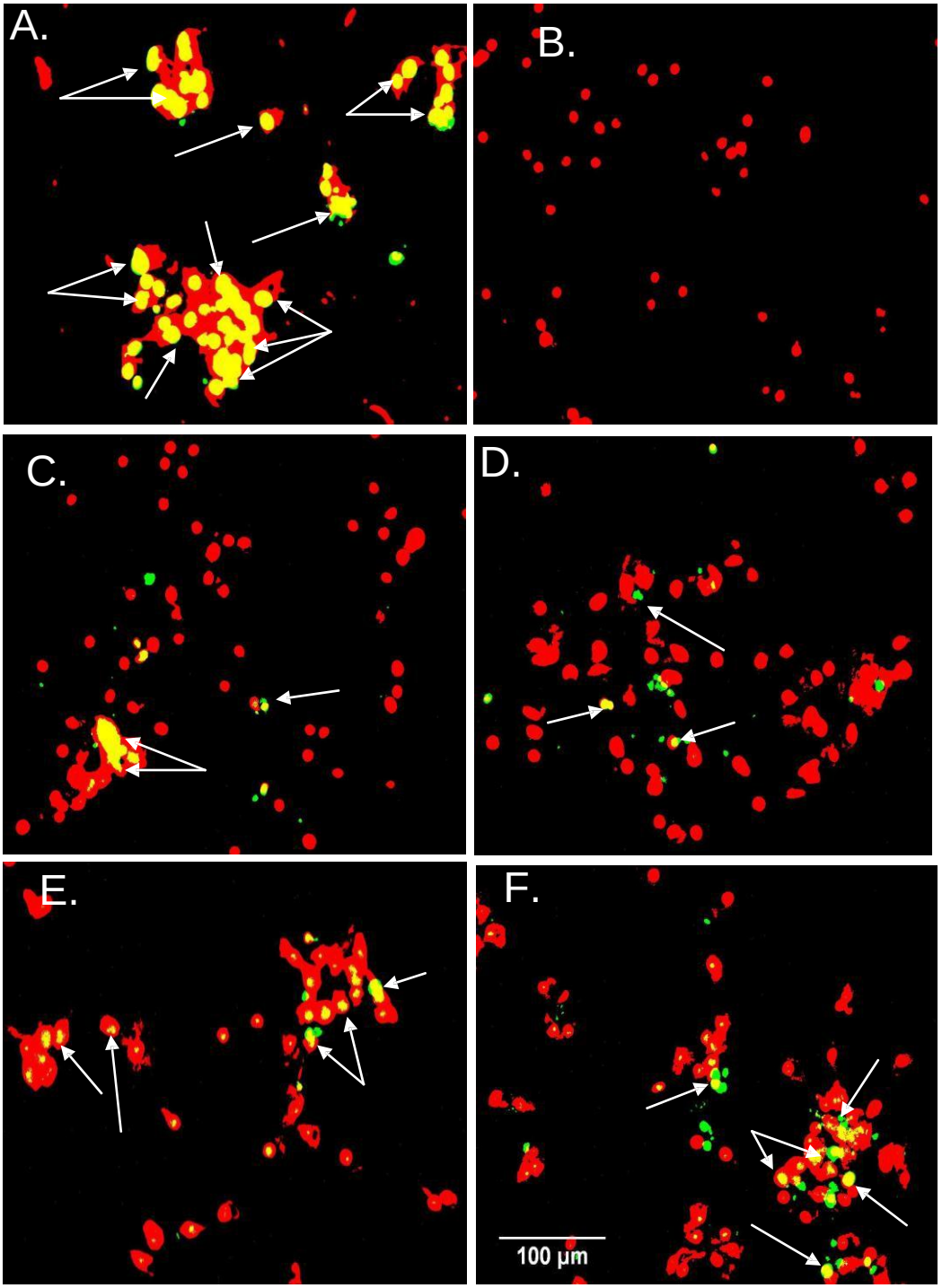


Figure 6.16: TUNEL staining of primary-derived human granulosa cells after treatment for 24h with tyrindoleninone

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), micro photographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction one; (E) after treatment with 0.01mg/mL fraction one; (F) after treatment with 0.05mg/mL fraction one. Scale bar 100µM.

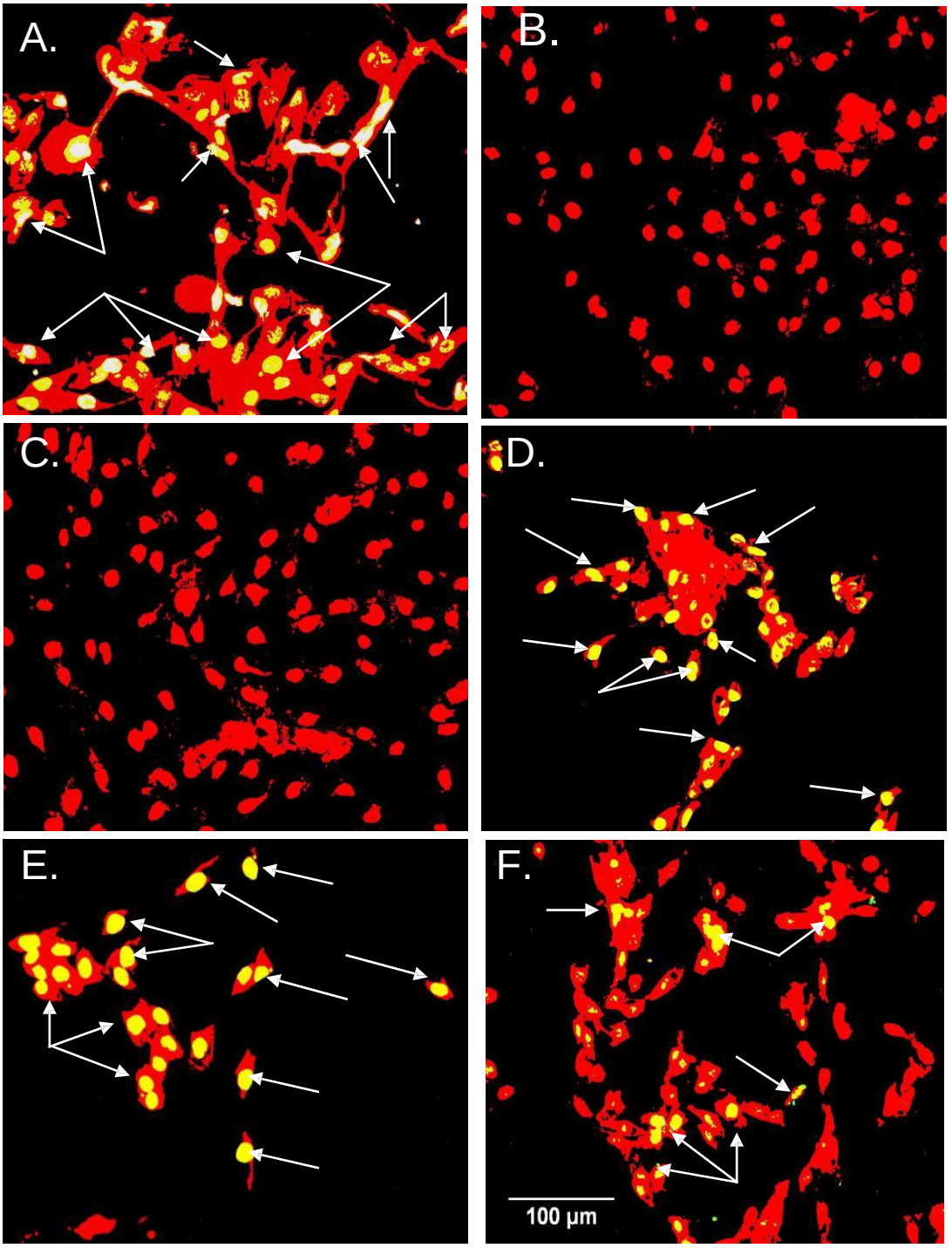


Figure 6.17: TUNEL staining of KGN cells after treatment for 24h with tyrindoleninone

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), micro photographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction one; (E) after treatment with 0.01mg/mL fraction one; (F) after treatment with 0.05mg/mL fraction one. Scale bar 100µM.

6.3.3.2 Fraction Two 6-Bromoisatin

When primary granulosa and KGN cells were treated with 6-bromoisatin at 0.05mg/mL for 4h, 27% of primary granulosa cells were TUNEL positive (Figure 6.18A & 6.19F), whereas 58% of KGN cells were positive and thus there were significantly more apoptotic cells than in the DMSO control ($p < 0.001$Figure 6.18B & 6.20F). After 24h of treatment with 6-bromoisatin (0.005mg/mL), 10% of primary-granulosa cells were TUNEL labelled (Figure 6.18A & 6.21D). In comparison, a statistically significant increase in TUNEL positive KGN cells of 53% relative to the DMSO control ($p < 0.001$Figure 6.18B & 6.22D) was observed after 24h.

Overall, both tyrindoleninone and 6-bromoisatin were more cytotoxic to the reproductive cancer lines in comparison to the primary-derived human granulosa cells. Cell numbers were inhibited in all three reproductive cancer cell lines at concentrations at least 10-fold less than that in the primary-granulosa cells. Furthermore, apoptosis was induced in the granulosa cancer cell line, KGN, at the lowest concentration of tyrindoleninone and 6-bromoisatin, in comparison to the corresponding primary cells at higher concentrations of 0.1mg/mL.

6.3.4 Detection of Apoptotic cells by DAPI staining

The results of the DAPI staining that were carried out to show condensed and fragmented DNA in apoptotic cells were highly subjective (Figures 6.23-25). The slides were difficult to assess or analyse as no clear trend was observed and the results were therefore considered unreliable for confirmation of apoptosis in the KGN cell line.

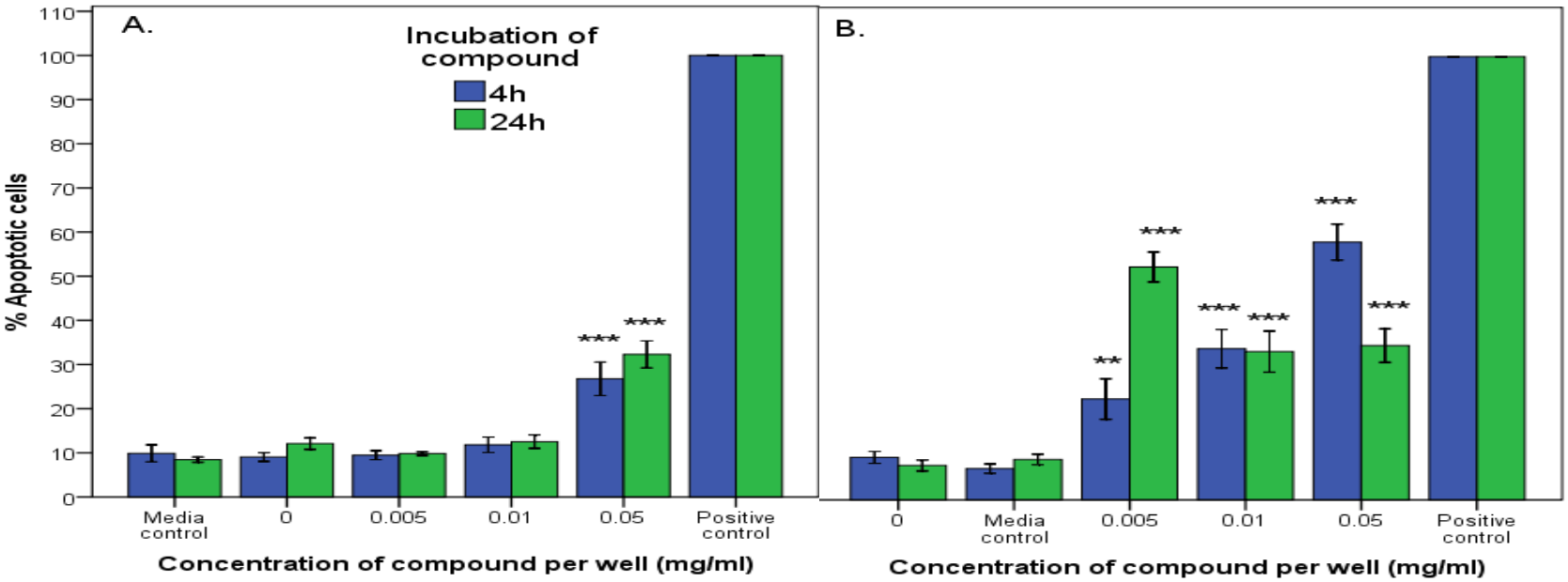


Figure 6.18: TUNEL-stained apoptotic primary-derived human granulosa (A) and KGN cells (B) after treatment with 6-bromoisatin for 4 and 24h

After an initial incubation period of 24h to allow cell adherence primary granulosa and KGN cells (30,000 cells per well) were treated with 6-bromoisatin for 4 and 24h at concentrations of 0.005, 0.1 and 0.5mg/mL at 37^{o}C + 5% CO_2. The positive control represents 1µg/mL DNase I. The cells were then stained for apoptotic bodies using the DeadEnd^{TM} Fluorometric TUNEL System (Promega) and fixed onto slides. Photo micrographs were taken of four randomly chosen microscope fields, and the number of TUNEL positive nuclei were calculated as a fraction of the total number of propidium iodide stained nuclei in each image. The average of the four fields was then calculated and the results are the mean ± 1 SEM of 3 separate repeated assays. Univariate analysis of variance with contrast (K Matrix) were conducted to determine the percentage of apoptotic cells from total cells induced by fraction two against the 1% DMSO control shown as 0 concentration at 4 and 24h. Significant difference between each treatment and the 1% DMSO control at 4 and 24h shown as $p < 0.01$ (**) and $p < 0.001$ (***).

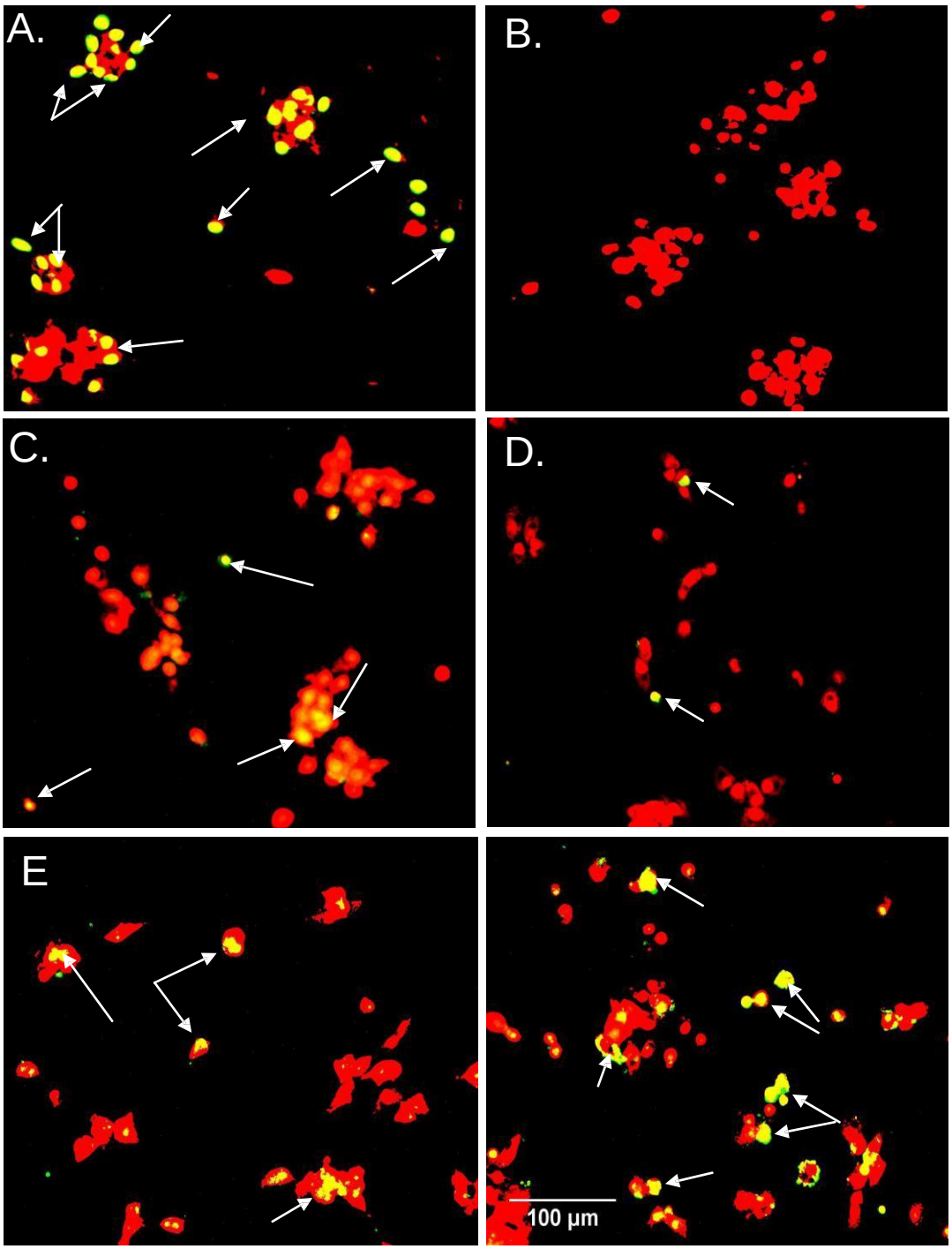


Figure 6.19: TUNEL staining of primary-derived human granulosa cells after treatment for 24h with 6-bromoisatin

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), microphotographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction two; (E) after treatment with 0.01mg/mL fraction two; (F) after treatment with 0.05mg/mL fraction two. Scale bar 100µM.

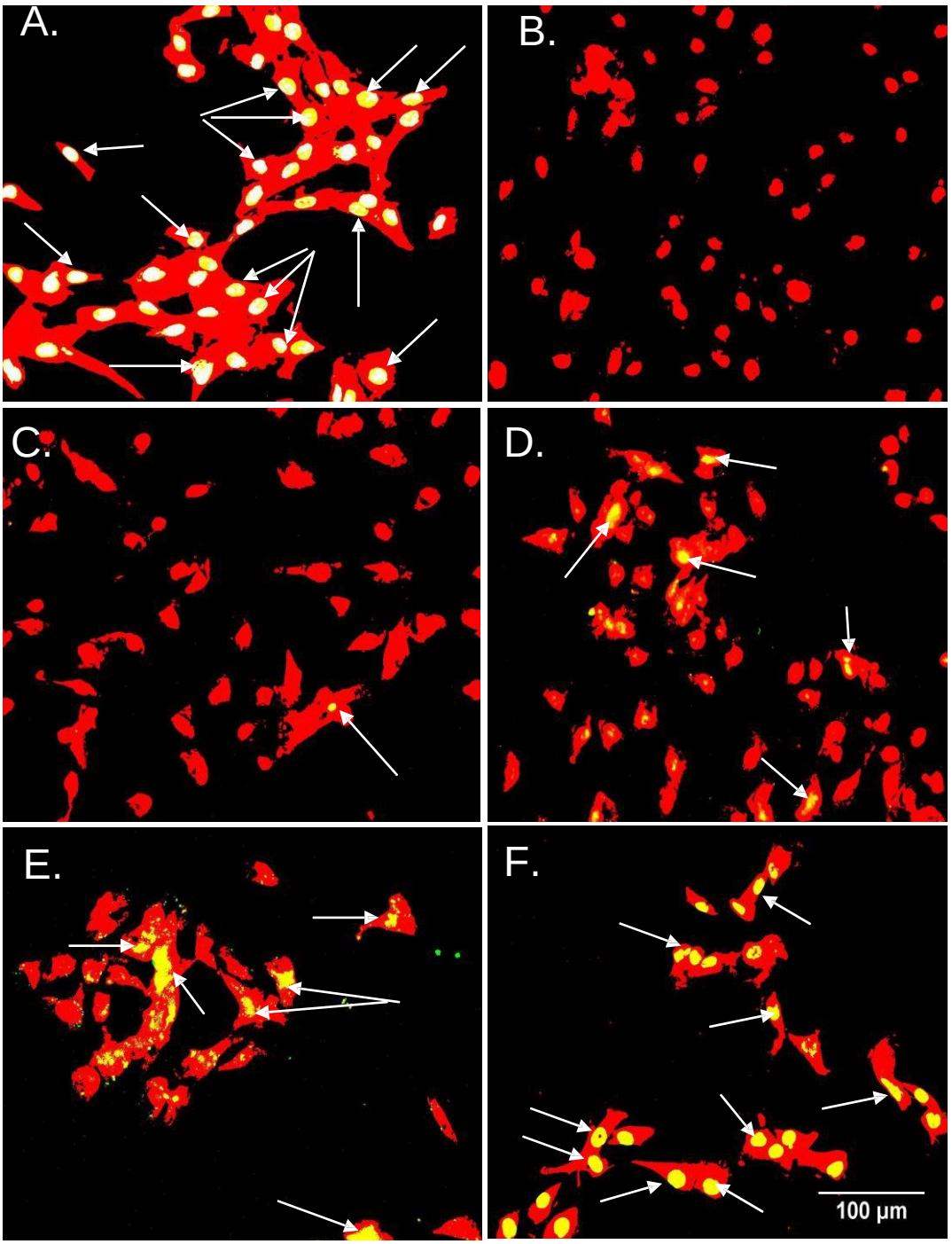


Figure 6.20: TUNEL staining of KGN cells after treatment for 4h with 6-bromoisatin

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), microphotographed on a Fluorescence Olympus BX50 Microscope at 200x magnification.TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction two; (E) after treatment with 0.01mg/mL fraction two; (F) after treatment with 0.05mg/mL fraction two. Scale bar 100µM.

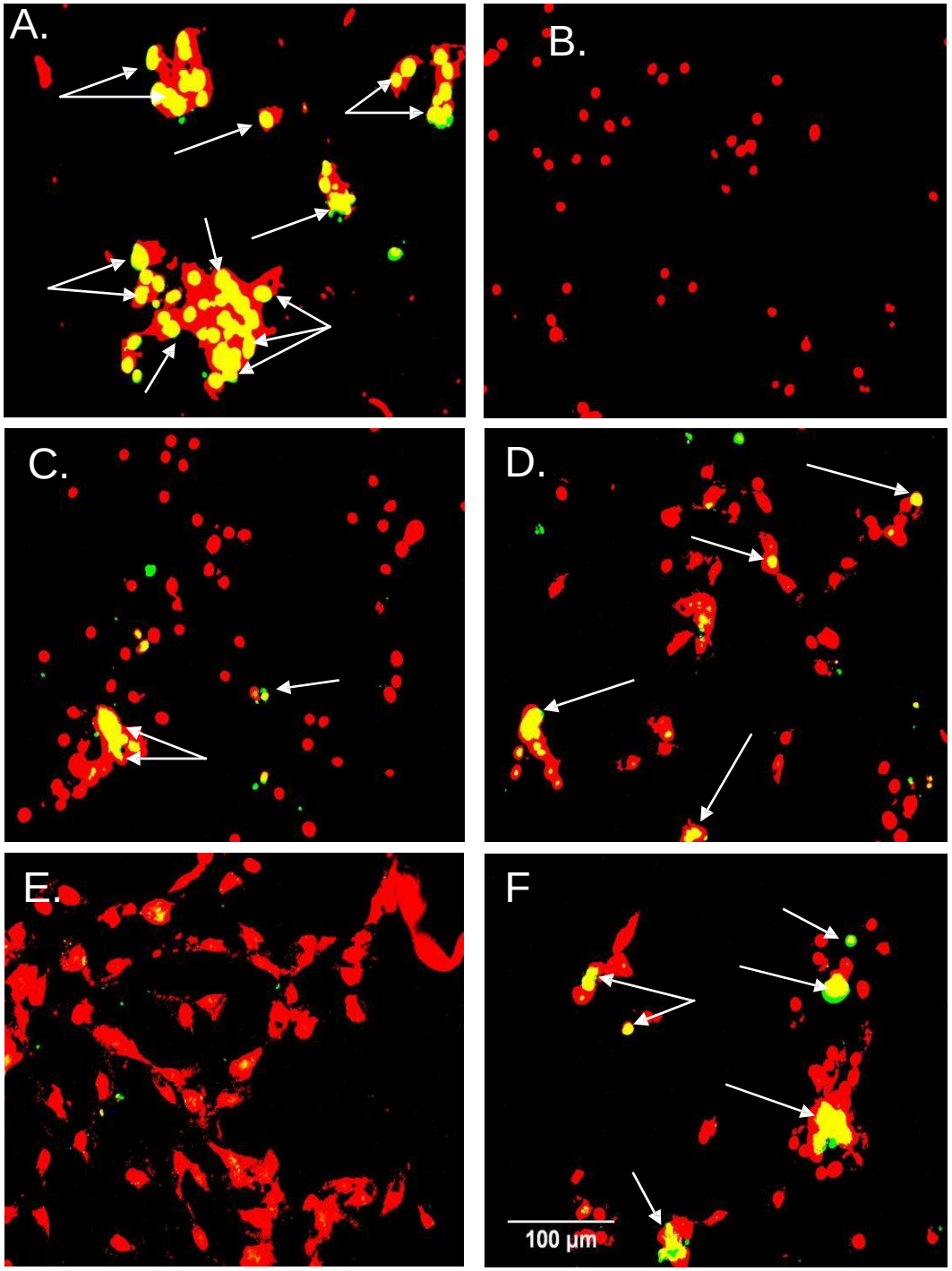


Figure 6.21: TUNEL staining of primary-derived human granulosa cells after treatment for 24h with 6-bromoisatin

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red), microphotographed on a Fluorescence Olympus BX50 Microscope at 200x magnification.TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction two; (E) after treatment with 0.01mg/mL fraction two; (F) after treatment with 0.05mg/mL fraction two. Scale bar 100µM.

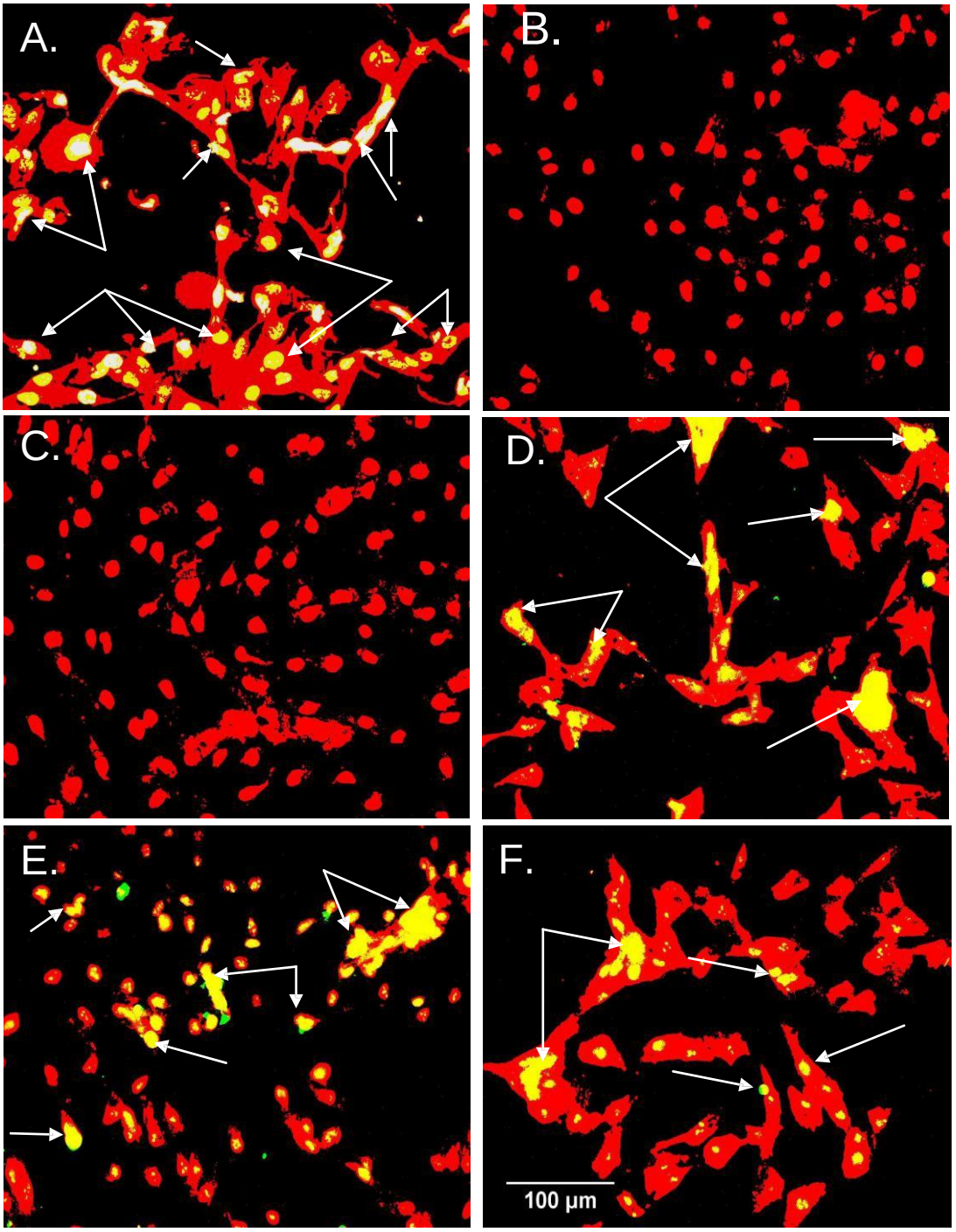


Figure 6.22: TUNEL staining of KGN cells after treatment for 24h with 6-bromoisatin

Pictures represent overlaid apoptotic stain (green) and nuclear stain (red) microphotographed on a Fluorescence Olympus BX50 Microscope at 200x magnification. TUNEL positive nuclei due to DNA fragmentation (yellow/white spots as indicated by arrows) in cells indicate cell death. Normal cells were stained (A) after treatment with DNAse I (positive control); (B) without terminal deoxynecleotide transferase (negative control); (C) after treatment with 1% DMSO control; (D) after treatment with 0.005mg/mL fraction two; (E) after treatment with 0.01mg/mL fraction two; (F) after treatment with 0.05mg/mL fraction two. Scale bar 100µM

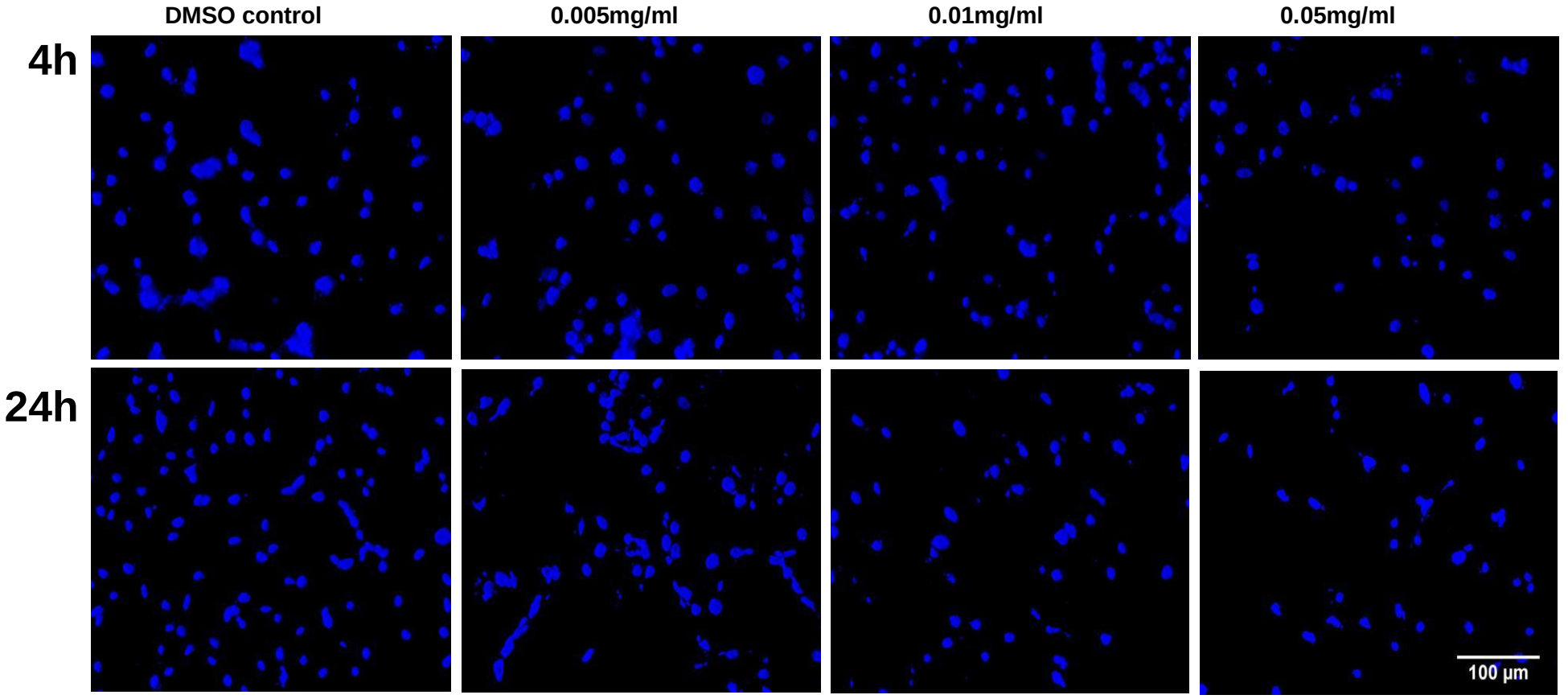


Figure 6.23: DAPI stining of KGN cells after treatment with tyrindoleninone at 200x magnification
DAPI staining of DNA condensation in KGN cells after treatment with tyrindoleninone (0.005, 0.01 and 0.05mg/mL) for 4 and 24h. Staining of DNA with DAPI was examined with a microscope using fluorescent mode at 200x magnification. Labelled DNA of viable cells scattered throughout the nucleus, bright condensation of chromatin reveals apoptotic cells. Scale bar 100µm.

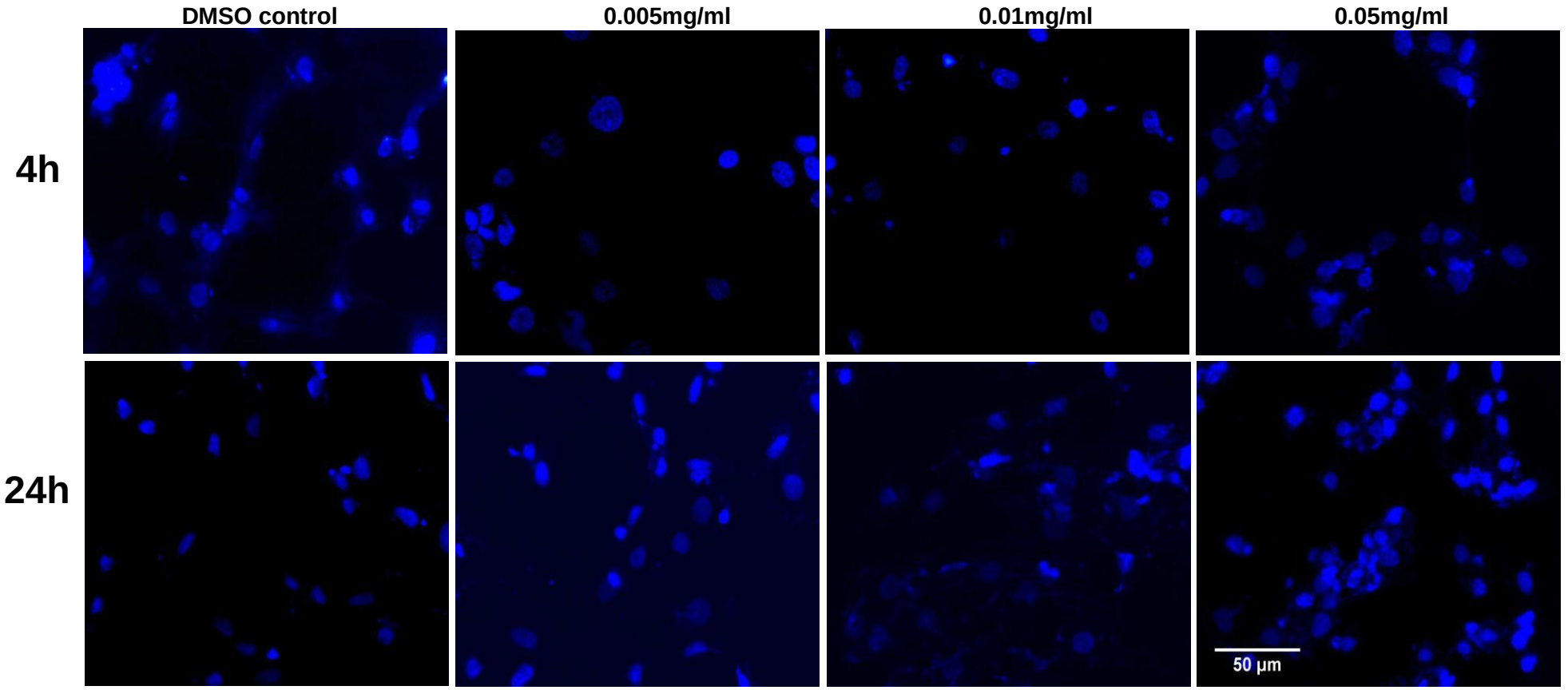


Figure 6.24: DAPI staining of KGN cells after treatment with tyrindoleninone 400x magnification.
DAPI staining of DNA condensation in KGN cells after treatment with tyrindoleninone (0.005, 0.01 and 0.05mg/mL) for 4 and 24h. Staining of DNA with DAPI was examined with a microscope using fluorescent mode at 400x magnification. Labelled DNA of viable cells scattered throughout the nucleus, bright condensation of chromatin reveals apoptotic cells. Scale bar 50µm.

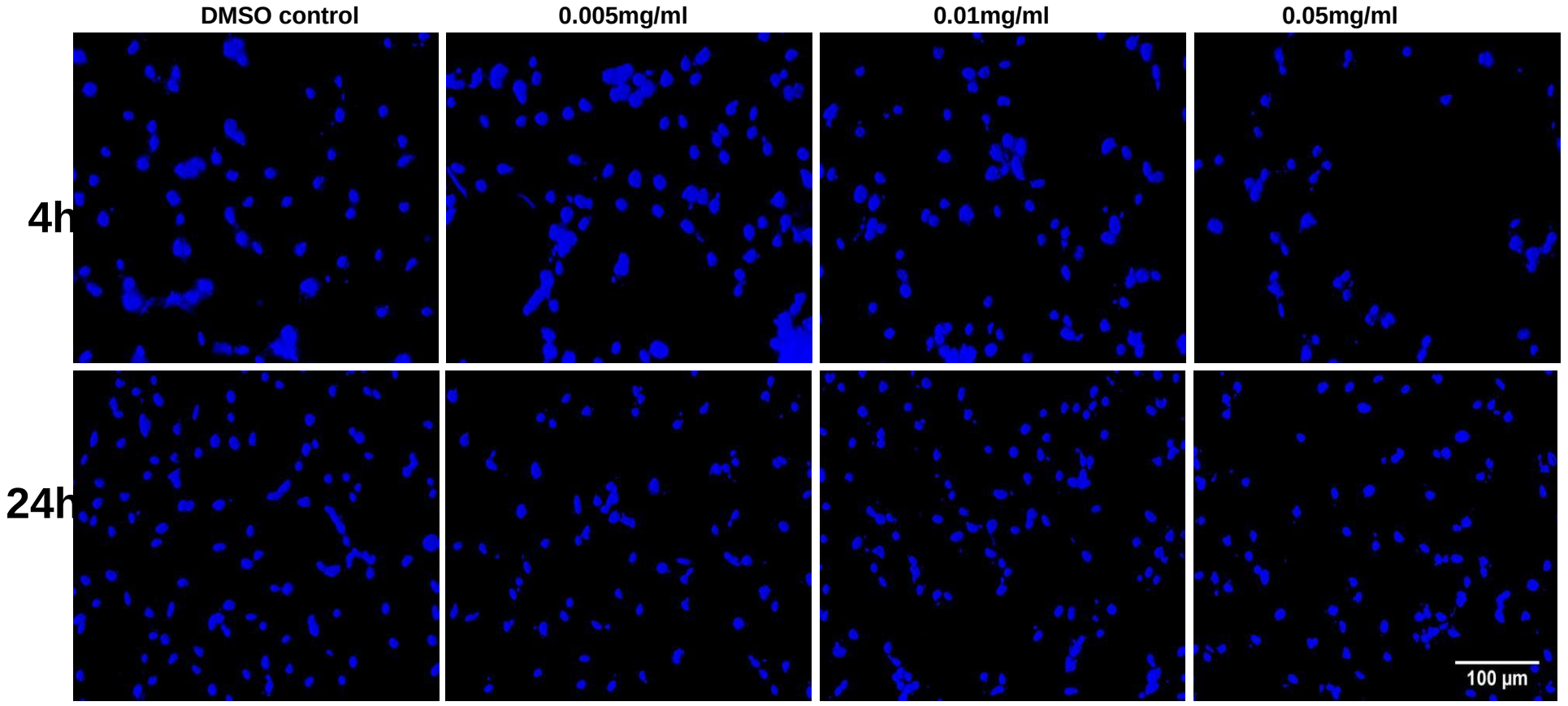


Figure 6.25: DAPI staining of KGN cells after treatment with 6-bromosatin 200x magnification
DAPI staining of DNA condensation in KGN cells after treatment with 6-bromoisatin (0.005, 0.01 and 0.05mg/mL) for 4 and 24h. Staining of DNA with DAPI was examined with a microscope using fluorescent mode at 200x magnification. Labelled DNA of viable cells scattered throughout the nucleus, bright condensation of chromatin reveals apoptotic cells. Scale bar 100µM

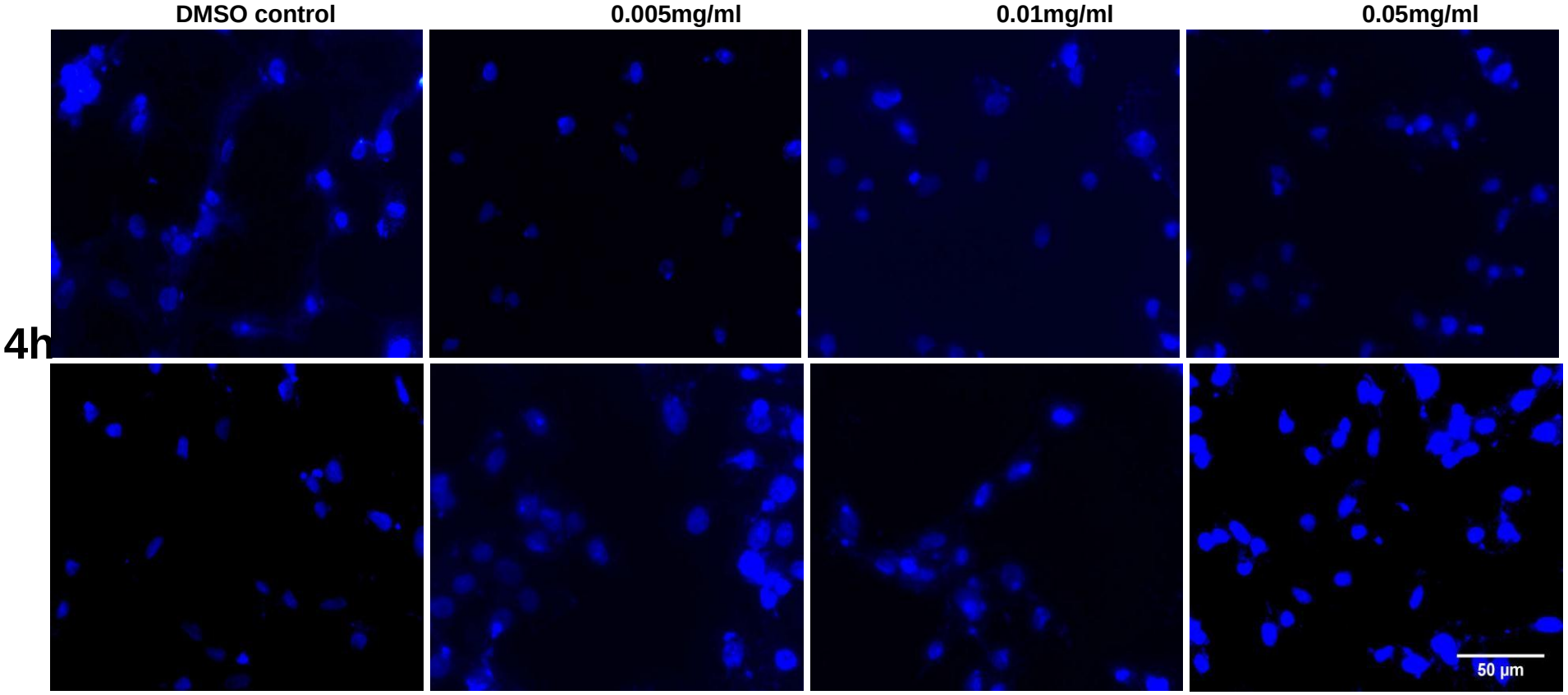


Figure 6.26: DAPI staining of KGN cells after treatment with 6-bromoisatin 400x magnification

DAPI staining of DNA condensation in KGN cells after treatment with 6-bromoisatin (0.005, 0.01 and 0.05mg/mL) for 4 and 24h. Staining of DNA with DAPI was examined with a microscope using fluorescent mode at 400x magnification. Labelled DNA of viable cells scattered throughout the nucleus, bright condensation of chromatin reveals apoptotic cells. Scale bar 50µM

Figure 6.26: DAPI staining of KGN cells after treatment with 6-bromoisatin 400x magnification
DAPI staining of DNA condensation in KGN cells after treatment with 6-bromoisatin (0.005, 0.01 and 0.05mg/mL) for 4 and 24h. Staining of DNA with DAPI was examined with a microscope using fluorescent mode at 400x magnification. Labelled DNA of viable cells scattered throughout the nucleus, bright condensation of chromatin reveals apoptotic cells. Scale bar 50µM

6.4 Discussion

The results of these experiments are consistent with other research confirming that the egg masses of *D. orbita* contain the same biologically active brominated compounds as the hypobranchial glands from the adult snails (Benkendorff, *et al*., 2001; Westley and Benkendorff, 2008). Semi-purification of the crude extract resulted in two relatively pure fractions. Fraction one contained predominately the brominated indolinone compound, tyrindoleninone, however traces of tyrindolinone were also detected at t_R 9.8 minutes on the diode array chromatogram. Tyrindoleninone is the more stable product as tyrindolinone can readiliy lose a methane thiol to give tyrindoleninone (Cooksey, 2001). Fraction two, consisted of the brominated isatin compound, 6-bromoisatin. Both tyrindoleninone and 6-bromoisatin decreased viable cell numbers in the KGN, JAr and OVCAR-3 reproductive cancer cell lines at much lower concentrations than in the primary-derived human reproductive cells, as determined by the crystal violet assay.

The up-regulation of caspase-3/7 in the KGN granulosa cancer cell line indicated that both tyrindoleninone and 6-bromoisatin induced cell death by apoptosis at low concentrations. In contrast, in the primary-derived granulosa cells, caspase-3/7 activity was only observed at a concentration at least 10-fold greater than in the KGN cells when treated with tyrindoleninone or 6-bromoisatin. Furthermore, the drop in caspase-3/7 signal at higher concentrations of tyrindoleninone, and 6-bromoisatin, coincided with a significant increase in LDH levels, which is consistent with secondary necrosis (Riss and Moravec, 2004; Pozhilenkova, *et al.*, 2008). TUNEL staining of the fragmented and condensed nuclei of KGN cells further confirmed activation of apoptosis in the presence of these compounds. The results of the DAPI

staining, however, were ambiguous and therefore were not used to confirm apoptosis by DNA fragmentation in the KGN cell line. Despite this, the increased selectivity towards the reproductive cancer cell lines, over the primary-derived reproductive cells, and the induction of cell death by apoptosis as opposed to necrosis, suggests that with further research these compounds could potentially be developed as a natural product for reproductive cancer therapy in the future.

Of the two fractions tested in the present research, tyrindoleninone (Fraction one) was the most active compound inhibiting the granulosa cancer cell line, KGN, by 50% at 0.04mg/mL in comparison to the primary-derived human granulosa cells (LC_{50}= >0.5mg/mL). In fact, tyrindoleninone was 5 times more active than 6-bromoisatin on the KGN cells after 4h treatment, as well as selectively inhibiting JAr (LC_{50} = 0.06mg/mL) and OVCAR-3 (LC_{50} = 0.08mg/mL) cancer cell lines. Benkendorff *et al*., (2011) have shown a 50% reduction in the U937 lymphoma cell line at 0.1mg/mL in the presence of tyrindoleninone for 4h. Other studies predicted an IC_{50} in cancer cell lines of 0.001mg/mL of tyrindoleninone after only 1h exposure (Vine, *et al.*, 2007a) and suggested that tyrindoleninone was much less cytotoxic to untransformed human mononuclear cells, with IC_{50} = 0.05mg/mL after 1h exposure. Differences between Vine, *et al*., (2007a) and the present study could be due to the difference in the cell types tested and how quickly and effectively the different cell lines were able to metabolise and eliminate the bioactive compound (Riss and Moravec, 2004). Furthermore, the relative purity of each fraction and the resulting concentration of the bioactive compound may also have contributed to the differences between these studies. Research has also shown that cell culture conditions, cell density and stock culture passage numbers can greatly affect the

outcome of an assay. Riss *et al.*, (2004) have shown that compounds can bind to constituents of the media reducing their effect on cancer cell lines. This difference between studies therefore, highlights the importance of maintaining consistent culture conditions to minimise variables in an assay.

Semi-purified and purified indole and isatin compounds are known to have cytotoxic effects in many cells types (Sze, *et al.*, 1993; Cane, *et al.*, 2000; Bacher, *et al.*, 2001; Chen, *et al.*, 2005c; Li, *et al.*, 2005; Vine, *et al.*, 2007a; Vine, *et al.*, 2007b; Matesic, *et al.*, 2008; Benkendorff, *et al.*, 2011) Research has demonstrated that isatins inhibit cell growth by targeting mitosis during cell division (Li, et al., 2005; Matesic, et al., 2008). Li *et al*., (2005), have shown that novel analogues of Combretastatin, a known inhibitor of tubulin binding, inhibit proliferation in breast cancer cell lines (MCF-7 and MDA-MB-231) and prostate cancer (PC-3). Matesic *et al*., (2008) have also shown that novel isatins bind to specific sites on tubulin in lymphoma (U937), leukaemia (Jurkat) and breast cancer (MCF-7) cell lines. These studies are useful in demonstrating the effects of indoles and isatins on biochemical changes in the cell however an inhibition of the cell cycle does not necessarily result in cell death. Cells arrested in the cell cycle are alive and often recover (Kroemer, *et al.*, 2009). Therefore, assays that activate executioner caspases such as caspase-3 are a more relevant determinant of cell death.

In the apoptotic pathway, caspase-3 triggers DNase activation, and therefore, precedes DNA fragmentation, which occurs near the end of the apoptotic pathway (Elmore, 2007). In our study, after 4h treatment with both tyrindoleninone, and 6-bromoisatin at low concentrations (0.005, 0.01, 0.05mg/mL), caspases-3/7 were

activated at a constant rate, while there was a dose-dependent increase in DNA fragmentation; which is consistent with the sequence of events in the apoptosis pathway (Glamoclija, *et al.*, 2005). Studies have shown that structurally similar indole and isatin compounds also induce apoptosis by the activation of caspase-3 at low concentrations, in a range of cell lines *in vitro* (Vine, *et al.*, 2007a; Vine, *et al.*, 2007b; Weng, *et al.*, 2007). For example 5,6,7-tribromoisatin activates caspase 3 and 7 at 0.003mg/mL after 5h in the Jurkat cell line (Vine, et al., 2007a). Other research suggests that indoles and isatins inhibit cell proliferation and activate apoptosis by binding and inhibiting signalling of extracellular protein kinases (ERKs; Can, et al., 2000). As ERK signalling pathways, such as the ERK/MARK phosphatase pathway, are essential for cell proliferation and survival (Steinmetz, *et al.*, 2004), it only follows that an inhibition of ERK would suppress growth and may even induce apoptosis in the cells.

After the KGN cells were treated for 24h with tyrindoleninone (Fraction one) and 6-bromoisatin (Fraction two), the series of apoptotic events were inconsistent. Both capsase-3/7 up-regulation and DNA fragmentation occurred concurrently at the lowest concentration tested (0.005mg/mL). As the executioner caspase-3 activates DNase activity in the apoptotic pathway and therefore precedes DNA fragmentation these results did not correspond to the know events of the apoptotic pathway (Kroemer, *et al.*, 2009). A possible explanation for this irregularity was that these apoptotic events may have been affected by the exposure time to the compounds. In other words, the maximum caspase-3/7 activity may have been reached at a lower exposure time (i.e. < 24h); somewhere between 4 and 24h. This may explain why

DNA fragmentation was detected at the lowest concentration of tyrindoleninone and 6-bromoisatin after 24h treatment.

Other studies showed that caspase-3 is active for a longer period than first thought. Glamačlija, *et al*. (2005), found that caspase-3 was detected for a longer period than DNA fragmentation in human granulosa follicular and luteal cells. Other researchers have suggested that the time between apoptotic events can vary between cell types and even within a single cell population (Goldstein, *et al.*, 2000). In fact, cells within the same population can undergo apoptotic events at different times (Goldstein, *et al.*, 2000). Goldstein, *et al*. (2000), exposed HeLa cells to UV in order to induce apoptosis, and found that cytochrome C release from a single cell occurs within 5 minutes of exposure, whereas cytochrome C release from the whole population was only detected after 8 hours treatment (Goldstein, *et al.*, 2000). Future research, therefore, should involve not only dose-dependent exposure, but also time-dependent exposure studies of these compounds, to determine the maximum signalling time of caspase-3/7 and therefore give a more accurate determinate of apoptotic events.

At higher concentrations of tyrindoleninone (≥0.1mg/mL) a dose-dependent increase in LDH was observed in the KGN, primary-derived granulosa cells and the JAr and OVCAR-3 cells. Our results therefore suggest that tyrindoleninone activates cell death by apoptosis at low concentrations, but causes secondary necrosis or necrosis at higher concentrations (Riss and Moravec, 2004; Pozhilenkova, *et al.*, 2008). In other words, at higher concentrations the apoptotic pathway was terminated and secondary necrosis may have been implemented. Other researchers have shown that a crude extract (0.5mg/mL) from the egg masses of *D. orbita* induces both apoptosis

and necrosis in the T-cell Jurkat cell line, and necrosis only in the HT29, colon cancer cell line (Benkendorff, *et al.*, 2011). Interestingly, *in vivo* studies have further demonstrated that high concentrations of crude *D. orbita* extracts (1mg/mL) induced a higher rate of apoptosis than low concentrations (0.125mg/mL), in cells of the distal colon *in vivo* (Westley, *et al.*, 2010). This is likely to be related to *in vivo* degradation and metabolism of the active compounds, thus requiring higher concentrations for oral delivery than is necessary to induce an effect *in vitro*. The differences between our results and that of others may also be related to the difference in testing the crude extract compared to testing semi-purified compounds in apoptosis assays. Furthermore, different concentrations of these muricid compounds possibly stimulate different cell death pathways in different cell lines and therefore, in the KGN, reproductive cells, apoptosis is being activated at low concentrations, whereas secondary necrosis or necrosis is activated at high concentrations.

This research confirms that tyrindoleninone and 6-bromoisatin are activating cell death in the KGN cancer cell line by apoptosis rather than necrosis however at low concentrations the mode of action is still unknown. The apoptotic pathway in the ovary has been well documented and both the Bcl-2 family of proteins and tumour necrosis factor-related apoptosis inducing ligand (TRAIL; a trans-membrane protein), and its receptors, have been implicated in apoptotic regulating pathways of ovarian and granulosa cancer cells (Woods, *et al.*, 2008a; Jääskeläinen, *et al.*, 2009). Furthermore, granulosa cells of primary and secondary follicles also express the TRAIL death receptors TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 (Jääskeläinen, *et al.*, 2009), although conflicting research has reported that normal

ovarian granulosa cells, are resistant to TRAIL-induced apoptosis (Wiley, *et al.*, 1995; Johnson, *et al.*, 2007).

Woods, *et al*., (2008), demonstrated that apoptosis in the KGN and COV434 human granulosa cancer cell lines, treated with cisplatin, a chemotherapeutic drug was enhanced with the addition of TRAIL and cell death was attributed to the extrinsic death receptor pathway. Therefore, it could be hypothesized that tyrindoleninone and 6-bromoisatin are inhibiting cell proliferation and activating apoptosis in the KGN granulosa cancer cell line via the TRAIL-induced pathway. However, further research would be required to identify the expression of the TRAIL mRNA and the TRAIL protein (possibly using real time PCR and/or immunohistochemical staining techniques), in both KGN cells and primary granulosa cells after treatment with the muricid compounds.

In conclusion, the natural brominated muricid compounds tyrindoleninone and 6-bromoisatin selectively inhibit the reproductive cancer cell lines at low concentrations, while having minimal effect on primary-derived female human granulosa cells. Furthermore, both tyrindoleninone and 6-bromoisatin induce cell death in the human granulosa cancer cell line, KGN, by apoptosis as opposed to necrosis at the lowest concentrations. Hence, our data, together with previous studies suggests that these naturally occurring compounds are selective towards human cancer cell lines both *in vitro* and *in vivo* and while preliminary investigations into the mechanisms of action have shown promising results, further and more in depth assays are needed to investigate the mode of action of these compounds on human reproductive cells.

Chapter 7 The Effects of the *D. orbita* Compounds on Primary-Derived Human Granulosa Cells

7.1 Introduction

The bioactive compounds from the Muricidae whelk, *D. orbita*, stimulate and inhibit hormone synthesis by primary-derived human granulosa cells (from women with normal reproductive physiology) and several reproductive cancer cell lines, both in the absence and presence of human choronic gonadotrophin (hCG; Chapter 5). Progesterone synthesis was either unaffected or inhibited in primary-derived granulosa cells however estradiol biosynthesis was often upregulated (Chapter 5). After 24 and 48h treatment, estradiol synthesis was significantly increased by primary-derived human granulosa cells when treated with fraction one (a mixture of the compounds) and fraction two (tyrindoleninone and tyrindolinone) often at the lowest concentrations. This hormetic dose-response or, stimulatory hormone response at low concentrations although unexpected has previously been reported (Calabrese, 2008; Nascarella, *et al.*, 2009). Furthermore, these muricid compounds inhibited KGN, JAr and OVCAR-3 cancer cell growth at concentrations at least 10-fold lower than in the primary-derived granulosa cells (Chapter 5).

In light of the fact that the secretion from the muricid family of whelks is also the source of the homeopathic remedy 'Murex purpurea' (Dunham, 1864) which is recommended for a range of gynaecological disorders, (Chapter 1; Boreicke, 2005), further investigations were undertaken to determine if theses natural muricid compounds could increase progesterone and estrogen synthesis by granulosa cells derived from women with normal and abnormal reproductive physiology both in the presence and absence of hCG.

Couples undergoing assisted reproductive technologies (ART) do so for numerous reasons based on either female or male infertility factors or both (Baird, *et al.*, 2000; Jose-Miller, *et al.*, 2007). Women with ovarian dysfunctions such as ovarian cysts and PCOS, along with women who have a history of pelvic inflammatory disease, fibroids, endometriosis, and unexplained infertility, are all classified as female infertility factors (Jose-Miller, *et al.*, 2007). Sperm defects such as decreased motility and erectile dysfunctions are classified as male factor infertility (Jose-Miller, *et al.*, 2007). For the benefit of this thesis, granulosa cells isolated from the follicular aspirates of women undergoing ART were separated into two distinct groups. These groups were classified as either cells from women with abnormal reproductive physiology that is, women who suffer infertility due to a female factor, and cells from women with normal reproductive physiology that is, women who are undergoing ART primarily due to a male factor.

Therefore, the main aims of this study were:

1. To investigate the cytotoxic effects of the semi-purified fractions containing bioactive compounds on primary-derived human granulosa cells from fertile women and compare with granulosa cells from infertile women (section 2.2.1.1 and Appendix II).
2. To examine the effects of the semi-purified fractions on basal progesterone and estradiol synthesis by primary-derived human granulosa cells from fertile women and granulosa cells from infertile women.
3. To examine the effects of the semi-purified fractions on human choronic gonadotrophin (hCG) stimulated progesterone and estradiol synthesis by

primary-derived human granulosa cells from fertile women and granulosa cells from infertile women.

Based on the positive effects these compounds have had on stimulating basal estradiol biosynthesis by primary-derived granulosa cells (Chapter 5) it was hypothesised that one or more of these semi-purified *D. orbita* fractions would stimulate basal estradiol synthesis at low or non-toxic concentrations in both primary-derived granulosa cells from fertile and infertile women.

7.2 Methods

7.2.1 Extraction, Isolation & Semi-purification of Bioactive Compounds from *Dicathais Orbita*

Compounds for the following experiments were extracted from *D. orbita* egg masses, semi-purified, identified and prepared for cell assays as previously described (section 5.2.1).

7.2.2 The Effects of the *D. orbita* Compounds on Human Primary-Derived Granulosa Cells from Women with Normal and Abnormal Reproductive Physiology

7.2.2.1 Cell Culture

Primary-derived human granulosa cells were isolated from women undergoing IVF and ICSI as previously described (sections 2.2.1.1). The cells were separated into two groups based on the infertility factor of the women (section 2.2.1.1; Appendix II). For the benefit of this thesis granulosa cells from women with normal reproductive physiology were combined into one group and granulosa cells from women with abnormal reproductive physiology into another group (section 2.2.1.1 and Appendix II). Granulosa cells were isolated from aspirates (section 2.2.1.1), collected from cases 25, 29, 30, 34, 35, 36 and 37 (2 Polycystic ovarian syndrome; 2 endometriosis and 3 idiopathic; n=7) and cases 27, 28 and 31 (1 tubular obstruction;

1 decrease sperm motility; 1 sperm defect; n=3; Appendix II). Viable cells were determined using trypan blue on a haemocytometer (section 2.2.2.3).

7.2.2.2 Cell Viability Assay to Test the Semi-purified D. orbita *Fractions*

Primary-derived human granulosa cells (10,000 cells per well) were seeded into sterile 96-well flat bottom plates (Interpath) in a final volume of 0.1mL per well of complete cell culture medium (section 2.1.2.1.3) and incubated at 37^{o}C + 5% CO_2 for 24h to allow differentiation and luteinization (Young, *et al.*, 2005a). All outside wells of plates contained 0.1mL of sterile PBS (section 2.1.3.5) to prevent evaporation in the inside treatment wells. Standard curve plates of 0-40,000 cells per well in doubling concentration, in four replicate wells, were setup for each cell group (fertile and infertile) and run in conjunction with each experiment (used to determine final cell number in the test plates).

After the initial cell adherence period, the spent medium was removed from all treatment wells and cells were exposed to 0.005, 0.01, 0.05, 0.1, 0.5 and 1mg/mL of the three semi-purified *D. orbita* fractions (section 5.2.1.3) prepared in cell culture medium (section 2.1.3.1.3) ± 1,000mIU/mL of hCG for 4, 24, 48 or 72h, in three replicate wells each, at 37^{o}C + 5% CO_2. Controls consisted of a medium only negative control (section 2.1.2.1.3), 1% DMSO solvent control, base media without serum (as a positive control) and 1,000mIU/mL hCG control (section 2.1.5.2).

At the end of each treatment period the supernatant was removed from all treatment wells and stored (-20^{o}C) for the measurement of both progesterone and estradiol by radioimmunoassay (section 2.2.5.2). The cells in the treatment wells were then rinsed with sterile 1x PBS (section 2.1.2.5). Cell viability was determined by the crystal

violet assay (section 2.2.2.1). The assay was repeated on seven separate occasions for cells from the infertile women and three for cells from the fertile women and the results shown as the mean ± 1 SEM.

7.2.2.3 Statistical Analysis

Two-way analysis of variance (ANOVA) using the sensitive contrast K Matrix analysis tests were conducted to examine the effects of the semi-purified *D. orbita* fractions on both cell viability and hormone synthesis using SPSS software package version 17 (Pallant, 2002). The alpha value was adjusted when necessary to a significance level of $p < 0.01$, when homogeneity of variance was violated using the Levene's Test of Equality of Error (Appendix VIII). The concentration that effectively inhibited cell numbers by 50% (LC_{50}) was calculated using nonlinear regression and sigmoidal curves with GraphPad Prism software version 5 (GraphPad Software Inc).

7.3 Results

7.3.1 The Effects of hCG on Granulosa Cell Proliferation and Hormone Synthesis

7.3.1.1 Granulosa Cells from Women with Normal Reproductive Physiology

The 1% DMSO control was found to significantly reduce granulosa cell viability from fertile women after 24h exposure in comparison to the media control (Figure 7.1A). After 48h, cell numbers increased after exposure to the 1% DMSO control, although this was not significantly different to the media control (Table 7.1; Figure 7.1A). hCG increased cell proliferation in granulosa cells at 48 and 72h exposure in the absence of 1% DMSO, although this was not significant (Table 7.1; Figure 7.1D).

Basal progesterone synthesis by granulosa cells from fertile women increased after 24h (Table 7.1; Figure 7.1B) and estradiol increased after 48h (Table 7.1; Figure

7.1C). Progesterone synthesis was unexpectedly low when primary granulosa cells from fertile women were stimulated with hCG for 4h in comparison to media only control (Table 7.1; Figure 7.1E). However, progesterone synthesised by granulosa cells from fertile women increased after 48h in the presence of hCG, in comparison to the media only control (Table 7.1; Figure 7.1E). hCG-stimulated estradiol synthesis by the granulosa cells from fertile women significantly increased after 24h however, after 48h hCG-estradiol decreased slightly in comparison to basal estradiol (Table 7.1; Figure 7.1F).

Granulosa cell viability from fertile women was reduced in the absence of serum (positive control; Table 7.1; Figure 7.1A), whereas basal progesterone synthesis by granulosa cells was only marginally decreased after 4, 24 and 48h (Table 7.1; Figure 7.1B). Estradiol synthesis by granulosa cells in the absence of serum however, was significantly decreased at 4h and although it did decrease after 24 and 48h, it wasn't found to be significantly lower than the media control (Table 7.1; Figure 7.1C).

7.3.1.2 Granulosa Cells from Women with Abnormal Reproductive Physiology

In the presence of hCG, the proliferation of primary-derived human granulosa cells from infertile women increased slightly after 48h, although not significantly (Table 7.1; Figure 7.2D). Basal progesterone and estradiol synthesis by granulosa cells from infertile women was similar to that of cells from fertile women except estradiol was lower after 48h (Table 7.1; Figure 7.2B & C). Progesterone synthesis was increased in hCG stimulated granulosa cells after 24 and 48h (Table 7.1; Figure 7.2E). Estradiol secretion was increased slightly after 24h by granulosa cells from infertile women when stimulated with hCG in comparison to the media controls and like granulosa cells from fertile women, there was a slight decrease in hCG stimulated estradiol synthesis after 48h by these cells (Table 7.1; Figure 7.2F).

Granulosa cells from infertile women were inhibited significantly in the absence of serum at ≤ 72h (Table 7.1; Figure 7.2A). Basal progesterone synthesis was inhibited significantly by granulosa cells from infertile women in the absence of serum, after 4, 24 and 48h (Table 7.1; Figure 7.2B). In comparison, basal estradiol synthesis was only significantly inhibited after 48h in the absence of serum (Table 7.1: Figure 7.2C).

Table 7.1: Effect of controls on primary granulosa cell numbers
Effect of hCG and no serum (positive) controls on cell viability, progesterone and estradiol synthesis in comparison to media only and 1% DMSO controls by granulosa cells from women with normal (fertile) and abnormal (infertile) reproductive physiology. Cell viability shown as final cell number per well; progesterone in ng/ml and estradiol in pg/ml (n=3 ± 1 Standard deviation).

Cell Type	Time (h)	Media	1% DMSO	hCG	hCG + 1% DMSO	No serum
Fertile	4					
Cell Number		12010 (±2621)	10781 (±1766)	13325 (±4427)	11824 (±2139)	2290 (±1234)
Progesterone		468 (±18)	314 (±162)	295 (±172)	415 ± (301)	338 (±358)
Estradiol		107 (±29)	221 (±113)	149 (±47)	144 (±42)	70 (±24)
Infertile	4					
Cell Number		10417 (±1630)	10278 (±1620)	11391 (±2138)	10504 (±1919)	4278 (±27110
Progesterone		461 (±185)	516 (±221)	510 (±243)	685 (±421)	367 (±213)
Estradiol		130 (±95)	138 (±168)	128 (±78)	139 (±113)	120 (±98)
Fertile	24					
Cell Number		15643 (±351)	10451 (±2162)	13136 (±1722)	12277 (±1580)	3731 (±1982)
Progesterone		847 (±312)	703 (±181)	1009 (±448)	1202 (±457)	604 (±328)
Estradiol		244 (±13)	192 (±83)	305 (±97)	265 (±74)	128 (±74)
Infertile	24					
Cell Number		10429 (±1256)	10358 (±1731)	11747 (±2872)	10862 (±1613)	4414 (±1967)
Progesterone		717 (±228)	865 (±231)	1197 (±412)	1115 (±390)	602 (±247)
Estradiol		164 (±140)	216 (±160)	219 (±173)	322 (±346)	170 (±219)
Fertile	48					
Cell Number		12710 (±1800)	18366 (±6575)	19650 (±3304)	16482 (±2868)	3725 (±1901)
Progesterone		820 (±311)	827 (±639)	1439 (±257)	1460 (±269)	483 (±286)
Estradiol		523 (±189)	280 (±191)	486 (±159)	256 (±112)	318 (±232)
Infertile	48					
Cell Number		11011 (±1908)	10640 (±1717)	12444 (±3362)	11049 (±2254)	3327 (±2764)
Progesterone		852 (±222)	665 (±194)	1124 (±393)	1226 (±386)	410 (±173)
Estradiol		209 (±144)	307 (±436)	201 (±104)	369 (±430)	88 (±155)
Fertile	72					
Cell Number		14010 (±2689)	14729 (±2507)	16790 (±4317)	13460 (±1966)	462 (±2094)
Infertile	72					
Cell Number		11023 (±1246)	10560 (±1874)	12670 (±4912)	12469 (±3864)	2564 (±3010)

7.3.2 The Effect of the *Dicathais orbita* Compounds on Primary-Derived Human Granulosa Cells

7.3.2.1 The Effect of Fraction one on Cell Viability and Hormone Synthesis

7.3.2.1.1 Granulosa Cells from Women with Normal Reproductive Physiology

The semi-purified *D. orbita* fraction one, which contained a mixture of brominated isatin and indole compounds (section 5.3.2.1), did not have any major effect on granulosa cell viability from fertile women, either in the absence or presence of hCG (Table 7.2; Figure 7.1A & D). Cell viability was only significantly reduced at 0.1mg/mL after 4h in the absence of hCG, and at 1mg/ml after 4h in the presence of hCG (Figure 7.1A and D).

Basal progesterone and estradiol synthesis by granulosa cells from the fertile women was not affected by fraction one (Table 7.2; Figure 7.1B and C). However, when stimulated with hCG, progesterone synthesis was inhibited after 24 and 48h (0.005 and 0.5mg/mL; Table 7.2; Figure 7.1E). Estradiol synthesis in comparison, was stimulated by fraction one in the presence of hCG at the lowest concentration tested (0.005mg/mL) after 48h incubation (Table 7.2; Figure 7.1F).

7.3.2.1.2 Granulosa Cells from Women with Abnormal Reproductive Physiology

Granulosa cell viability from infertile women were significantly reduced by fraction one both in the absence and presence of hCG (Table 7.2; Figure 7.2A and D). Cell viability was reduced to 77% of control when exposed to fraction one for 4h without hCG at 0.1mg/mL (Table 7.2; Figure 7.2A), whereas in the presence of hCG, 0.5mg/ml of fraction one caused a reduction in cell numbers (to 73% of control; Figure 7.2D). After 72h exposure LC_{50} was 0.4mg/ml both in the presence and absence of hCG (Table 7.2; Figure 7.2A and D).

Basal progesterone was stimulated at the lowest concentration (0.005mg/mL) after 4h, but inhibited after 48h, at all concentrations tested (Table 7.2; Figure 7.2B). Basal estradiol synthesis by granulosa cells from the infertile women was not affected by fraction one (Table 7.2; Figure 7.2C). hCG stimulation had no significant effect in comparison to the control on either progesterone or estradiol synthesis by granulosa cells from infertile women (Table 7.2; Figure 7.2E and F).

Table 7.2: Results of fraction one on primary-derived human granulosa cells
Effects of Fraction one (F1) ± hCG on primary-derived human granulosa cell viability, progesterone and estradiol synthesis from women with normal and abnormal reproductive physiology (shown as fertile or infertile). Cell viability shown as $LC_{50,}$ the concentration that killed 50% of the cells, & hormone data shown as the concentration that was significantly different to the 1% DMSO control. (+) = concentrations that stimulated hormone synthesis; (-); concentrations = that inhibited hormone synthesis; N/T = not tested; n/e = no effect.

Cell Source	Time (h)	LC_{50} (mg/mL)		Effect on Hormone Synthesis			
				Progesterone		Estradiol	
		F1	F1 + hCG	F1	F1 + hCG	F1	F1 + hCG
Fertile	4	>1	>1	n/e	n/e	n/e	n/e
Infertile		>1	>1	0.005 (+)	n/e	n/e	n/e
Fertile	24	>1	>1	n/e	0.005, 0.5 (-)	n/e	n/e
Infertile		>1	>1	n/e	n/e	n/e	n/e
Fertile	48	>1	>1	n/e	0.005, 0.05, 0.5 (-)	n/e	0.005, 0.05 (+)
Infertile		1	1	0.005, 0.05, 0.5 (-)	n/e	n/e	n/e
Fertile	72	>1	>1	N/T	N/T	N/T	N/T
Infertile		0.4	0.4	N/T	N/T	N/T	N/T

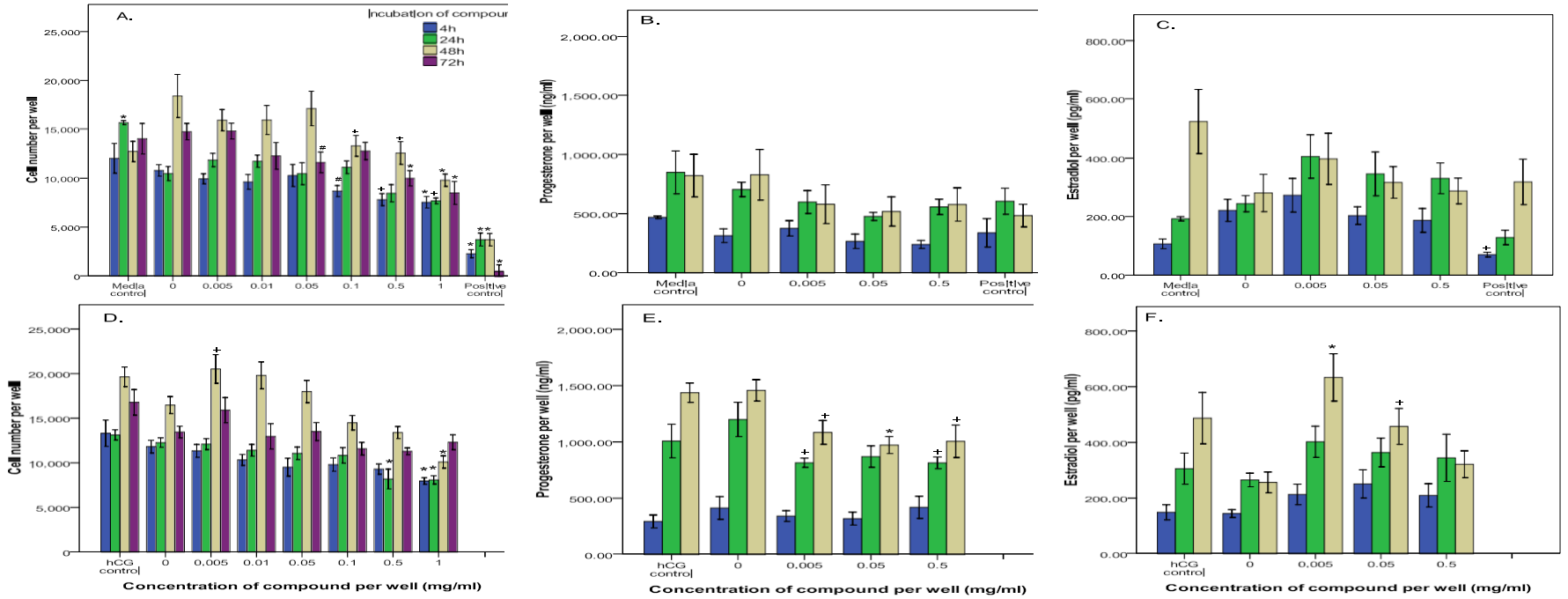


Figure 7.1: The effect of fraction one on primary granulosa cells derived from women with normal physiology

The effect of the semi-purified *D. orbita* fraction one on primary-derived human granulosa cells from women with normal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period, granulosa cells (10,000cells/well) were treated with fraction one ± hCG for 4, 24, 48 and 72h. The positive control was DMEM/F12 base media in the absence of FBS. Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis was assessed by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*)

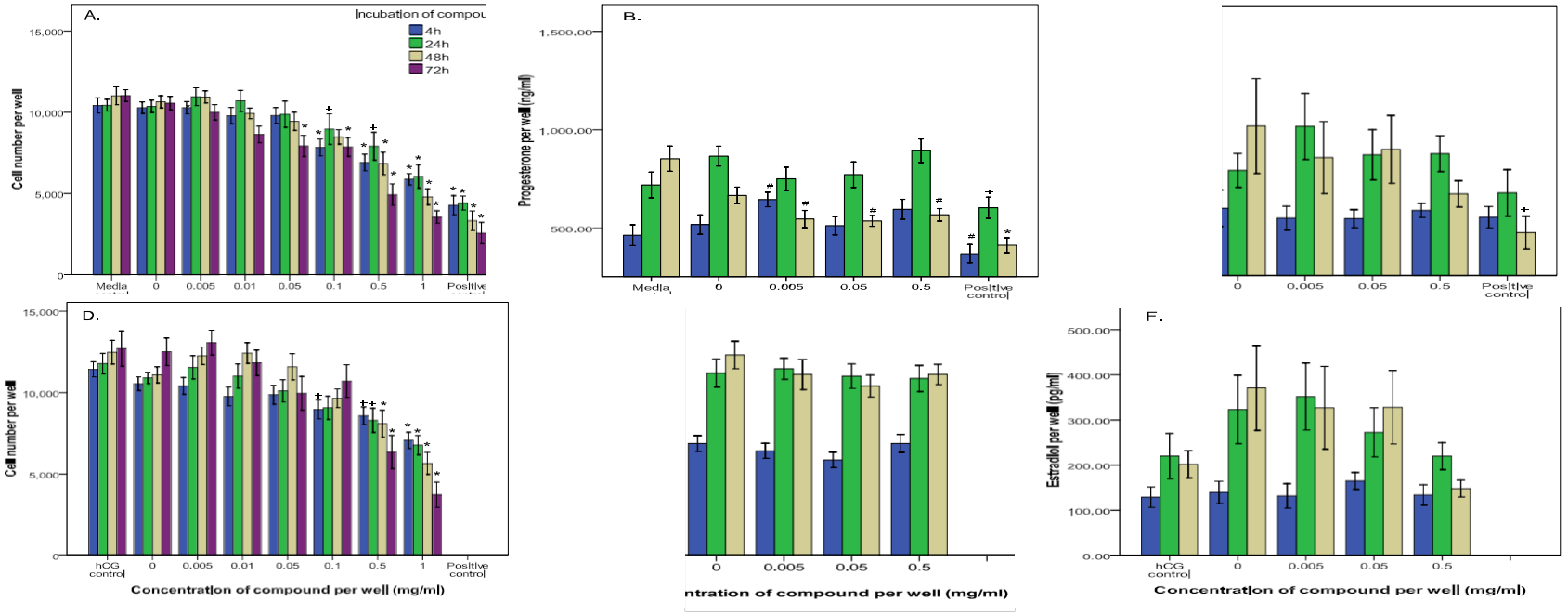


Figure 7.2: The effect of fraction one on primary human granulosa cells derived from women with abnormal physiology

The effect of the semi- purified *D. orbita* fraction one on primary-derived human granulosa cells from women with abnormal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction one ± hCG for 4, 24, 48 and 72h. The positive control was DMEM/F12 base media in the absence of FBS.Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 7; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction one on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*).

7.3.2.2 The Effect of Fraction Two on Cell Viability and Hormone Synthesis

7.3.2.2.1 Granulosa Cells from Women with Normal Reproductive Physiology

Fraction two, which contained a mixture of tyrindoleninone and tyrindolinone (section 5.3.2.1), had an inhibitory effect on the viability of granulosa cells from women with normal reproductive fertility both in the presence and absence of hCG (Table 7.3; Figure 7.3A). Fraction two significantly reduced granulosa cell viability to 81% of control at 0.5mg/ml after 4h in the absence of hCG (Figure 7.3A) and to 57% of control in the presence of hCG (Figure 7.3D). With increased exposure periods to fraction two, granulosa cell viability was reduced further (24h LC_{50} 0.5mg/ml and 0.3mg/ml with and without hCG; Table 7.3; Figure 7.3A and D).

Basal progesterone synthesis was inhibited after 24h exposure to 0.5mg/mL (Table 7.3; Figure 7.3B), while basal estradiol synthesis was not affected (Table 3; Figure 7.3C). When stimulated with hCG, fraction two inhibited progesterone synthesis by granulosa cells from fertile women at 0.05mg/mL after 48h and at 0.5mg/mL after 24 and 48h (Table 7.3; Figure 7.3E). In comparison, estradiol synthesis was stimulated by fraction two with hCG at 0.005mg/mL after 48h and 0.05mg/ml after 24h by granulosa cells (Table 7.3; Figure 7.3F).

7.3.2.2.2 Granulosa Cells from Women with Abnormal Reproductive Physiology

Fraction two reduced the cell viability of granulosa cells from women with abnormal reproductive physiology at all incubation periods tested, in both the absence and presence of hCG (Table 7.3; Figure 7.4A & D). After 4h cell viability was significantly reduced to 80% of control at 0.05mg/mL of fraction two without hCG (Figure 7.4A) and, to 75% of control at 0.1mg/mL in the presence of hCG (Figure 7.4D). Longer exposure periods and higher concentrations of fraction two, in the

absence and presence of hCG, reduced granulosa cell viability further (Figure 7.4A and D).

Basal progesterone synthesis by granulosa cells from infertile women was only inhibited at 0.5mg/ml after 24 and 48h with fraction two (Table 7.3; Figure 7.4B) and basal estradiol was not inhibited at any concentration or time point tested (Table 7.3; Figure 7.4C). hCG stimulated progesterone synthesis by granulosa cells was inhibited at 0.05mg/mL after 4 and 48h and, at 0.5mg/ml at all incubation periods (Table 7.3; Figure 7.4E). However, there was no significant difference between hCG stimulated estradiol synthesis by granulosa cells from infertile women in comparison to control (Table 7.3; Figure 7.4F).

Table 7.3: Effects of fraction two on primary-derived human granulosa cells
Effects of Fraction two (F2) ± hCG on primary-derived human granulosa cell viability, progesterone and estradiol synthesis from women with normal and abnormal reproductive physiology (shown as fertile or infertile). Cell viability shown as $LC_{50,}$ the concentration that killed 50% of the cells, & hormone data shown as concentration that was significantly different to the 1% DMSO control. (+) = concentrations that stimulated hormone synthesis; (-) = concentrations that inhibited hormone synthesis; N/T = not tested; n/e = no effect

Cell Source	Time (h)	LC_{50} (mg/L)		Effect on Hormone Synthesis			
				Progesterone		Estradiol	
		F2	F2 + hCG	F2	F2 + hCG	F2	F2 + hCG
Fertile	4	>1	0.7	n/e	n/e	n/e	n/e
Infertile		0.3	0.4	n/e	0.05, 0.5 (-)	n/e	n/e
Fertile	24	0.5	0.3	0.5 (-)	0.5 (-)	n/e	0.05 (+)
Infertile		0.3	0.4	0.5 (-)	0.5 (-)	n/e	n/e
Fertile	48	0.2	0.5	n/e	0.005, 0.5 (-)	n/e	0.005 (+)
Infertile		0.3	0.3	0.5 (-)	0.5 (-)	n/e	n/e
Fertile	72	0.2	0.4	N/A	N/A	N/A	N/A
Infertile		0.1	0.2	N/A	N/A	N/A	N/A

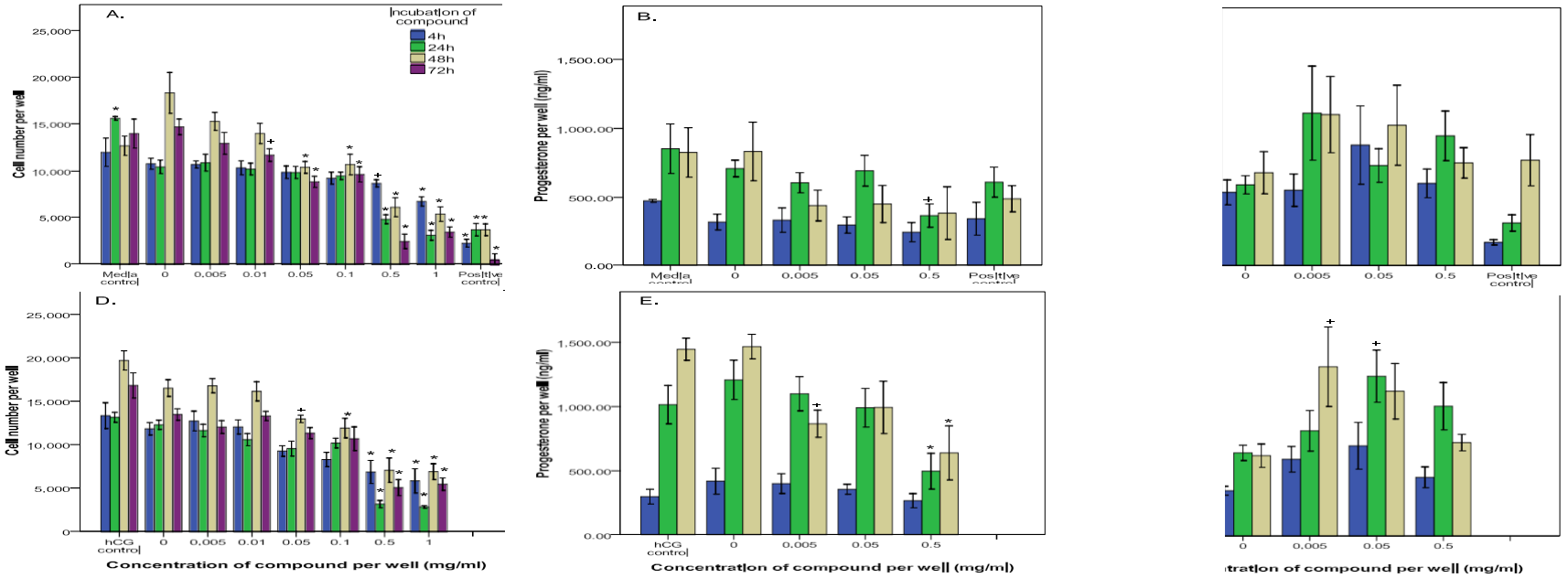


Figure 7.3: The effect of fraction two on primary granulosa cells derived from women with normal physiology

The effect of the semi-purified *D. orbita* fraction two on primary-derived human granulosa cells from women with normal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction two ± hCG for 4, 24, 48 and 72h. . The positive control was DMEM/F12 base media in the absence of FBS. Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis was assessed by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*)

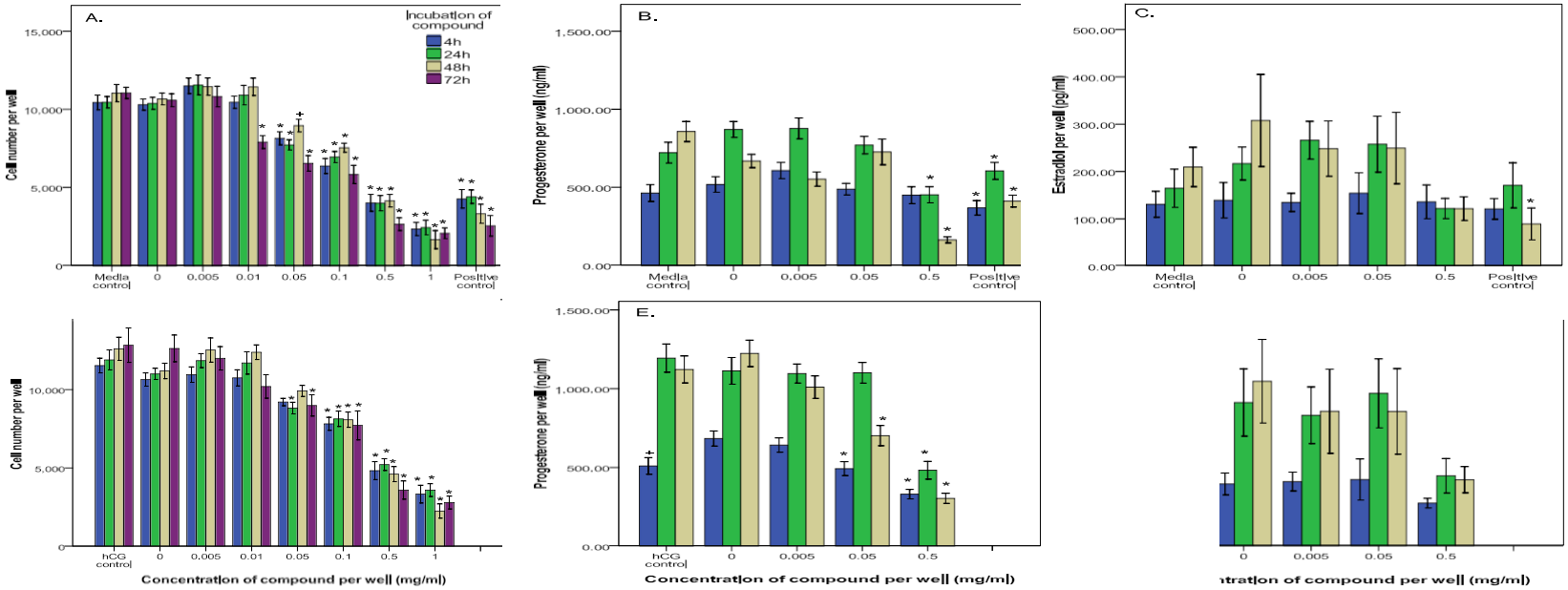


Figure 7.4: The effect of fraction two on primary granulosa cells derived from women with abnormal physiology

The effect of the semi- purified *D. orbita* fraction two on primary-derived human granulosa cells from women with abnormal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction two ± hCG for 4, 24, 48 and 72h. . The positive control was DMEM/F12 base media in the absence of FBS. Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis was assessed by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 7; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction two on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*)

7.3.2.3 The Effect of Fraction Three on Cell Viability and Hormone Synthesis

7.3.2.3.1 Granulosa Cells from Women with Normal Reproductive Physiology

Fraction three (5.3.2.1), which contained the compound 6-bromoisatin, inhibited the of viability in granulosa cells from women with normal reproductive physiology after 4h in the absence of hCG (Table 7.4; LC_{50} 0.6mg/mL; Figure 7.5A). However, cells exposed for 72h (Table 7.4; LC_{50} > 1mg/mL; Figure 7.5A). In the presence of hCG, fraction three only significantly reduced granulosa cell viability after 4h at 1mg/ml (45% of control; Figure 7.5D).

Both basal progesterone and estradiol synthesis by granulosa cells from fertile women were not affected when treated with fraction three (Table 7.4; Figure 7.5B and C). In comparison, in the presence of hCG, progesterone synthesis was inhibited after 48h at all concentrations of fraction three (Table 7.4; Figure 7.5E), whereas estradiol synthesis was stimulated after 48h at 0.005 and 0.05mg/ml of fraction three with hCG (Table 7.4; Figure 7.5F).

7.3.2.3.2 Granulosa Cells from Women with Abnormal Reproductive Physiology

Granulosa cells from women with abnormal reproductive physiology were inhibited by fraction three in both the presence and absence of hCG after 4h at 0.05mg/mL, with cells reduced to 85% of control (Figure 7.6A and D). Higher concentrations and longer incubation periods reduced cell numbers further after 72h (LC_{50} 0.3mg/mL without hCG and LC_{50} 0.4mg/mL with hCG; Table 7.4 and Figure 7.6A and D).

In a similar manner to granulosa cells from fertile women, both basal progesterone and estradiol synthesis by granulosa cells from infertile women were not affected by fraction three (Table 7.4; Figure 7.6B and C). However, when granulosa cells from

the infertile women were stimulated by hCG, progesterone synthesis was inhibited after 48h at all incubation periods tested (Table 7.4; Figure 7.6E). Estradiol synthesis by granulosa cells was also inhibited by fraction three in the presence of hCG but only at 0.5mg/mL and after 48h exposure which, coincided with a decrease in cell numbers (Table 7.4; Figure 7.6F).

Table 7.4: Effects of fraction three on primary-derived human granulosa cells
Effects of Fraction three (F3) ± hCG on primary-derived human granulosa cell viability, progesterone and estradiol synthesis from women with normal and abnormal reproductive physiology (shown as fertile and infertile). Cell viability shown as $LC_{50,}$ the concentration that killed 50% of the cells, & hormone data shown as concentration that was significantly different to the 1% DMSO control. (+) concentrations that stimulated hormone synthesis; (-) concentrations that inhibited hormone synthesis; N/T = not tested; n/e = no effect.

Cell Source	Time (h)	LC_{50} (mg/mL)		Effect on Hormone Synthesis			
				Progesterone		Estradiol	
		F3	F3 + hCG	F3	F3 + hCG	F3	F3 + hCG
Fertile	4	0.6	>1	n/e	n/e	n/e	n/e
Infertile		0.9	0.8	n/e	n/e	n/e	n/e
Fertile	24	>1	>1	n/e	n/e	n/e	n/e
Infertile		0.5	0.7	n/e	n/e	n/e	n/e
Fertile	48	0.7	>1	n/e	0.005, 0.05, 0.5 (-)	n/e	0.005, 0.05 (+)
Infertile		0.6	0.7	n/e	<0.5 (-)	n/e	0.5 (-)
Fertile	72	>1	>1	N/A	N/A	N/A	N/A
Infertile		0.3	0.4	N/A	N/A	N/A	N/A

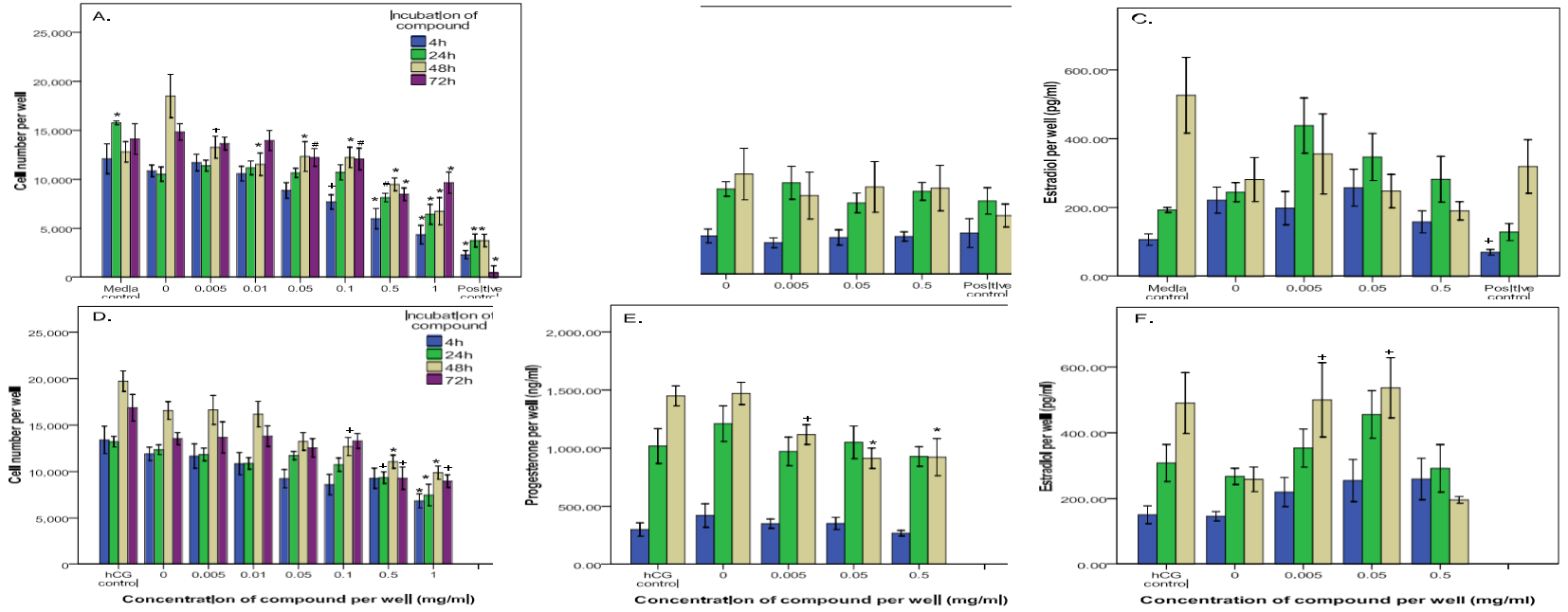


Figure 7.5: The effect of fraction three on primary granulosa cells derived from women with normal physiology

The effect of the semi- purified *D. orbita* fraction three on primary-derived human granulosa cells from women with normal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction three ± hCG for 4, 24, 48 and 72h. . The positive control was DMEM/F12 base media in the absence of FBS.Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis was assessed by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 3; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*)

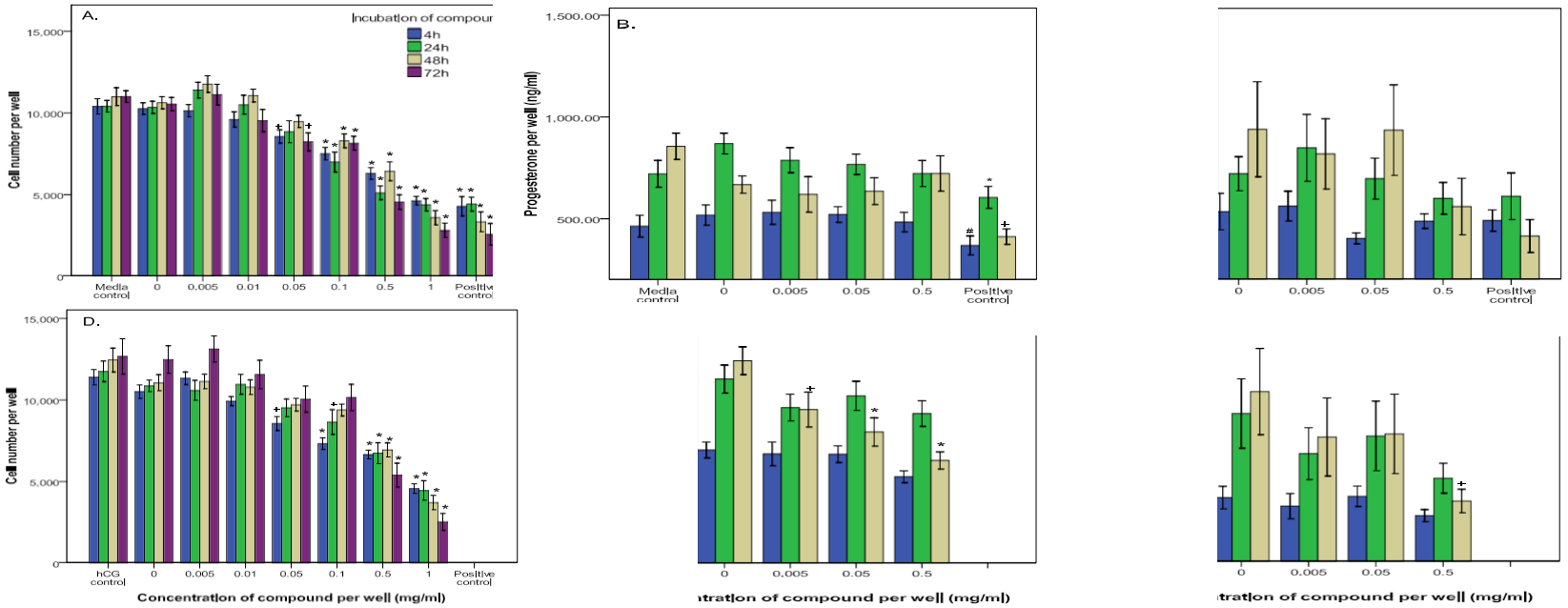


Figure 7.6: The effect of fraction three on primary granulosa cells derived from women with abnormal physiology

The effect of the semi- purified *D. orbita* fraction three on primary-derived human granulosa cells from women with abnormal reproductive physiology (fertile). Cell viability (A & D), progesterone synthesis (B & E) and estradiol synthesis (C & F) were tested in the absence (A, B & C) and in the presence of hCG (D, E, F). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with fraction three ± hCG for 4, 24, 48 and 72h. The positive control was DMEM/F12 base media in the absence of FBS. Cell viability was determined by the Crystal Violet assay at 570nm, with reference absorbance 630nm, and hormone synthesis was assessed by the radioimmunoassay. The results are the mean from three separate repeat assays (n = 7; ± 1 SEM). Univariate analysis of variance with contrast (K Matrix) were conducted to compare the effects of the concentration of fraction three on cell number per well, and hormone synthesis against the 1% DMSO control at different incubation periods. Significant difference between each treatment concentration and the 1% DMSO control at each incubation period shown as $p < 0.05$ (#), $p < 0.01$ (+) and $p < 0.001$ (*)

7.4 Discussion

The present study compared the effects of the semi-purified *D. orbita* fractions on primary-derived human granulosa cells from women undergoing ART who either had normal (fertile) or abnormal (infertile) reproductive physiology. The effects of these muricid compounds on cell viability and endocrine functions, in the presence and absence of hCG were investigated.

Overall, the granulosa cells from women with abnormal reproductive physiology were more susceptible to all three semi-purified muricid fractions both in the presence and absence of hCG, showing a reduction in viable cell numbers at lower concentrations than granulosa cells from the corresponding fertile group. As all three semi-purified fractions either had no effect, or, inhibited basal and hCG-stimulated progesterone synthesis by granulosa cells from women with both normal and abnormal reproductive physiology, whereas estradiol was stimulated at low non-toxic concentrations in hCG-stimulated cells from fertile women but had no effect or was inhibited in granulosa cells from the infertile group. In comparison to the beneficial effects the structurally similar synthetic compounds (Chapter 3 & 4) had on both progesterone and estradiol synthesis, these results suggests that the muricid compounds would not be beneficial for either fertile or infertile women at the concentrations tested. However, the muricid compounds were not tested at the same low concentrations as the synthetic compounds, in which basal estradiol was stimulated. Published research has actually shown that a biphasic or 'hormetic' effect in the low-dose zone is plausible (Calabrese, 2008). Future work therefore, needs to examine the muricid fractions at lower concentrations (in the µg/mL range) on primary-derived human granulosa cells.

Unexpectedly, in this study granulosa cells derived from infertile women synthesized similar quantities of both basal progesterone and estradiol in the absence of treatment, to that of cells from fertile women. Granulosa cells from fertile women undergoing ovarian stimulated IVF regimes are known to synthesize large quantities of progesterone and estrogen *in vitro* (Bar-Ami, *et al.*, 1993). After an initial 18-24h period in culture, granulosa cells differentiate and begin to luteinize (Tapanainen, *et al.*, 1987; Bar-Ami, *et al.*, 1993; Lobb, *et al.*, 1998). However, granulosa cells derived from infertile women undergoing IVF for infertility conditions such as, PCOS, and endometriosis are often found to have a suppressed capacity to secrete progesterone or estrogen, or both, in culture (Mason, *et al.*, 1994; McAllister, 1995; Harlow, *et al.*, 1996).

The anomaly of high levels of hormone synthesis by granulosa cells from the infertile women in this research may be attributed to fact that the source of granulosa cells in this study came from a mixed cohort of women with a range of infertility factors. These cells were derived from women with PCOS, endometriosis and unknown fertility (idiopathic), and the results were pooled together. Other studies have specifically analysed granulosa cells from only one cohort of infertility against granulosa cells from normal (fertile) women. McAllister, *et al.*, (1995) and Mason, *et al.*, (1994) studied the differences between PCOS and normal granulosa cells, while, Harlow, *et al.*, (1996) compared granulosa cells from endometriosis subjects to normal granulosa cells. Granulosa cells from long follicular cycles (endometriosis) are known to secrete reduced amounts of estradiol and progesterone, they have a lower expression of aromatase, reduced number of LH receptors and a reduced LH surge (Harlow, *et al.*, 1996). PCOS suffers have normal plasma FSH levels but

increased levels of both androgens and LH, and can also have a reduction in serum estradiol and progesterone (Erickson, *et al.*, 1990; Mason, *et al.*, 1994; McAllister, 1995). The infertility factor of four of the women in this study was either PCOS or endometriosis and three cases were of unknown infertility therefore the idiopathic cases may have been able to synthesis small amounts of progesterone and estrogen up to 24h.

In a similar manner to Chapter 4, the results of the infertile group were examined separately (Appendix VII) to identify any differences between the groups. From the results it was evident that granulosa cells from the endometriosis cases continued to proliferate up to 48h in comparison to the other two groups, PCOS and idiopathic. Furthermore, the granulosa cells from the endometriosis group synthesised the highest quantity of basal progesterone after 24 and 48h, which contradicts previous findings (Harlow, *et al.*, 1996). Granulosa cells from both idiopathic and endometriosis groups were found to produce similar quantites of estradiol after 48h. In comparison, the granulosa cells from the PCOS group produced minimal amounts of estradiol (Appendix VII), which contradicted previous observations (Chapter 4). These inconsistencies between progesterone and estradiol synthesized from granulosa cells derived from infertile women from different infertility factors highlights the fact that each infertility case is different. Therefore, reliable and reproducible results require large groups when using primary-derived cells. Furthermore, to obtain a more accurate result, future studies should analyse the results from different infertile sources separately rather than pooling the results together.

Of the three fractions tested, the more purified fractions two and three reduced granulosa cell numbers derived from infertile women, at lower concentrations and exposure times than the granulosa cells from fertile women. This differential effect between granulosa cells from the two groups may be the result of abnormal folliculogenesis and luteal phases in granulosa cells from the infertile women, resulting in immature cells. For example, in PCOS granulosa follicles, follicular growth becomes arrested at the small antral phase, resulting in the failure of growth by the dominant follicle which then disrupts folliculogenesis and ovulation (Amer, 2006). In normal follicle growth the dominant follicle will undergo further growth stimulated by FSH, eventually leading to ovulation (Speroff and Fritz, 2005). This abnormal follicular growth in some infertile women would also reduce maturation of granulosa cells resulting in a failure of granulosa cells to completely differentiate and luteinize. Thus, granulosa cells from infertile women may have been immature and therefore more susceptible to the muricid compounds as vital pathways and enzymes required to metabolise and eliminate these compounds may not be completely developed. Unlike the fertile group, granulosa cells from the infertile group may not have been able to overcome the toxic effects of the muricid compounds.

The semi-purified fractions had varying effects on both basal and hCG-stimulated hormone synthesis. Cells from infertile women were more sensitive to fraction one than cells from fertile women as basal progesterone was inhibited at all concentrations tested after 48h. In contrast, hCG-stimulated progesterone production by cells from fertile women was inhibited by fraction one, but not the cells from infertile women. Fractions two and three also caused a significant decrease in hCG-stimulated progesterone synthesis in both groups of granulosa cells, after 48h at low

concentrations, which could not be attributed to cell death. However, if these semi-purified compounds were tested at a concentration at least 10-fold lower (as with the synthetic compounds, Chapter 4), the results may have been different. In contrast to the progesterone results, estradiol synthesis was up-regulated in hCG-stimulated granulosa cells from fertile women when treated with fraction one, two and three. However, all three compounds had no effect on estradiol synthesis by granulosa cells from infertile women. These results suggest that one or a mixture of the brominated indole or isatin compounds may have up-regulated a specific steroidogenic enzyme for estradiol synthesis in the granulosa cells from fertile women, while inhibiting progesterone synthesis.

In vivo, both progesterone and estradiol biosynthesis are reliant upon the availability of cholesterol uptake into the mitochondria, involving the steroidogenic acute regulatory protein (StAR) followed by a number of sequential steps involving the P450 family of enzymes and beta hydroxylated dehydrogenase (β-HSD) enzymes (Figure 1.8, Chapter 1; Speroff and Fritz, 2005). In the present study it is unlikely that the muricid fractions inhibited progestereone while at the same time stimulating estradiol by acting on StAR or P450scc because this would have inhibited pregnenolone corresponding to the inhibition of both progesterone and estradiol. It is more probable that either P450c17 and/or $P450_{arom}$ were up-regulated and/or, 3β-HSD was down-regulated, therefore driving the reaction towards the formation of estradiol over progesterone. As progesterone and estradiol are both essential in the establishment and maintenance of pregnancy (Speroff and Fritz, 2005), the inhibitory effect of progesterone biosynthesis at low concentrations in hCG-stimulated granulosa cells could be indicative of an *in vivo* response during pregnancy.

Therefore, more studies are required to investigate the effects of these compounds on steroidogenesis.

Other naturally derived indole compounds including indole-3-carbinol (I3C) have been shown to be both estrogen antagonist and agonist (Kojima, *et al.*, 1994; Jin, *et al.*, 1999; Gao, *et al.*, 2002; Auborn, *et al.*, 2003). I3C has been shown to block ovulation and disrupt the preovulatory surge of LH and FSH by disrupting the hypothalamic axis inhibiting progesterone synthesis but not plasma estradiol, in a gonadotrophin stimulated rat model (Gao, *et al.*, 2002). Gao, *et al.*, (2002) further demonstrated that when rats were given hCG, the inhibition of ovulation by I3C was only partially reversed. The muricid compounds may have acted in a similar manner to the I3C compound, thereby inhibiting progesterone synthesis and not estradiol. This may also explain why the muricid compounds up-regulated hCG-stimulated estradiol synthesis and not progesterone synthesis because like I3C in the presence of hCG, progesterone synthesis was in some way still inhibited by one or more of the indole and isatin-like muricid compounds whereas estradiol was not affected.

In summary, this study has shown that the granulosa cells from women with abnormal reproductive physiology were more susceptible to the toxic effects of the semi-purified *D. orbita* fractions than the granulosa cells from women with normal reproductive physiology. Furthermore, exposure to all three fractions significantly inhibited progesterone synthesis by granulosa cells from both infertile and fertile women. In comparison, hCG-stimulated estradiol synthesis was up-regulated in granulosa cells from fertile women, in the presence of all three fractions at the lowest concentration tested. The muricid compounds however did not stimulate basal

estradiol synthesis by granulosa cells from either fertile or infertile women as hypothesized, based on the findings with similar synthetic compounds (Chapter 3 and 4), however it is important to note that the muricid compounds were tested in mg/mL range, whereas the synthetic compounds were tested in µg/ml and a hormetic dose response was observed with greater stimulation of steroidogenesis at the lower concentrations. Therefore further studies of these muricid compounds are really required to determine any beneficial effects of these naturally derived muricid compounds.

Chapter 8 Final Discussion

8.1 Summary of Research

The Australian marine mollusc *Dicathais orbita* produces bioactive brominated compounds that selectively kill human female reproductive cancer cells *in vitro*. Cell death is induced by apoptosis in reproductive cancer cells and not by necrosis (Table 8.1). The synthetic compounds and natural muricid compounds do, however, stimulate progesterone synthesis in a cell and concentration specific manner. At low concentrations in the microgram range, estradiol synthesis was upregulated in some human female reproductive cancer cells and in primary-derived human reproductive cells, in a cell and concentration specific fashion. Overall, the stimulatory effects on estradiol synthesis together with the anti-proliferative effects on cancer cells highlights the potential of these marine compounds as a natural product for female human reproductive cancers and possibly for menopause.

Table 8.1: Summary of the Cell Assays

Five complementary assays measuring different endpoints were used to determine the LC_{50} of all compounds tested in this research on the un-stimulated reproductive cells after 24h exposure. MTT is a cell viability assay that measures the ability of the enzymes of the mitochondria to reduce a substrate to the product formazan; crystal violet is also a cell viability assay in which the stain is only taken up into the DNA of live cells; the caspase -3/7 assay identifies cells undergoing apoptosis by measuring the activity of the death effector caspases -3 and 7; TUNEL is fluorescent labelling at the 3'OH end of DNA after the DNA has been cleaved during apoptosis and the results represent the concentration that caused 50% TUNEL labelled cells; LDH is an indicator of necrosis and measures LDH that has been released from damaged cells undergoing necrosis indicating cell damage and membrane rupture; n/t = not tested; JAr = choriocarcinoma cell line derived from a trophoblastic tumour of the placenta; OVCAR-3 = cell line derived from an adenocarcinoma; KGN = cell line derived from a granulosa cell carcinoma; GC Fertile = primary-derived human granulosa cells from women with normal reproductive physiology; GC Infertile = primary-derived human granulosa cells from women with abnormal reproductive physiology.
n/t = not tested

Compound Tested	Cell Type	LC_{50} (mg/ml)				
		MTT	Crystal Violet	Caspase 3/7	TUNEL	LDH
Dicathais orbita compounds						
Tyrindoleninone	JAr	0.03	0.05	n/t	n/t	0.05
	OVCAR-3	0.06	0.09	n/t	n/t	0.1
	KGN	0.02	0.04	0.005	<0.005	0.05
	GC Fertile	0.4	>0.5	0.1	>0.05	0.5
	GC Infertile	n/t	0.3	n/t	n/t	n/t
6-bromoisatin	JAr	0.1	0.04	n/t	n/t	0.05
	OVCAR-3	0.1	0.06	n/t	n/t	0.1
	KGN	0.1	0.05	0.005	<0.005	0.5
	GC Fertile	0.4	0.5	0.05	>0.05	0.5
	GC Infertile	n/t	0.5	n/t	n/t	n/t
Synthetic compounds						
5-bromoisatin	JAr	n/t	0.01	n/t	n/t	n/t
	GC Fertile	n/t	0.04	n/t	n/t	n/t
	GC Infertile	n/t	0.02	n/t	n/t	n/t
Indirubin	JAr	n/t	>0.1	n/t	n/t	n/t
	GC Fertile	n/t	>0.1	n/t	n/t	n/t
	GC Infertile	n/t	>0.1	n/t	n/t	n/t
6'6-dibromindirubin	JAr	n/t	>0.1	n/t	n/t	n/t

8.2 Comparison of Cell Based Assays for Drug Screening

8.2.1 Cell Culture Assays

In vitro high through-put cell-based screening assays are an essential tool in the early preclinical stage of drug discovery (Seiler, *et al*., 2004). If cell-based assays were applied to reproductive screening of novel compounds they would not only serve to compliment and support existing *in vivo* studies, but potentially would also save time and money compared to expensive animal studies. Cell-based assays can give a profile of the immediate effects and safety of novel compounds, along with providing valuable information on the effects of these drug candidates on different cell signalling pathways (Stockwell 1999). The use of a range of cell based assays that measure different endpoints improves the reliability and validity of preclinical data (Riss and Moravec, 2004).

The series of assays adopted in this study were complementary because they targeted different cell pathways and provided useful information on the general cytotoxicity and mode of action of the compounds tested. Initial results demonstrated that tyrindoleninone inhibited the metabolic functions (as detected by the MTT assay) in the KGN, JAr and OVCAR-3 cancer cell lines (Chapter 5; Table 8.1) before cell death occurred (as detected by the crystal violet; Chapter 6; Table 8.1). The apoptosis assays (performed on the KGN cell line; Chapter 6; Table 8.1) confirmed that these bioactive muricid compounds induced apoptosis and cell death at the lowest concentrations tested and therefore supported the results of the MTT assay (Table 8.1).

Metabolic enzymatic assays such as the MTS (Chapter 3) and MTT (Chapter 3 and 5; Table 8.1) assays, were developed to measure the biochemical rate at which cells can convert a substrate to a product in the mitochondria of the cell (Mosmann, 1983; Cory, *et al*., 1991). In comparison, cell staining assays such as the crystal violet assay (Chapter 3, 4, 6 and 7; Table 8.1), which stain the DNA of live cells were developed to measure the actual number of live cells left in culture after treatment with a specific compound (Gillies, *et al*., 1986). The MTT and crystal violet assays (Table 8.1) show that cell physiology is affected differently by assay conditions. Both assays show a clear trend that the muricid compounds are cytotoxic to reproductive cancer cells, but the results of each assay indicated different potencies of the compounds. For example, the MTT assay generated lower LC_{50}'s than the crystal violet assay when used to determine cytotoxicity of tyrindoleninone as opposed to 6-bromoisatin (Table 8.1). Because the MTT assay is measuring a physiological event in cells, whereas the crystal violet assay stains the DNA of live cells, it could be speculated that the different results are due to the difference between compound structures and how they interact biochemically with enzymes in the cells in the MTT assay. These results therefore reiterate the importance of developing standard procedures by measuring different physiological endpoints across several different *in vitro* cell-based assays.

Cell viability assays are complimented by apoptosis and necrosis assays, including the Caspase 3/7, TUNEL, DAPI staining and LDH assays (Chapter 6; Table 8.1), to determine mode of cell death (Gavrieli, *et al*., 1992; Liu, *et al*., 2004; Ren, *et al*., 2004; Travert, *et al.*, 2006; Niles, *et al.*, 2007; Benachour and Seralini, 2009). The results of this research clearly show that the muricid compound induced apoptosis

(by the activation of caspase -3 and 7 and TUNEL staining; Table 8.1) of the cells at lower concentrations (Table 8.1) than those associated with cell death by cell membrane damage, (necrosis; Table 8.1). Furthermore, the concentrations that induced necrosis (measured by LDH release), were similar, or were higher than the concentrations that were detected by the crystal violet cell viability assay. Benkendorff *et al* (2011) found evidence that crude extracts of *D. orbita* induced apoptosis in Jurkat lymphoma cells using flow cytometry. More recent research has shown that tyrindoleninone and 6-bromoisatin induce apoptosis in CaCo2 cells by flow cytometry (Esmaeelian *et al*., unpublished) and apoptosis was induced in damaged colon cells *in vivo* when mice were treated with a crude *D. orbita* extract (Westley, *et al.*, 2010). These apoptosis and necrosis assays therefore served to provide more information about cell death mechanisms induced in cells exposed to the muricid compounds.

Semi-purified and purified indole and isatin compounds are known to have cytotoxic effects in many cell types and their effects on cell viability have been assessed using a variety of different assays (Sze, *et al.*, 1993; Cane, *et al.*, 2000; Bacher, *et al.*, 2001; Chen, *et al.*, 2005b; Li, *et al.*, 2005; Vine, *et al.*, 2007a; Matesic, *et al.*, 2008; Benkendorff, *et al.*, 2011), but this has made comparison of the results difficult. For example, when the crystal violet assay was used, it was found that tyrindoleninone significantly reduced cell viability in the reproductive cancer cells at lower concentrations (LC_{50} = 0.04- 0.09mg/ml or 0.02-0.06mg/ml, after 4 and 24h exposure respectively; Table 8.1; Chapter 6) than have previously been described. Using the MTS assay, Benkendorff *et al* (2011) noted that LC_{50} only occurred at 0.1mg/mL after 4h exposure to an impure fraction containing tyrindoleninone, in the

MCF-7 breast, CaCo2 colon and U937 lymphoma cell lines. However, Vine *et al* (2007) also used the MTS assay and reported an LC_{50} of 0.001mg/mL for U937 lymphoma cells after 1h exposure to tyrindoleninone. It is difficult to draw any conclusions from this 100-fold difference in LC_{50}'s across the three studies because too many factors varied, including cell line, compound purity, assay type and exposure time.

A number of contributing factors may explain the different activity of tyrindoleninone identified between these separate studies. In particular, cell types can vary considerably in the way they absorb, metabolise and excrete compounds (Riss and Moravec, 2004). For example, the choriocarcinoma JAr cell line expresses genes involved in hepatic excretion which enable these cells to transport, metabolise and eliminate a range of compounds fast and effectively (Serrano, *et al*., 2007). This may explain why the JAr cells were less sensitive to tyrindoleninone than the lymphoma cells in the Vine *et al* (2007) study. However, the JAr cells were more sensitive to tyrindoleninone than OVCAR-3 cells and similar to KGN cells (JArs 0.03mg/mL; OVCAR-3 0.06mg/mL; KGN 0.02mg/mL after 24h; Table 8.1), but neither OVCAR-3 or KGN cells have reported mechanisms for toxic metabolism and excretion. It is also possible that the suspension cells, like the lymphomas used by Vine *et al* (2007) are more sensitive to cytotoxins in cell culture than adherent cells, such as the reproductive cancer cells used in this study. However, Benkendorff *et al* (2011) reported a similar LC_{50} against the lymphoma U937 and a range of solid tumour cell lines, including MCF-7 breast and CaCo2 colon cells. Nevertheless one consistent outcome that is reported in all of these previous studies on tyrindoleninone is the higher sensitivity of primary cell lines in comparison to transformed tumour

cells. The premise that tyrindoleninone has broad spectrum, yet specific activity is further corroborated in the present study, by showing activity against a range of reproductive cancers, with 10-fold higher specificity towards human cancer cell lines than against primary-derived human granulosa cells (Table 8.1).

A second factor that could explain the different activity of tyrindoleninone in the different studies is the extraction process and semi-purification of the muricid compounds. Different concentrations of tyrindoleninone in the purified fractions may have contributed to its effect on different cell types. For example, Benkendorff *et al* (2011) produced a fraction containing only 62.7% tyrindoleninone which therefore was not as pure as the tyrindoleninone fraction (95%) that was tested on the reproductive cells in the present study (Figure 2B, Chapter 5). The purity of tyrindoleninone was not reported in Vine *et al* (2007) however the original study by Vine (2002) noted the presence of a contaminating plasticizer in the fraction tested, which may have increased the solubility and availability of the compound to the lymphoma cells (Benkendorff, *et al.*, 2011). Therefore, the purity of compound and contaminating factors may explain the differences between the results of tyrindoleninone across these three studies.

Thirdly, the assay used to screen for cytotoxicity may contribute to some of the differences in activity between studies. Benkendorff *et al* (2011) and Vine *et al* (2007) both adopted the MTS assay for screening tyrindoleninone, with vastly different results, whereas in this study the MTT and crystal violet assays were used. The MTS assay often produces inconsistent results and is not as sensitive as the MTT assay (Pabbruwe, *et al.*, 2005). To determine the most reproducible assay in this

study initially the MTS, MTT and crystal violet assays were compared to find the most sensitive, accurate and reliable cell viability assay for the JAr cell line (Chapter 3). Both the MTT and crystal violet assays proved to be more reproducible, and less prone to experimental error than the MTS assay, as has previously been reported (Pabbruwe, *et al*., 2005). Overall, the MTT assay produced a standard curve with a longer linear range and higher R^2 value than the crystal violet assay for the JAr cell line.

As the MTT and crystal violet cell-based assays produced comparable results they were used to determine the effects of the natural semi-purified muricid compounds from *D. orbita* on both primary-derived female human reproductive cells and three female human reproductive cancer cell lines (Chapter 5, 6 & 7). The two different cell viability assays generated similar results (Table 8.1). Cell growth was inhibited and there was a decrease in cell numbers across all three reproductive cancer cell lines when exposed to the muricid compounds compared to the primary granulosa cells (Table 8.1). These cell viability assays measure different mechanisms of action and hence different end points, therefore these two assays were reproducible and both confirmed the results of the cytotoxicity of the muricid compounds on female human reproductive cancer cells (Table 8.1).

8.2.2 Hormone Detection Assays

Drug candidates may not induce cell death at lower concentrations however they may still affect different cell functions. An important metabolic function of reproductive cells is the synthesis of steroid hormones. Insufficient progesterone or estradiol secretion is known to disrupt a female's normal ovarian cycle, which can

then lead to more serious disorders such as endometriosis or polycystic ovarian syndrome (Chapter 1; Robker and Richards, 1998). Therefore, an added benefit of *in vitro* reproductive toxicity assays is the ability to rapidly examine the effects of drug candidates on different aspects of hormone synthesis. Female cancer cell lines (KGN, JAr and OVCAR-3), along with primary-derived human granulosa cells all secrete the hormones progesterone and estradiol in cell culture and will respond to stimulation by the gonadotrophins, cAMP or hCG (Chapter 1). Therefore these cell types were ideal for examining the effects of muricid compounds on female hormone synthesis.

Two techniques for analyzing hormone synthesis, the radioimmunoassay (RIA) and enzyme-linked immuno-sorbent assay (ELISA), were compared to determine the most reliable and sensitive assay for detecting hormones. In this study, the ELISA assay proved to be the more sensitive assay for measuring progesterone synthesis by the JAr cells (Chapter 2). There was a lower coefficient of variation between repeats of the ELISA using the optimised cell culture conditions (CV = 15%), compared to the RIA (CV = 45%). The added benefit of the ELISA assay in preference to the RIA was the safety of the assay with the use of non radioactive material (Ohno, *et al.*, 2004). Unfortunately, the progesterone ELISA standard curve range was only 0-4ng/L but the human primary-derived granulosa cells produced ≥ 1000ng/mL progesterone and the error introduced by dilution of granulosa cell conditioned medium was too high to allow use of the ELISA. The RIA assay did however prove to be sensitive enough to detect the low concentrations of basal and hCG/db-cAMP stimulated progesterone and estradiol secreted by the JAr, OVCAR-3 and KGN cells. Consequently, this assay was chosen to measure hormone secretion on all

reproductive cells after treatment with test compounds, so that a direct comparison could be made between all cell types (Table 8.2 and 8.3).

In vivo, hCG and its secondary messenger cAMP play an essential role in regulating and maintaining hormone synthesis via the steroidogenic pathway (Chapter 1; Vander, *et al*., 1998). hCG/LH facilitates the development and proliferation of the follicular granulosa cells (Mandi, *et al.*, 2007). The first step of hormone synthesis is the binding of hCG or LH to the corresponding cell surface receptor on the reproductive granulosa cells (Leung and Adashi, 2004). LH or hCG binding to the LH/hCG receptor activates cAMP and subsequently a signalling cascade which eventually leads to the uptake of cholesterol into the cell and then into the mitochondria (Chapter 1; Leung and Adashi, 2004). A number of reproductive disorders including polycystic ovarian syndrome and endometriosis have been associated with deficient progesterone and/or estradiol synthesis during the normal reproductive cycle caused by receptor dysfunction and/or binding (Speroff and Fritz, 2005; Amer, 2006; van Kaam, *et al.*, 2007). Both basal and hCG/db-cAMP-stimulated primary and reproductive cancer cells produced progesterone and estradiol (Chapters 3, 4, 5 & 7), including the primary granulosa cells derived from women with abnormal reproductive physiology (Chapter 4 & 7), in the absence of test compounds.

In this study, a mixed cohort of infertile women with different fertility problems donated granulosa cells that were designated as having 'abnormal reproductive physiology' (Chapter 4 & 7). When the data were separated according to the cause of infertility, however, it was noted that the granulosa cells from the four different

infertility groups (ovarian cyst, polycystic ovarian syndrome (PCOS), endometriosis and idiopathic; Appendix VI) in the first study and three different groups (PCOS, endometriosis and idiopathic; Appendix VII) in the second study synthesised different quantities of progesterone and estradiol. For example, the PCOS group produced ten times more basal progesterone after 48 and 72h than either the endometriosis or idiopathic groups and five times more than the ovarian cyst group in the first cohort of assays (Appendix VI). In comparison in the second set of assays granulosa cells from the endometriosis group synthesised the most basal progesterone, but similar amounts to the idiopathic group when hCG stimulated (Appendix VII). Overall the idiopathic group secreted more basal and hCG stimulated estradiol than either the endometriosis or PCOS groups (Appendix VII). Future studies, therefore, should define the reproductive pathology of infertile donor women. This would then aid in making this *in vitro* model more relevant to possible *in vivo* responses.

Low concentrations ($1x10^{-5}$mg/ml) of the synthetic compounds (5-bromoisatin, indirubin and 6'6-dibromoindirubin) stimulated progesterone secretion in the JAr cancer cell line after 24h exposure (Table 8.2; Chapters 3), but had varying effects on the primary-derived human granulosa cells (Table 8.2 and 8.3; Chapter 4). At shorter incubation periods (24h) progesterone secretion by primary granulosa cells was either not affected or was inhibited, but after longer incubation periods (48 and 72h) progesterone was often stimulated in granulosa cells derived from woman with normal reproductive physiology, but inhibited in granulosa cells derived from four mixed cohort women with abnormal physiology (Table 8.3; Chapter 7). In contrast, the synthetic compounds had no effect on estradiol synthesis by granulosa cells

derived from woman with normal reproductive physiology at any time point (Table 8.3; Chapter 7). These data suggest that 'something' changed after 24h exposure. Common protein upregulation is known to occur in this timeframe, and raises the intriguing possibility that the synthetic compounds affected protein expression in a way that increased progesterone, but not estradiol synthesis. The primary function of luteinised granulosa cells is to produce progesterone rather than estradiol, and *in vivo* progesterone synthesis increases during the four days after ovulation, whereas estradiol synthesis remains more or less constant (Devoto, *et al.*, 2002). It may therefore be possible that the synthetic compounds enhanced this physiological mechanism whereas the granulosa cells derived from women with abnormal reproductive physiology proved unable to respond to this stimulation, suggesting a potential target for clinical remediation.

Table 8.2: Summary of the effects of the compounds tested in this study on hormone synthesis

Summary of the effects of the bioactive muricid compounds (4, 24 & 48h), the synthetic compounds and the homeopathic remedy (24h) on both basal and hCG/cAMP stimulated progesterone and estradiol synthesis by primary granulosa cells from women with normal and abnormal physiology, and the reproductive human female cancer cell lines KGN, JAr and OVCAR-3. Results show the concentration that caused a statistically significant stimulation (+) or inhibition (-) of hormone synthesis (mg/ml). P4 = progesterone; E2 = estradiol; GC fertile = primary granulosa cells derived from women with normal reproductive physiology; GC infertile = granulosa cells derived from women with abnormal reproductive physiology; n/t = not tested; n/e = no significant effect. Data in table derived from results of chapters 3, 4 and 5.

Compound Tested	Cell Type	Concentration (mg/ml) that stimulated (+) or inhibited (-)			
		Basal P4	Stimulated P4	Basal E2	Stimulated E2
4h Tyrindoleninone	GC Fertile	n/e	n/e	0.05 (-)	n/e
	KGN	≤0.5 (-)	≤0.5 (-)	0.05 (+)	0.005 (-)
	JAr	≤0.5 (-)	0.5 (-)	0.005 (+)	0.5 (-)
	OVCAR-3	≤0.5 (-)	0.5 (-)	n/e	0.05 (+)
4h 6-bromoisatin	GC Fertile	0.005 (-)	n/e	0.005,0.5 (-)	<0.05 (-)
	KGN	≤0.5 (-)	≤0.5 (-)	0.005, 0.5 (+)	n/e
	JAr	≤0.5 (-)	0.5 (-)	0.005 (+)	0.5 (-)
	OVCAR-3	0.05 (-)	0.05 (-)	n/e	n/e
24h Tyrindoleninone	GC Fertile	0.5 (-)	n/e	0.005, 0.05 (+)	0.005, 0.05 (-)
	KGN	≤0.5 (-)	≤0.5 (-)	n/e	0.5 (+)
	JAr	0.5 (-)	≤0.5 (-)	0.005, 0.05 (+)	0.005 (+), 0.5 (-)
	OVCAR-3	≤0.05 (-)	0.005 (-)	≤0.05 (+)	0.005 (-)
24h 6-bromoisatin	GC Fertile	n/e	n/e	n/e	n/e
	KGN	≤0.5 (-)	≤0.5 (-)	0.05, 0.5 (+)	0.05 (-)
	JAr	n/e	≤0.5 (-)	0.05, 0.5 (+)	n/e
	OVCAR-3	n/e	n/e	n/e	≤0.5 (-)
48h Tyrindoleninone	GC Fertile	n/e	0.5 (-)	0.005(+),0.5(-)	0.005, 0.5 (-)
	KGN	≤0.5 (-)	≤0.5 (-)	n/e	0.005 (-)
	JAr	0.05,0.5 (-)	≤0.5 (-)	n/e	0.05 (+)
	OVCAR-3	n/e	n/e	n/e	n/e
48h 6-bromoisatin	GC Fertile	n/e	n/e	n/e	≤0.5 (-)
	KGN	≤0.5 (-)	≤0.5 (-)	n/e	n/e
	JAr	0.005,0.5 (-)	≤0.5 (-)	n/e	n/e
	OVCAR-3	n/e	0.05,0.5 (-)	n/e	0.005 (-)
	GC Fertile	n/e	n/t	n/e	n/t
	GC Infertile	n/e	n/t	$1x10^{-5}$ (-)	n/t
24h 6'6-dibromoindirubin	JAr	0.01 (+)	n/t	n/t	n/t
24h Indirubin	JAr	0.01 (+)	n/t	n/t	n/t
	GC Fertile	n/e	n/t	n/e	n/t
	GC Infertile	n/e	n/t	≤0.1 (-)	n/t
24h 5-bromoisatin	JAr	0.01 (+)	n/t	n/t	n/t
	GC Fertile	0.1 (-)	n/t	n/e	n/t
	GC Infertile	n/e	n/t	≤0.1 (-)	n/t

Table 8.3: Summary of the effects of the compounds tested in this study on hormone synthesis

Summary of the Summary of the effects of the bioactive muricid compounds and synthetic compounds on basal and hCG/cAMP stimulated progesterone and estradiol synthesis by primary granulosa cells from women with normal and abnormal physiology 4, 24 and 48h exposure. Results show concentration that significantly stimulated (+) or inhibited (-) hormone synthesis in mg/ml. P4 = progesterone; E2 = estradiol; GC fertile = primary granulosa cells derived from women with normal reproductive physiology; GC infertile = granulosa cells derived from women with abnormal reproductive physiology; n/t = not tested; n/e = no significant effect. Data from Chapters 4 & 7.

Compound Tested	Cell Type	Concentration (mg/ml) that stimulated (+) or inhibited (-)			
		Basal P4	Stimulated P4	Basal E2	Stimulated E2
***Dicathais orbita* compounds**					
4h Tyrindoleninone	GC Fertile	n/e	n/e	n/e	n/e
	GC Infertile	n/e	0.05, 0.5 (-)	n/e	n/e
4h 6-bromoisatin	GC Fertile	n/e	n/e	n/e	n/e
	GC Infertile	n/e	n/e	n/e	n/e
24h Tyrindoleninone	GC Fertile	0.5 (-)	≤0.5 (-)	n/e	0.05 (+)
	GC Infertile	0.5 (-)	≤0.5 (-)	n/e	n/e
24h 6-bromoisatin	GC Fertile	n/e	n/e	n/e	n/e
	GC Infertile	n/e	n/e	n/e	n/e
48h Tyrindoleninone	GC Fertile	n/e	0.005,0.5 (-)	n/e	0.005 (+)
	GC Infertile	0.5 (-)	0.5 (-)	n/e	n/e
48h 6-bromoisatin	GC Fertile	n/e	0.005,0.05, 0.5 (-)	n/e	0.005, 0.05 (+)
	GC Infertile	n/e	≤0.5 (-)	n/e	0.5 (-)
48h Indirubin	GC Fertile	$1x10^{-4}$, 0.1(+)	n/t	n/e	n/t
	GC Infertile	n/e	n/t	n/e	n/t
48h 5-bromoisatin	GC Fertile	$1x10^{-4}$, $1x10^{-3}$ (+), 0.1 (-)	n/t	n/e	n/t
	GC Infertile	n/e	n/t	n/e	n/t
72h Indirubin	GC Fertile	0.01 (+)	n/t	n/e	n/t
	GC Infertile	≤0.01 (-)	n/t	n/e	n/t
72h 5-bromoisatin	GC Fertile	n/e	n/t	n/e	n/t
	GC Infertile	$1x10^{-5}$, $1x10^{-3}$,0.01 0.1 (-)	n/t	0.1 (-)	n/t

The effects of the natural muricid compounds on hormone synthesis by the primary-derived human granulosa cells from women with normal reproductive physiology produced inconsistent results across this study. After 24 and 48h exposure to

tyrindoleninone (Table 8.2; 0.005mg/ml; Chapter 5; n=3) basal estradiol synthesis was upregulated in granulosa cells derived from women with normal reproductive physiology, but when tested again on granulosa cells from three different women under the same conditions (Table 8.3; Chapter 7) also with normal reproductive physiology, it had no effect on estradiol synthesis.

These varying results were also seen when 6-bromoisatin was tested on the primary-derived human granulosa cells from women with normal reproductive physiology. After 4h 6-bromoisatin (0.005mg/ml; Table 8.2; Chapter 5; n=3) inhibited basal progesterone synthesis in granulosa cells derived from women with normal reproductive physiology. When 6-bromoisatin was tested a second time on granulosa cells from three different women with normal reproductive physiology and under the same conditions (Chapter 7), there was no effect on either basal or hCG stimulated progesterone synthesis but instead, hCG stimulated progesterone was inhibited after 48h (Table 8.3; Chapter 7; n=3). Similar to progesterone, basal and hCG estradiol was shown to be inhibited in primary granulosa cells from women with normal reproductive physiology in the presence of 6-bromoisatin (0.005, 0.05 and 0.5mg/mL) after 4 and 48h exposure (Table 8.2; Chapter 5) When tested a second time on primary granulosa cells derived from three different women with normal reproductive physiology (Table 8.3; Chapter 7), under the same conditions there was no effect on either basal or stimulated estradiol. In fact, hCG stimulated estradiol synthesis was significantly stimulated (Table 8.3; Chapter 7). In summary, tyrindoleninone either upregulated, or had no effect on basal estradiol synthesis by normal granulosa cells. 6-bromoisatin, however, down-regulated or had no effect on basal progesterone and estradiol synthesis by normal granulosa cells. These two

muricid-derived compounds had somewhat opposing effects on basal steroidogenesis by normal granulosa cells but inter-individual variation obscured the results. To minimise these inconsistencies in the future, larger groups of primary cells should be tested to obtain a more accurate profile of how these muricid compounds are affecting progesterone and estradiol synthesis.

The effects of muricid brominated indole compounds on progesterone and estradiol synthesis by female reproductive cells has never previously been reported therefore it is difficult to speculate how these compounds are acting on the steroidogenic pathway. However, previous studies of the effects of a similar indole compound to the muricid compounds, indole-3-carbinol (I3C) have demonstrated that I3C interferes with the estrogen receptor on the MCF-7 (breast cancer cell line) and prevents estrogen signalling resulting in decreased progesterone synthesis (Auborn, *et al.*, 2003). It is therefore probable that the muricid compounds are also interfering with, or preventing binding of the progesterone and estrogen receptors on the cell surface.

In conclusion, this study has demonstrated that *in vitro* reproductive toxicity screening should include a selection of cell-based assays in order to determine the effects of a new drug candidate on specified aspects of female reproduction. The adverse effects of a drug compound on reproduction are highly complex (Spielmann, 2005). Hence, the question really needs to be addressed as to whether sufficient data can be gained from these *in vitro* assays to determine whether a compound should be designated toxic to human reproduction. Clearly, cell-culture assays can only provide an account of toxicity on some aspects of reproduction (Edwards, *et al.*, 2008). The

vast complexity of the female reproductive cycle along with various receptor targets and interaction restricts the data that can be obtained from *in vitro* studies. Further, animal studies will always be required for examining the toxicity of new compounds pre and post natal, and on multiple generations (OECD, 2004). However, this research, although limited has identified that cell-based assay can investigate the effects of new compounds on hormone synthesis by reproductive cells. The data compiled in the present study clearly demonstrate that cell-based assays can provide useful information on cell cytotoxicity, mode of action and aspects of basal and gonadotrophin stimulated steroidogenesis. These assays therefore enable highly toxic and potential endocrine disruptive compounds to be screened out early in the drug development process, thus facilitating more focussed animal studies with associated savings in time and money.

8.3 Challenges in the Discovery and Development of New Drug Candidates from Nature

8.3.1 Pharmaceuticals versus Nutraceuticals

The discovery of new drug candidates from the marine environment offers many advantages over synthetically derived drug candidates (Blunt, *et al.*, 2008; Mayer, *et al.*, 2009). These include their structural diversity and novel properties of secondary metabolites, which often selectively target cancer cells over their primary-cell counterparts (Blunt, *et al*., 2008). However, along with these advantages there are a number of disadvantages in the development of compounds from marine sources.

Purification of the bioactive compound of interest often poses great difficulties, although the activity and quality often justifies the research (Faulkner, 2000). Only compounds that can be completely purified or synthetically reproduced can be

developed into pharmaceuticals (ICH, 2000). This ensures consistency of the product from batch to batch (Good Manufacturing Practice), an essential element that is required by the Trades and Goods Manufacturing (TGA, 2012) and Food and Drug Administration (FDA) for approval of a new drug (ICH, 2000). 6-bromoisatin is now synthetically available although chemists have failed to chemically synthesize the bioactive compound tyrindoleninone (Vine, 2007; Benkendorff, *et al.*, 2011). However, chemists have been able to semi-purify tyrindoleninone (98%) from tyrindolinone (Baker and Duke, 1973a), albeit these results have not been repeatable (Appendix III). Tyrindoleninone may therefore be a difficult compound to target for future drug development.

In comparison to pharmaceuticals, nutraceuticals (food supplements) do not have to be purified for human consumption (Straus, 2000). Therefore, nutraceuticals may be an alternative to conventional pharmaceuticals and in fact are often the first choice of treatment by consumers, especially women (Piscitelli, *et al.*, 2000; Straus, 2000). For example research has shown that the dietary supplement black cohosh, is effective for relieving menopause symptoms (Dennehy, 2006). Another nutraceutical, Indole-3-Carbinol (IC3), derived from cruciferous vegetables, has been shown to stimulate estradiol synthesis in MCF-7 cells, which may also be beneficial during menopause (Auborn, *et al.*, 2003; Dennehy, 2006).

The biggest obstacle to overcome in the development of nutraceuticals, is the availability and quantity of the raw product required for continuous supply and production (Benkendorff, 2009). Often large supplies of specimens are required to produce an adequate amount of purified product. For example, in this research

hypobranchial glands were dissected from 300 specimens of *D. orbita* yielding 5.072g of crude extract, and producing only 0.256g of purified tyrindoleninone and 0.823g of 6-bromoisatin (Chapter 5). In comparison, 285g of *D. orbita* egg capsules yielded 3.726g of crude compound resulting in 0.120g of purified tyrindoleninone and 0.105g of 6-bromoisatin (Chapter 6). These quantities were sufficient for cell culture assays, however *in vivo* studies would require much larger quantities. Westley *et al* (2010) conducted an animal study using the crude *D. orbita* extract administering either 1.0mg/g daily per mouse or 0.125mg/g (n=10) over a 4-week period. It was calculated that 200g of raw material were required to obtain the crude extract (Westley, *et al.*, 2010). A much larger and constant supply of this mollusc would be required for commercial use in humans. In fact, previous research has demonstrated the possibility of growing this muricid species in aquaculture, which could potentially solve the supply problem (Woodcock and Benkendorff, 2008; Benkendorff, 2009).

8.3.1.1 Solvent Issues to Overcome in the Extraction Process

The choice of solvent and its toxicity level is another problem that needs to be overcome in the development of the *D. orbita* compounds as a nutraceutical. A number of different solvent systems have been used to extract the brominated compounds from *D. orbita* (Baker and Sutherland, 1968; Baker and Duke, 1973b; Benkendorff, *et al.*, 2000; Benkendorff, *et al.*, 2011). These solvents include chloroform, methanol, ethanol, benzene, methanethiol, dimethyl disulfide and/or a combination of these. In 1973, Baker and Duke successfully purified tyrindoleninone from a crude hypobranchial gland extract of *D. orbita* using the volatile gas methanethiol (2%) bubbled through benzene on aluminium plates. This gas is now regarded hazardous and its use is highly restricted. After several attempts to

reconstruct the same experiment albeit with a more stable solvent, dimethyl disulfide, the present research failed to produce purified tyrindoleninone (Appendix III). It was therefore concluded that equal volumes of chloroform and methanol (v/v) followed by semi-purification using dichloromethane (DCM) produced the most successful purified fraction possible in this research (Chapter 5 & 6).

Solvents used to extract and subsequently purify compounds can themselves be cytotoxic to humans (ICH, 2011). In fact, according to the International Conference of Harmonisation (ICH, 2011) on guidelines for residual solvent requirements for registration of pharmaceuticals for human use, DCM is a Class 2 solvent, a classification considered inherently toxic for human consumption (ICH, 2011). Therefore, if DCM was used in the purification of the *D. orbita* compounds for commercialisation it would need to have a final concentration of 600ppm or be $\leq$ 6.0 mg/day in the final product, for human consumption (ICH, 2011). An alternative to the toxicity problem of DCM may be to select a different, less toxic solvent system in the purification of the *D. orbita* compounds, and indeed this has already been investigated. Recent research in our laboratory has identified an improved system of extracting tyrindoleninone and 6-bromoisatin from a crude *D. orbita* extract using ethyl acetate in hexane (Esmaeelian Per. Com). Hexane is still a Class 2 solvent, whereas ethyl acetate is classed as a Class 3 solvent by the standards published by the ICH; which are solvents that are regarded as less toxic and pose a lower risk to human health (ICH, 2011). Therefore, crystallisation from ethyl acetate or perhaps a similar solvent minus the hexane could solve the solvent toxicity problem for human consumption of these bioactive compounds in the future.

8.3.2 Pharmaceuticals from Synthetic Isatin & Indole Compounds

The synthetic compounds indirubin and 6'6-dibromoindirubin were not found to have any significant effect on cell proliferation in the reproductive cancer cells, despite their proven biological activity (Hoessel, *et al.*, 1999). However, indirubin has been chemically synthesized and adapted to produce a range of synthetic derivatives (Meijer, *et al.*, 2006). These synthetic derivatives have improved solubility and biological selectivity (Leclerc, *et al.*, 2001; Nam, *et al.*, 2005; Meijer, *et al.*, 2006; Kim, *et al.*, 2007). Since the discovery of indirubin and indoles, a number of the synthetic derivatives have inspired interest as pharmaceutical agents in the treatment of cancer and as such have been patented over the last 7-years (Karabelas, *et al.*, 2002; Wang, *et al.*, 2003; Wang, *et al.*, 2005; Carson, *et al.*, 2006; Kim, *et al.*, 2009). A series of indirubin compounds, known as cyclic-dependent kinases (CDK) and glycogen synthase kinase-3 (GSK-3) inhibitors (AGM011, AGM012, AGM014, AGM029, AGM030 and AGM011; US patent # 7,572,923), have shown unique biological activity against these enzymes of the cell cycle. These indirubin derivatives have anti-proliferative properties and induce apoptosis in a range of lung, stomach, colon, abdominal and leukaemia cancer cell lines, and *in vivo*, the compound AGM011 suppresses solid tumors in rats by 80% (Kim, *et al.*, 2009). A number of indirubin derivatives are currently used for treating leukaemia, lung and prostate cancer (Hoessel, *et al.*, 1999; Nam, *et al.*, 2005; Kim, *et al.*, 2007). In light of this, it would seem probable that these improved synthetic derivatives may also be biologically active against female reproductive cancer cell lines rather than the parent compound indirubin. However, because indirubin had both inhibitory and stimulatory effects on hormone synthesis in granulosa cells all indirubin derivatives

should be tested for potential effects on female reproductive physiology prior to commercialisation of any new drug.

8.4 Potential New Product for Female Reproductive Cancers

Gynaecological cancers are less common than lung, breast and bowel cancer in women however these cancers often result in higher mortalities due to late detection and advanced state of the disease when diagnosed (Wray, *et al*., 2007). In particular, ovarian cancer presents minimal warning signs and becomes drug resistant to platinum-based drugs such as cisplatin and paclitaxel (standard treatments for ovarian cancer following surgery) (Luo, *et al.*, 2010). Consequently, the disease often returns with fatal results (Chen, *et al.*, 2005b; Wray, *et al.*, 2007). Hence, there is a need to investigate new drug treatments for these gynaecological diseases.

History has demonstrated that often the most effective anticancer drug candidates have originated from nature (Chapter 1) including novel compounds isolated from the marine environment (Nagle, *et al.*, 2004; Taraboletti, *et al.*, 2004; Chin, *et al.*, 2006; Williams, *et al.*, 2007; Carter and Keam, 2010; von Schwarzenberg and Vollmar, 2010). Consistent with previous studies (Vine, *et al.*, 2007a; Benkendorff, *et al.*, 2011), the muricid compounds from *D. orbita* have *in vitro* anticancer properties and also induce apoptosis in reproductive cancer cell lines. Therefore, there is potential to develop a new marine anti-cancer natural treatment for reproductive cancers from these Muricid extracts.

One main issue with the development of new anti-cancer compounds is the stability and bioavailability of the active compound which are required to last long enough to

act on the designated targets *in vivo* (Grabley and Thiericke, 1999). In this study, a crude sample of *D. orbita* extract added to tissue culture plates at 37^{o}C for 1-24h demonstrated that tyrindoleninone was oxidised to 6-bromoisatin after only 1h (Appendix IV). 6-bromoisatin however, was still detected after 24h exposure to 37^{o}C and 5% CO_2 (Appendix IV). Recently, an *in vivo* study using a crude *D. orbita* extract (containing a mixture of 6-bromoisatin and tyrindoleninone) fed to mice found that 6-bromoisatin was more stable than tyrindoleninone in the gastric environment (Westley, *et al.*, 2010). In fact, tyrindoleninone was readily oxidised to 6-bromoisatin or reduced to tyriverdin (Westley, *et al.*, 2010). Westley *et al* (2010) did not identify any major toxic effects of the crude *D. orbita* extract to the mice, while at the same time the extract was still found to induce apoptosis in stimulated cancer cells in the colon. Oral delivery may not be so optimal for reproductive cancers, so further investigation into alternative delivery routes and bioavailability studies are required.

Selectively targeting cancer cells over primary cells is a major advantage in the development of new drug candidates specifically when trying to obtain approval of the drug by the FDA (Simmons, *et a*l., 2005). Ideally cell death induced by apoptosis rather than necrosis, is the desired mode of cell death (Chapter 1 & 6), as unlike necrosis, apoptosis is a form of cell death that does not cause trauma to the surrounding tissue (Jin and El-Deiry, 2005; Elmore, 2007). Together with an upregulation of caspase-3 and -7, DNA fragmentation (identified by TUNEL assays; Chapter 6) and microscopic observations of apoptotic changes (surface blebbing and apoptotic bodies Chapter 5), it was confirmed that cell death was induced primarily

by apoptosis in the reproductive cancer cell lines at microgram concentrations of tyrindoleninone and 6-bromoisatin (Chapter 6).

From the results of this research alone, it is difficult to determine the pathway of apoptosis and mode of action of muricid bioactive compounds. Previous research using the KGN reproductive granulosa cancer cell line (Manabe, *et al*., 2004; Woods, *et al*., 2008a; Jääskeläinen, *et al*., 2009) found a number of different pathways that could be involved in apoptosis in the KGN cells (Chapter 6). For example, the death domain ligand TRAIL has been shown to commonly occur via both extrinsic and intrinsic pathways in the KGN cancer cell line but not in the corresponding primary granulosa cells (Woods, *et al*., 2008b; Jääskeläinen, *et al*., 2009). Therefore, it could be speculated that tyrindoleninone and 6-bromoisatin stimulated cell death in the KGN cancer cells by TRAIL-induced apoptosis, however further research would be needed to confirm this assumption including assays such as western blotting using TRAIL antibodies.

8.5 Alternative Hormone Replacement Therapy

Estrogen, which belongs to a family of naturally occurring steroid hormones (Chapter 1 Figure 1.8) is responsible for a number of physiological functions in both women and men (Nelson and Bulun, 2001). Traditionally thought of as an essential hormone in female reproduction, estrogen is in fact found in many other peripheral tissues of the body, such as the adipose tissue (Purohit and Reed, 2002). Besides reproductive functions, estrogen is also responsible for the maintenance of bone mass and cognitive functions, regulation and synthesis of lipoproteins, maintenance of heart and blood vessels and the regulation of insulin, it is therefore an essential hormone in both males and females (Nelson and Bulun, 2001).

During the reproductive age in women, the hormones progesterone and estrogen are produced by the granulosa cells of the ovaries via the steroidogenic pathway (Figures 1.8 and 1.9 Chapter 1; Speroff and Fritz, 2005). However, with the decrease in ovarian hormonal production at menopause, there is a marked reduction in hormone biosynthesis, and the main site of estrogen synthesis moves from the ovaries to peripheral tissues (Nelson and Bulun, 2001; Purohit and Reed, 2002). After menopause, plasma estrogen levels drop from 40-400pg/ml, pre-menopause, to just 10-20pg/ml post-menopause (Speroff and Fritz, 2005). With this considerable decrease in plasma estrogen after menopause, women become more susceptible to disorders associated with low estrogen levels, including osteoporosis, coronary heart disease and dementia (WHO, 1981). Consequently, hormone replacement therapy (HRT), either as estrogen alone or, a mixture of estrogen and progestin (a synthetic substitute that has similar effects to that of progesterone) is often recommended to women during menopause (WHO, 1981; Blanc, *et al.*, 1998).

Estrogen biosynthesis in peripheral tissue is well established and involves aromatases, estrogen dehydrogenases and estrogen sulphatases, which in adipose tissue are distributed throughout the entire body including the breast tissue (Simpson and Zhao, 1996; Nelson and Bulun, 2001; Purohit and Reed, 2002). However, research shows that the entire complement of enzymes required for estrogen synthesis are also found in malignant tissue (Purohit and Reed, 2002). With the introduction and increased use of HRT for post-menopausal women, the occurrence of estrogen dependent cancers such as endometrial and breast cancers, have significantly increased (Verkooijen, *et al.*, 2009). The first study to identify the connection between HRT and breast cancer risk was conducted in 1995 (Colditz, *et*

al., 1995). Since that time, well documented studies have confirmed the increased risk of breast cancer and other estrogen dependent cancers with the continued use of HRT (Verkooijen, *et al.*, 2009). This ultimately resulted in a dramatic decrease in sales of HRT world-wide (Verkooijen, *et al.*, 2009). Estrogen has an adverse role in relation to estrogen receptive cancers, however it also exerts many beneficial effects for menopausal women (Purohit and Reed, 2002). Consequently, the FDA recommends that women should only use HRT for a minimal time and at the lowest concentration that produces a beneficial effect (FDA, 2009).

In light of the potential adverse effects of HRT, studies now indicate that women often choose complementary and alternative therapies (CAM) for the treatment of symptoms of menopause (Pinkerton, *et al.*, 2009). Previous research has shown that a number of phytoestrogens, such as soyabean isoflavones can display both anti-estrogen and pro-estrogen effects and therefore may protect against breast cancer while at the same time being beneficial for symptoms of menopause, including osteoporosis (Michnovicz, 1996; Eichholz, *et al.*, 2002). Indole-3-carbinol (I3C), a structurally similar compound to the muricid compounds, derived from cruciferous vegetables has been shown to be both an estrogen antagonist and agonist (Michnovicz and Bradlow, 1991; Auborn, *et al.*, 2003). There are also a wide range of herbal remedies that have been shown to relieve the symptoms of menopause, including ginseng, which has a slight estrogenic effect (Eichholz, *et al.*, 2002; Pinkerton, *et al.*, 2009). Saponins or 'ginsenosides', the active ingredient in ginseng, are steroid-like compounds that have a 3β-hydroxyl ring and a large hydrophobic side chain, which are the two main structural components of estrogenicity (Katzenellenbogen, 1995). In this research, low concentrations of natural brominated

indoles upregulated estradiol synthesis by hCG stimulated primary-derived granulosa cells from women with normal reproductive physiology. If this stimulation of estradiol also occurs in post-menopausal cells, these are potential applications for menopause.

Previous studies have already identified that these semi-purified *D. orbita* compounds are anti-proliferative against the human female breast cancer cell line, MCF-7 (Benkendorff, *et al.*, 2011). However, this study is the first to identify that brominated indoles from Muricidae molluscs inhibits the endometrial cancer cell line JAr and can stimulate estradiol synthesis in human primary-derived granulosa cells. Further research testing the effects of brominated indoles on estradiol synthesis in cells from post-menopausal peripheral tissue together with *in vivo* animal studies, could be of interest. These studies would provide data on the effects of these compounds on both hormone synthesis and cell cytotoxicity.

8.6 Final Conclusion

In conclusion, purified brominated indole compounds from the Australian muricid whelk, *D. orbita*, are biologically active against a series of female human reproductive cancer cell lines, while at the same time these compounds stimulate endogenous estradiol synthesis by healthy primary-derived female human granulosa cells. This significant result highlights the potential to develop a new nutraceutical, as a naturally derived treatment for menopause, from these muricid compounds that may also have the added benefit of inhibiting female estrogen-dependent cancers. In contrast, the synthetic brominated indoles, was not effective at inhibiting cell proliferation in the female cancer cell lines. However, at very low concentrations

these compounds did stimulate progesterone synthesis and therefore warrant further investigation for treating gynaecological disorders given that the homeopathic remedy is currently sold for this purpose.

APPENDIX I: Manufactures

Reagent	Company	Address	Catalogue No	Lot No.
Acetate plate sealer	Sigma-aldrich	Missouri, USA	CLS3095	3100
Acetic acid	AJAX Chemicals	Melbourne Victoria	2789	802479
Albumin from bovine serum (BSA)	Sigma-aldrich	Missouri, USA	A7906	074K0567
2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt	Sigma-aldrich	Missouri, USA	A1888	015K0795
Benzene, Chromasolv Plus, for HPLC ≥ 99.9%	Sigma-aldrich	Missouri, USA	270709	00440TH
Calcium chloride ($CaCl_2$)	Sigma-aldrich	Missouri, USA	C-3881	118H2075
Caspase-Glo 3/7 assay kit	Promega	Madison, USA	G8091	286191
Chloroform	BDH	Kilsyth, Victoria	10077.6B	34962
Chloroform Chromasolv® for HPLC ≥99.8%, amylene stabilized	Sigma-aldrich	Missouri, USA	34854	7017A
Choronic gonadotrophin human (hCG)	Sigma-aldrich	Missouri, USA	CG5	036K1439
Citric acid	May & Baker	West Footscray, Victoria	D1204	D1204
Clarion Mounting Media-organic	Sigma-aldrich	Missouri, USA	C0487	036K1191
Crystal violet	Aldrich	Sydney NSW	548-62-9	DN00129MG
CytoTox-ONE Homogenous Membrane Integrity kit	Promega	Madison, USA	G7891	280087
Dead-End TUNEL assay kit	Promega	Madison, USA	G3250	283785
4'6'-diamidino-2-phenylindole dihydrochloride	Sigma-aldrich	Missouri, USA	32670	1424953

(DAPI)				
N6, 2-O-dibutyrladenosine 3’5’-cyclic monophosphate Sodium (cAMP)	Sigma-aldrich	Missouri, USA	D0627	048K7001
Dichloromethane (DCM) HPLC grade	Sigma-aldrich	Missouri, USA	650463	02634ME
Dimethyl disulfide 99%	Aldrich	Sydney, NSW	471569-250ML	06709ME
Dimethyl sulfoxide	BDH	Kilsyth, Victoria	10323	37635
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	BDH	Kilsyth, Victoria	10248	36730
Trans-dehydroandrosterone	Sigma-aldrich	Missouri, USA	D4000	100K0099
DMEM/HAMS-F12 media without HEPES	Invitrogen corporation	CA, USA	12500-062	627978
Donkey anti-Sheep/Goat IgG	Chemicon	Boronia, Victoria	AB7134	8214576370
Estradiol ^{125}I	MP Biomedicals	Solon, OH, USA	07-138226	TE0801
Ethanol	Merck	Kilsyth, Victoria	4.10230.9010	42542
Ethlenediamine-tetracetic acid (EDTA)	Sigma-aldrich	Missouri, USA	ED2SS	73H1037
Fetal Bovine Serum (FBS)	Invitrogen Corporation	CA, USA	10099-141	614642
Glacial Acetic Acid	APS Chemicals	Sydney, NSW	2789	H9AO22
Glucose	Sigma-aldrich	Missouri, USA	G-7021	87H13255
L-glutamine	Sigma-aldrich	Missouri, USA	G-8540	096K0115
Glycerol	APS Chemicals	Sydney NSA	242-500ml	KNAI
Goat polyclonal to 17β estradiol 3 (Abcam)	Sapphire Bioscience	Waterloo, NSW	ab50660	407516
HEPES. 99.5% cell culture tested	Sigma-aldrich	Missouri, USA	H4034	104K5419
Human chorionic gonadotrophin	Sigma-aldrich	Missouri, USA	CG5	036K1439

Histoclear	National Diagnostic	Atlanta, Georgia, USA	HS-200	11-05-17
Hydrochloric acid (HCl)	Ajax Fine Chemicals	Sydney NSW	1789	A1367
Hydrogen peroxide 30% solution	Sigma-aldrich	Missouri, USA	5230499	01990
Indirubin	APIN Chemicals	Abingdon, Oxon	20338i	10921
Insulin solution from bovine pancrease	Sigma-aldrich	Missouri, USA	I0516	077K8405
Insulin solution human	Sigma-aldrich	U Missouri, USA	I9278	116K8405
Lyphochek Immunoassay Plus Controls	BIO RAD	Gladsville, NSW, Australia	370	40110
Lymphoprep	Axis-Shield	Cambridgeshire,UK	1114544	1009859
Magnesium Chloride ($MgCl_26H_20$)	Sigma-aldrich	Missouri, USA	2670	026K01421
Merthiolate (Thimerosal)	Sigma-aldrich	Missouri, USA	30416	84F-0343
Methanol	Merck	Kilsyth, Victoria	010365	AH611267
Methanol HPLC grade	Sigma-aldrich	Missouri, USA	270547	08158DH
Minisart Sterile 0.2µm filters	Sartorius stedim	Dandenong South, Victoria	16534	90750103 exp 2012-08
β-Nicotinamide adenine nucleotide	Sigma-aldrich	Missouri, USA	N1511	086K7052
Nitroblue tetrazolium	BIO RAD	Gladsville, NSW, Australia	170-6532	298-83-9
Nunc Lab-Tek II - CC2 Chamber Slide ™ System (8-well)	Sigma-aldrich	Missouri, USA	S6815	032007 8 0
Nunc Maxisorb plates (ELISA plates)	Sigma-aldrich	Missouri, USA	M9410	06996MD
Paraformaldehyde	BDH	Kilsyth, Victoria	29447	318 K19151648
Penicillin/streptomycin solution	Thermo Scientific	Scoresby, Melbourne, Vic	SV30010	JTJ32977
Potassium chloride (KCl)	APS	Sydney, NSW	383	F9A224
Progesterone	Sigma-aldrich	Missouri, USA	P8783	034K01985
Progesterone ^{125}I	MP Biomedicals	Solon, OH, USA	07-170126	PG0803

Propidium Iodide (PI)	Sigma-aldrich	Missouri, USA	P4170	037K3676
RPMI-1640 Medium	Sigma-aldrich	Missouri, USA	R1383	017K8316
Selenium sodium selenite	Sigma-aldrich	Missouri, USA	S5261	229865
Sheep anti-rabbit Imunoglobulin IgG	Chemicon	Boronia, Victoria	AB7130	07121436762
Sodium Bicarbonate $NaHCO_3$	Pfizer	West Ryde, NSW	S084PA	CE63
Sodium Bicarbonate $NaHCO_3$	Sigma-aldrich	Missouri, USA	S8875	49H0407
Sodium carbonate (Na_2CO_3)	Chem-Supply	Gillman, Adelaide, SA	SA099	242777
Sodium carbonate (Na_2CO_3)	May & Baker	West Footscray, Victoria	6124	6124
Sodium chloride (NaCl)	Ajax Chemicals	Auburn, NSW	465	70334315
Sodium chloride (NaCl)	Sigma-aldrich	Missouri, USA	S9625	104K0176
Sodium dihydrogen orthophosphate (NaH_2PO_4)	BDH	Kilsyth, Victoria	10245	59137
Sodium dodecyl Sulphate (SDS) 20%	Sigma-aldrich	Missouri, USA	L4509	046K0085
Sodium hydrogen carbonate ($NaHCO_3$)	BDH	Kilsyth, Victoria	10247	5829
Thiazolyl Blue Tetrazolium (MTT)	Sigma-aldrich	Missouri, USA	M5655	03330DH
TLC aluminium silica gel 60F254 plates	Merck	Darmstadt, Germany	1:05554.0001	HX616604
3H methyl thymidine	GE Healthcare	Sydney, NSW	TRK758	300
Transferin Apo-human	Sigma-aldrich	Missouri, USA	T1147	068K1571
0.25% Trypsin-EDTA Solution	Sigma-aldrich	Missouri, USA	T4049	126K2338
Tris-HCl	Sigma-aldrich	Missouri, USA	T1503	074K5442
Triton X-100	Sigma-aldrich	Missouri, USA	T-8787	110K0251
Tween 20	Sigma-aldrich	Missouri, USA	P1379	094K0052
Water for injections (sterile)	AstraZeneca	Export Park, Adelaide, SA	825700	B308035 Sep 2011

APPENDIX II: Classification of Primary-derived Human Granulosa Cells

Primary-derived Human Granulosa Cell Identification

Primary-derived human granulosa cells were obtained from women undergoing assisted reproductive technology (ART) at Flinders Medical Centre, Bedford Park, Adelaide, South Australia, with informed consent approved by the Flinders Clinical Research Ethics Committee (approval number 260/067). The women were either undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) depending on their infertility factor. The aim of this section was to group the granulosa cells derived from the women (hereafter classified as cases or groups) into two groups, classified as normal or abnormal reproductive physiology based on the infertility status of each woman and not on the procedure they were undergoing (IVF or ICSI) using the following criteria and previously published literature (Jose-Miller, *et al.*, 2007):

1. Normal reproductive physiology or fertile were classified as granulosa cells derived from cases whose partners had male sperm defects, decreased motility and/or erectile dysfunctions, and women who had previously had ectopic pregnancies or a tubal obstruction. The ectopic preganancies and tubal obstruction cases were considered fertile because the granulosa cells and hormone synthesis should not be affected by these disorders.

2. Abnormal reproductive physiology or infertile cases were defined as granulosa cells from cases with, polycystic ovarian syndrome (PCOS), single ovarian cyst, endometriosis, pelvic inflammatory disease, abnormal morphology and/or idiopathic infertility. These cases were considered infertile for this thesis because all

of these disorders could potentially have an affect on hormone synthesis and concentration.

During IVF or ICSI procedures hyper-stimulation of multiple follicles was induced in all cases with varying concentrations of FSH (Table 1) prior to oocyte retrieval. The oocytes were separated from the follicular aspirates and the granulosa cells were isolated from the aspirates as described in section 2.2.1.1. Granulosa cells cannot be classified as fertile or infertile however for the benefit of this thesis, in Chapters 4 and 7 infertile and fertile refers to the original infertility factor of the case donor.

Case study notes were reviewed and the following information recorded, the date of birth, the number of treatment cycles, the maximum estradiol (E_2) synthesized, the number of follicles greater than 15, the follicle stimulating hormone (FSH) starting dose, the FSH total dose, the number of eggs aspirated the number of mature oocytes, the procedure they were undergoing (IVF or ICSI), the number of granulosa cells isolated and the fertility factor, as defined in the Table 1.

To identify primary-derived human granulosa cells isolated from follicular aspirates of cases undergoing IVF and ICSI treatments (section 2.2.1.1), the 3-βHSD assay was performed (section 2.2.1.1.1; n=3). To further assess if female primary-derived human granulosa cells proliferate in cell culture, a dual staining technique was employed using ^{3}H-methyl tritiated thymidine and 3-βHSD stain (section 2.2.1.1.2; n=1).

Results

Blue 3-βHSD positive staining was localised to the mitochondrial membrane of the cells (Figure 1), whereas the nucleus was stained red after the 3-βHSD assay (Figure 1).

The incorporation of tritiated thymidine into the DNA was localised to the nuclei (Figure 2) however, both the 3-βHSD and neutral red staining were either rinsed out during the micro-autoradiography of the slides (Figure 2).

Discussion

The 3β-HSD enzyme is the enzyme which catalyzes the conversion of pregnenolone to progesterone in the hormone producing cells, granulosa cells (Bar-Ami and Gitay-Goren, 2000). Women who are undergoing assisted reproductive technologies (ART) are administered human choronic gonadotrophin (hCG) 36h prior to oocyte retrival resulting in an elevated increase in progesterone (P4) secretion; which can be observed in cell culture (Bar-Ami and Gitay-Goren, 2000). Therefore, localised staining techniques, such as dehydrogenase enzyme staining can be performed to identify lutenized granulosa cells (Fischer and Kahn, 1972). The results of the staining herein clearly show that the cultured granulosa cells were actively synthesizing hormones with the localised blue staining of the 3β-HSD enzyme (Figure 1).

After granulosa cells have been cultured for 18-24h they differentiate and lutenize and begin to synthesize the hormones progesterone and estradiol in culture (Young, *et al.*, 2008), therefore granulosa cells would not be expected to proliferate in cell culture. Interestingly though, the thymidine labelled cells (Figure 2) indicates the

opposite, the granulosa cells appear to still be proliferating in culture, however as neither the 3β-HSD staining or neutral red staining of the nucleus was successful in the dual staining technique, it was impossible to identify that these cells were actually granulosa cells. Other studies have shown that cultured granulosa cells will proliferate in culture when exposed to growth factors such as insulin-like growth factor (IGF-1) along with FSH or LH (Yong, *et al.*, 1992). Therefore, an improved staining technique of the granulosa cells needs to be developed to confirm the proliferation of cells.

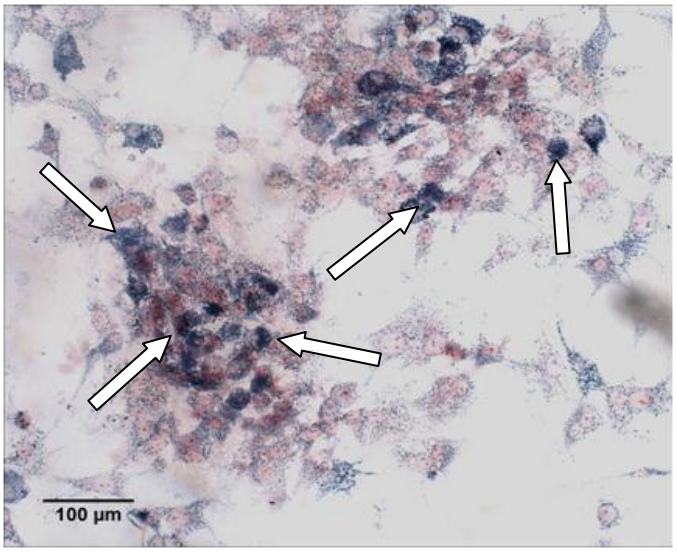


Figure 1: Primary-derived human granulosa cells stained to localise the 3-βHSD enzyme as indicated by the arrows. Cells were isolated from follicular aspirates and plated into the wells of Nunc Lab-Tek II-CC2 Chamber Slides at 30,000 cells per chamber in 0.3ml of complete assay medium and incubated at 37^{o}C + 5% CO_2 to allow cells to adhere to the wells. The media was removed and 3-βHSD staining performed. Cells were observed and images taken on an Olympus BX50 Brightfield Microscope.

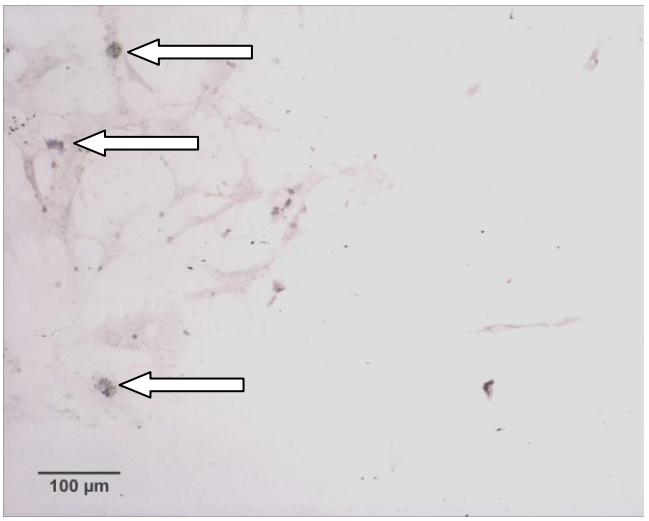


Figure 2: Primary-derived human granulosa cells dual stained with ^{3}H-methyl tritiated thymidine and 3-βHSD stain. Cells were isolated from follicular aspirates and plated into the wells of Nunc Lab-Tek II-CC2 Chamber Slides at 30,000 cells per chamber in 0.3ml of complete assay medium and incubated at 37^{o}C + 5% CO_2 to allow cells to adhere to the wells. 6h prior to end of each incubation period, the media were removed and fresh media containing 1µCi/ml ^{3}H tritiated thymidine added and slides were re-incubated. At end of incubation, medium were removed and the cells were stained with 3-βHSD and fixed to the slides with 10% formalin. Micro-autoradiography of the slides was then performed. Cells were observed and images taken on an Olympus BX50 Brightfield Microscope. Arrows indicate tritiated thymidine incorporation into DNA.

Table 1: A listing of all the women used in this study with information on their fertility factor and IVF status. Reference to the experiment their donated granulosa cells were used in is provided in the far right column.

Case #	Age	Procedure	Fertility Issue	Classified Fertility factor	Max E_2	Follicles > 15	FSH start dose	FSH total dose	Number oocytes aspirated	Number of granulosa isolated	Fertile or in-fertile	Experiment used in
1	29	ICSI	Decreased motility	Male Factor	5090.00	6.00	150.00	1500.00	9.00	2900000	Fertile	Chapt.4
2	37	IVF	Ovarian Cyst	Female Factor	5849.00	5.00	300.00	3300.00	11.00	2500000	Infertile	Chapt.4
3	39	IVF	Idiopathic	Female Factor	2599.00	3.00	150.00	1500.00	5.00	1700000	Infertile	Chapt.4
4	26	IVF	Endometriosis	Female Factor	4719.00	3.00	150.00	1650.00	6.00	2200000	Infertile	Chapt 4
5	31	ICSI	Tubular obstruction/ sperm defect	Both	4577.00	14.00	150.00	1500.00	19.00	3100000	Fertile	Chapt 4 & 3β-HSD staining Appendix II
6	39	ICSI	Decreased motility	Male factor	24940.00	11.00	250.00	2750.00	12.00	3000000	Fertile	Chapt.4
7	30	IVF	Tubular obstruction	Female Factor	25608.00	10.00	150.00	1350.00	20.00	2600000	Fertile	Chapt.4 & 3β-HSD staining Appendix II
8	35	ICSI	Decreased motility	Male factor	-	9.00	125.00	950.00	25.00	4300000	Fertile	Chapt. 4 & 3β-HSD staining Appendix II
9	30	IVF	Tubular obstruction	Female factor	3579.00	9.00	150.00	1400.00	9.00	3000000	Fertile	Chapt. 4& 3β-HSD staining Appendix II

10	38	ICSI	Sperm defect	Male factor	3042.00	6.00	150.00	1500.00	3.00	3900000	Fertile	Chapt. 4 & 3β-HSD staining Appendix II
11	34	IVF	Tubular obstruction	Female factor	2186.00	3.00	150.00	1650.00	8.00	2400000	Fertile	3β-HSD staining
12	38	ICSI	Sperm defect	Male factor	4958.00	3.00	250.00	3000.00	7.00	1300000	Fertile	Not used
15	21	ICSI	Tubular obstruction	Female factor	19120.00	3.00	150.00	1425.00	9.00	1600000	Fertile	Chapt.5
16	28	IVF	Ovarian cyst	Female Factor	6,534.00	6.00	300.00	3000.00	4.00	1600000	Infertile	Chapt. 4
17	35	IVF	Unexplained	Female Factor	3948.00	4.00	150.00	1500.00	6.00	1600000	Infertile	Chapt.4
18	41	IVF	PCOS/Tubular obstruction	Female Factor	5964.00	5.00	450.00	5400.00	11.00	2000000	Infertile	Chapt.4
19	37	ICSI	PCOS	Female Factor	7,599.00	8.00	350.00	3850.00	9.00	2200000	Infertile	Chapt. 4
20	35	IVF	Abn Morphology	Male Factor	2,558.00	10.00	450.00	5400.00	12.00	8700000	Fertile	Appendix III
21	29	ICSI	Idiopathic	Female Factor	3,706.00	5.00	150.00	1800.00	9.00	4700000	Infertile	Not used
22	34	ICSI	Decreased motility	Male Factor	6,064.00	7.00	150.00	1800.00	10.00	5800000	Fertile	Chapt.5
23	36	ICSI	Decreased motility	Male Factor	1,688.00	4.00	150.00	1500.00	6.00	5000000	Fertile	Chapt. 5
24	42	ICSI	Triple sperm defect.	Male Factor	18,794.00	8.00	350.00	3850.00	11.00	520000	Fertile	Chapt.5
25	38	ICSI	Recommended by Doctor	Neither	13,257.00	8.00	300.00	3000.00	7.00	680000	Infertile	Chapt.7
26	28	ICSI	Sperm defect	Male	8340.00	6.00	150.00	1800.00	14.00	460000	Fertile	Not used

				factor								
27	40	ICSI	Tubular obstruction	Female factor	7,036.00	9.00	450.00	5400.00	21.00	780000	Fertile	Chapt.7
28	24	IVF	Decreased motility	Male factor	7,109.00	9.00	150.00	1650.00	11.00	780000	Fertile	Chapt.7
29	41	IVF	Idiopathic	Female factor	25,596.00	14.00	300.00	28875.00	41.00	1100000	Infertile	Chapt.7
30	41	ICSI	Endometroisis	Female factor	14,938.00	14.00	150.00	2100.00	17.00	1100000	Infertile	Chapt.7
31	38	ICSI	Sperm defect	Male Factor	7,199.00	7.00	300.00	4500.00	12.00	900000	Fertile	Chapt.7
32	42	ICSI	Decreased motility	Male factor	3,706.00	5.00	15.00	1800.00	9.00	500000	Fertile	Not used
33	35	ICSI	Idiopathic	Female factor	5,127.00	4.00	300.00	3600.00	5.00	760000	Infertile	Not used
34	40	IVF	Idiopathic	Female factor	7,347.00	1.00	200.00	2600.00	8.00	1100000	Infertile	Chapt.7
35	35	ICSI	PCOS	Female factor	5,515.00	-	75.00	975.00	12.00	1300000	Infertile	Chapt.7
36	32	ICSI	PCOS/decreased motility	Both	6,590.00	6.00	150.00	1800.00	11.00	1100000	Infertile	Chapt.7
37	38	ICSI	Endometriosis /decreased motility	Both	10,517.00	-	150.00	1650.00	10.00	1500000	Infertile	Chapt.7
38	40	ICSI	Sperm defect	Male Factor	2,408.00	5.00	300.00	3000.00	19.00	880000	Fertile	Not used
39	32	IVF	Sperm defect	Male factor	54,329.00	27.00	200.00	1850.00	30.00	960000	Fertile	Chapt.6
40	41	ICSI	Decreased motility	Male factor	2,264.00	2.00	300.00	3000.00	1.00	740000	Fertile	Chapt.6
41	37	IVF	Sperm donor	Male factor	7389.00	5.00	300.00	4500.00	12.00	5800000	Fertile	Chapt.6

42	29	ICSI	Sperm defect	Male factor	5,329.00	3.00	150.00	1750.00	10.00	2700000	Fertile	Chapt.6
43	33	ICSI	Decreased motility	Male factor	3,765.00	14.00	150.00	1800.00	8.00	4160000	Fertile	Chapt.6

APPENDIX III: Concentration of 6-bromo-2,2-dimethylthioindolin-3-one (Tyrindolinone) & 6-bromo-2-methylthioindoleninone (Tyrindoleninone).

Introduction:

Preliminary cell culture experiments in this project using semi-purified *Dicathais orbita* fractions of a mixture of tyrindolinone and tyrindoleninone had indicated that fractions containing higher concentrations of tyrindolinone were more cytotoxic to primary-derived human granulosa cells than fractions containing higher concentrations of tyrindoleninone. Therefore, it only followed that an experiment should be conducted to try and separate these two compounds so that they could be tested separately on primary-derived human granulosa cells.

The aim of this experiment therefore was to separate the bioactive compounds tyrindolinone and tyrindoleninone from a crude extract isolated from the hypobranchial glands of *D. orbita.* Once isolated and purified, tyrindoleninone and tyrindolinone were tested on primary-derived human granulosa cells and the JAr, choriocarcinoma cancer cell for cytotoxicity.

Methods:

Extraction and purification

Bioactive compounds from *D. orbita* were extracted from the hypobranchial glands of frozen whelks collected in September 2008, from an abalone farm in Pt Lincoln, South Australia and transported frozen to Flinders University, and from the egg masses collected from specimens in re-circulating tanks at Flinders University, South

Australia. The hypobranchial branchial glands were removed from frozen whelks (15.15g; 150 specimens), first by removing shell by cracking it gently in a vice. The columnar muscle was then cut away from the shell with a sharp scalpel and the whole visceral mass gently removed from the shell. The three regions of the hypobranchial gland were cut away from the remaining tissue; easily distinguished as a yellow, sometimes greenish region. The isolated hypobranchial gland tissue was placed immediately into HPLC grade chloroform: methanol (1:1 v/v) in the dark with stirring at 4^{o}C; which was replaced four times over a 48h period.

The resulting solution was filtered at the tap through a büchnel funnel and then added to a separating funnel with MilliQ water (~50mL), to facilitate separation of the aqueous and organic layers. The lower organic layer was collect and filtered through glass wool to remove any traces of remaining tissue. Chloroform was removed under reduced pressure (474mbar; 40^{o}C) to give an oily red/brown residue; which was re-dissolved in ~1mL of dichloromethane DCM (100%). The DCM was dried off under a stream of N_2 gas and the final oily red product (0.3013g) was stored in amber vials at -20^{o}C to protect from light and oxygen until required.

The egg capsules (22.24g) were cut open and soaked immediately in HPLC grade chloroform and methanol (1:1 v/v) in the dark with stirring at 4^{o}C for 4h. The solvent mix was then decanted and replaced another 2 times over a 24h period. A small amount of MillQ water (~50-100ml) was added to the combined extracts and the non-polar chloroform layer was separated from the polar water layer. The yellow chloroform layer was then evaporated to dryness under reduced pressure as above (474mbar; 40^{o}C) to give a brown/red oil (0.6253g), and stored at -20^{o}C until purified.

To facilitate the concentration of 6-bromo-2,2-dimethylthioindolin-3-one (tyrindolinone) and 6-bromo-2-methylthioindoleninone (Tyrindoleninone) an adaption of the Baker and Duke (1973) (Baker and Duke, 1973a) protocol was adopted using dimethyl disulfide instead of the volatile gas, methanethiol.

A crude sample extracted from the hypobranchial glands (0.3g) was passed through an alumina column (30g) and eluted with anhydrous benzene (490mL) and dimethyl disulfide (2%: 10mL) under a stream of N_2 gas. The solvents in the collected fractions were removed under reduced pressure (335 and 236mbar; 50^{o}C) and the reddish oil re-dissolved in hexane (40mL) and benzene (10mL). The green solid (tyriverdin compound) was then filtered at the tap and the sample was placed on ice to facilitate crystal formation. After 1h and no evidence of crystal formation, the hexane/benzene was removed under reduced pressure as above and the red oil (0.051g) was stored at -20^{o}C.

After examination of the results (Table 1 and Figure 1), a decision was made to increase the concentration of dimethyl disulfide to 10%; the hypothesis was that, the increase in dimethyl disulfide would drive the reaction towards the product tyrindolinone. A second crude *D. orbita* sample, extracted from the egg capsules (0.6253g) was purified on an alumina column under N_2 gas and fractions were eluted using anhydrous benzene (450ml) and dimethyl disulfide (10%: 50mL). The solvents were removed under reduced pressure as above and the red oil dissolved in benzene (10mL) and hexane (40mL). The tyriverdin solid was filtered at the tap as described above, and placed on ice to induce crystallization of the product. After ~1h and no crystal formation the resulting solvent was evaporated under reduced pressure (as

above), chemical analysis was performed, and the red oil product (0.080g) stored at -20^{o}C. A third crude sample was also passed through an alumina column eluting fractions with benzene only (100%) using a crude sample from the hypobranchial glands (0.498g) and treated as per the above two column. The resulting red oil product (0.0237g) was used as a control to determine the effectiveness of the concentration of tyrindolinone using 2 and 10% dimethyl disulfide in benzene.

Chemical analysis

Thin layer chromatography (TLC) analysis using aluminium silica gel 60F 254 plates (Merck) was performed on the crude and semi-purified product with DCM (100%). Liquid chromatography mass spectrometry (LC/MS) was also performed on crude and semi-purified *D. orbita* samples. Analysis of samples by LC/MS employed an electrospray ionisation mass spectrometry (ESI) and UV detection. The analysis was performed on a Micromass Quattro micro (Waters, Manchester, UK) tandem quadrupole mass spectrometer. LC separation was provided by a Waters liquid chromatography (Waters, Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector. The mobile phase was split after the UV detector using a low volume T-piece; 300µL was directed to the electrospray source of the mass spectrometer and the rest to waste. Data was acquired by the Masslynx Software (v4.0) for both the MS and UV data. UV detection wavelengths were 300 and 600nm respectively. Liquid chromatography incorporated a HPLC Phenomenex, Synergi Hydro-RP, 250 x 4.6 mm, 4µm particle column. Two solvents were employed; Solvent A, 0.1% aqueous formic acid and Solvent B, 0.1% formic acid in acetonitrile at a flow rate of 1.0mL/min and injection volume of 20µL.

Compound preparation for assays

Two Fractions, one from the purification using benzene only, containing predominately tyrindoleninone (Figure 1D) and, the second from the purification using dimethyl disulfide (2%) in benzene, containing predominately tyrindolinone (Figure 1B) were tested on primary-derived human granulosa cells and the uterine cancer cell line, JAr. The fractions were thawed, weighed and prepared fresh on day of experiment. To facilitate solubility of organic compounds in culture medium fraction extracts were dissolved in dimethylsulphoxide (DMSO; 100%) at 100 x the final concentration (final concentrations 0.005-1mg/mL). Final experimental wells contained a final concentration of 1% DMSO.

Preliminary cell culture analysis of Tyrindoleninone and Tyrindolinone

Primary-derived human granulosa cells were obtained from women undergoing IVF and ICSI fertility treatments at Flinders Reproduction Medicine, in the Flinders Medical Centre due to a male factor with informed consent and approved by the Flinders Clinical Research Ethics Committee (approval number 260/067). Granulosa cells were isolated from the follicular aspirates by density gradient on a percoll column as (Young, *et al.*, 2005a). Collected granulosa cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM): HAMS F12 (GIBCO, Invitrogen Corporation) supplemented with 10% FBS (GIBCO Invitrogen Corporation), penicillin/streptomycin 5000IU/ml & 5000ug/ml respectively (Thermo Scientific), insulin (5ug/mL), transferin Apo-human (5ug/mL), selenium sodium selenite (5ng/mL) (ITS) and buffered with 1.2g/L $NaHCO_3$ (Pfizer).

The JAr (Patillo, et al., 1971) cell line was obtained from the Global Bioresource Centre™ (ATCC) and maintained in RPMI-1640 medium supplemented with 10 &

20% FBS respectively (GIBCO, Invitrogen Corporation), sodium pyruvate (1mM), HEPES (10mM), glucose (4.5g/L), L-glutamine (2mM), and penicillin and streptomycin (5000IU/ml & 5000ug/ml respectively) (Thermo Scientific), and buffered with 1.5g/L $NaHCO_3$ (Pfizer). JAr cells were maintained in $75cm^2$ sterile tissue culture flasks (NUNC, Thermo Fisher Scientific) at 37^oC in a humidified atmosphere with 5% CO_2 and subcultured every 2-3 days as required.

JAr cells were detached from flasks at 80% confluence by adding 2-3mls of 1 x 0.25% Trypsin/EDTA solution to flask and incubating at 37^oC for ~5 minutes. To detached cells (noted as a milky suspension), ~2-3mL of complete medium was then added. Cell suspension was centrifuged (78 x g) for 5 minutess and pellet resuspended in 5ml complete medium. Cells were counted on a haemocytometer and cell number determined using the trypan blue exclusion assay as previously described (Freshney, 2005).

Primary derived-human granulosa cells and JAr and cell line (2×10^4 cells/well) were seeded into flat-bottom 96 well plates (NUNC, Thermo Fisher Scientific) and incubated to allow cell adherence at 37^oC and 5% CO_2 (24h primary granulosa cells and 2h for JAr cell line). Standard curve plates of primary cells ($0\text{-}4 \times 10^4$ cells/well) and JAr cells ($0\text{-}8 \times 10^4$ cells/well) were setup alongside treatment plates. After cell adherence periods, spent media was removed and *D. orbita* semi-purified fractions were added (0.0005-1mg/mL) in triplicate. Media only and 1% DMSO, controls were also added in triplicate wells per plate. Positive controls of medium only without FBS for primary granulosa cells, and hydrogen peroxide (10,000µg/mL) for JAr were also plated in triplicate. Plates were incubated at 37^oC and 5% CO_2 for 4

and 24h. Media was removed from treatment plates at the end of designated incubation periods. Wells were rinsed with sterile phosphate buffered saline (PBS) and 50µL of crystal violet (0.5%) was added to all treatment wells for a 10 minute period. Plates were then rinsed with deionised water at the tap, dried and then 50µL acetic acid (33%) added to all treatment wells for 10 minutes. Plates were then measured on a spectrophotometer at 570nm with reference absorbance of 630nm. Absorbances in treatment plates were then converted to final cell number using the equation of the line from corresponding standard curve plates.

No statistical analyses could be performed because the experiment was only performed on one occasion. The results therefore are the mean ± standard error of mean of three replicate wells.

Results and Discussion

Chemical analysis

Thin layer chromatography (TLC) of the crude sample revealed a number of brominated indole compounds (Table 1) including an orange spot (R_f = 0.65), orange/yellow spot (R_f = 0.56), light orange spot (R_f = 0.32) and dark orange spot (R_f = 0.26). In comparison, the TLC purified fraction using dimethyl disulfide (2%) in benzene produced only 2 distinct spots; one orange/red spot (R_f = 0.77) and one yellow spot (R_f =0.65). By increasing the concentration of dimethyl disulfide to 10% only 1 yellow spot was recorded on the TLC (R_f = 0.63). The benzene control TLC indicated to main compounds in the sample with two spots on the TLC; an orange spot (R_f = 0.51) and a yellow spot (R_t = 0.62).

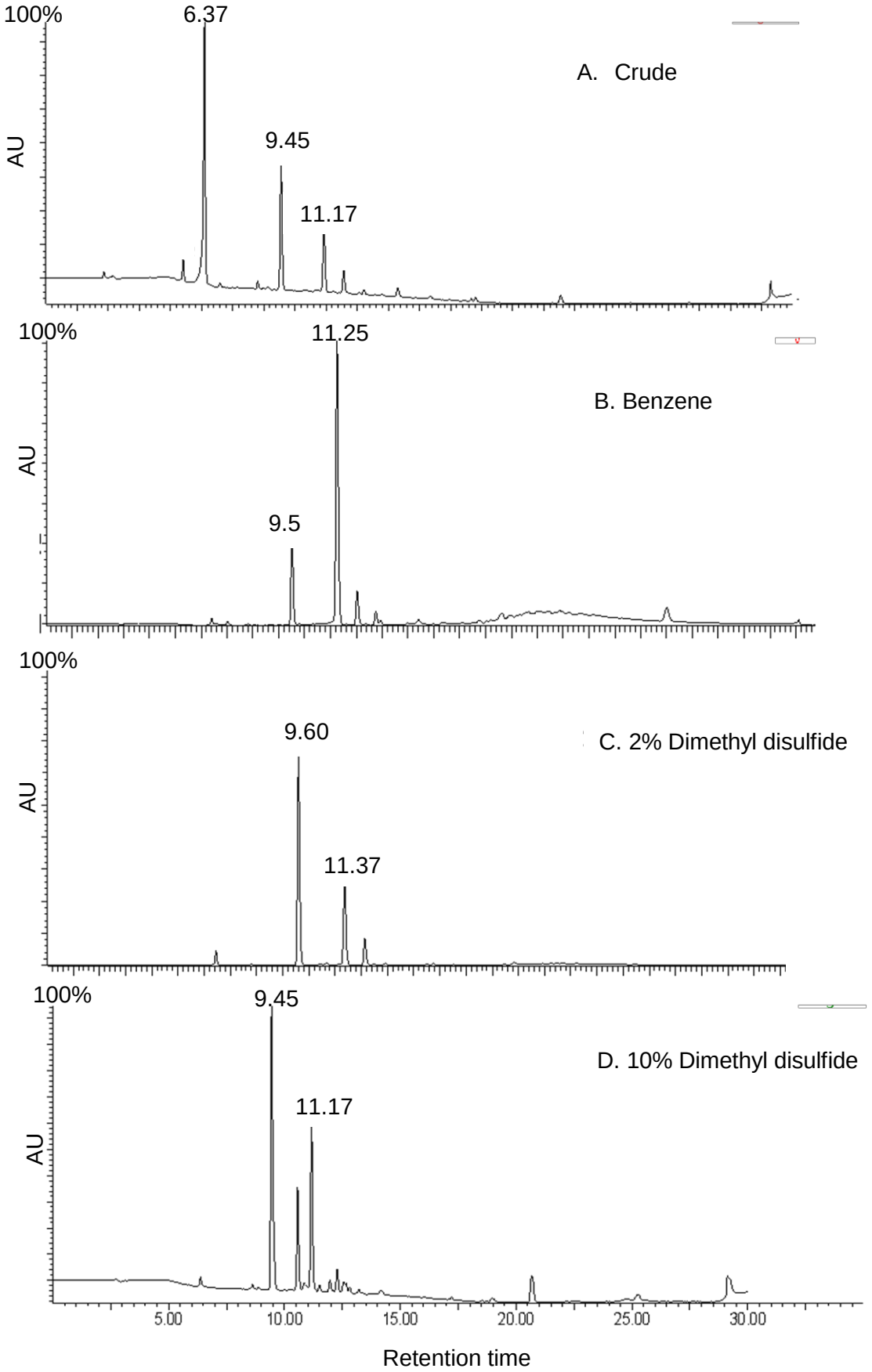
Table 1: Thin layer chromatography analysis of compounds from the crude and semi-purification extracts from *D. orbita* egg masses and hypobranchial glands run on DCM (100%).

Colour	Rf value (cm)	Crude Sample	Dimethyl disulfide (2%)/benzene	Dimethyl disulfide (10%)/benzene	Benzene (100%)
Orange	0.65	+	-	-	-
Orange/yellow	0.56	+	-	-	+
Orange/red	0.77	-	+	-	-
Light orange	0.32	+	-	-	-
Yellow	0.65	-	+	+	+
Dark orange	0.26	+	-	-	-

LC/MS confirmed that the crude sample did contain a mixture of brominated indole compounds (Figure 1A) including tyrindoxyl sulphate UV Diode array minor peak R_t = 5.52 minutes and at ESI/MS *m/z* 336, 338, 6-bromoisatin major peak R_t = 6.37 minutes and at ESI/MS *m/z* 224, 226, tyrindolinone, the second largest peak R_t = 9.45 minutes and at ESI/MS *m/z* 302, 304 and tyrindoleninone R_t = 11.17 minutes and *m/z* 256, 258 (Figure 1A). The control using benzene only (Figure 1B) resulted in a dominant peak which eluted off the HPLC column at R_t = 11.25 minutes and *m/z* 256, and corresponded to molecular mass of tyrindoleninone. A smaller peak at R_t = 9.52 minutes and *m/z* 302 corresponded to the molecular mass of tyrindolinone. A very small peak registered at R_t = 12.01 minutes and *m/z* 417 correlated to the molecular mass of tyriverdin, indicating that the green solid was not completely filtered in the purification process.

Purification of a crude sample with 2 % dimethyl disulfide in benzene was partially successful in concentrating tyrindolinone however, the results confirmed that

tyrindoleninone was also present (Figure 1C). The dominant peak at R_t = 9.6 minutes and at *m/z* 302, 304 corresponded to tyrindolinone and base peak *m/z* 257 correlated to the loss of a methane thiol group from the ring. The second smaller peak at R_t = 11.37 minutes and at ESI/MS *m/z* 256 corresponded to the molecular mass of tyrindoleninone. Interestingly, increasing the concentration of dimethyl disulfide to 10% unfortunately did not push the reaction further to tyrindolinone, as was originally hypothesised (Figure 1D). In fact, the LC/MS results indicated a number of smaller peaks which were confirmed to be a small amount of contaminating solvent that had not been evaporated in the purification process. However, the dominate compound at R_t = 9.45 minutes (Figure 1D) was confirmed to be tyrindolinone with the base peak at *m/z* 257 corresponding to the molecular mass of tyrindoleninone as loss of the methane thiol group, a much smaller peak at *m/z* 302 corresponding to the molecular mass of tyrindolinone. The second peak at R_t = 11.17 minutes and *m/z* 257 confirmed the peak to be tyrindoleninone.


100%
6.37
A. Crude
9.45
11.17
AU
100%
11.25
B. Benzene
9.5
AU
100%
9.60
C. 2% Dimethyl disulfide
11.37
AU
100%
9.45
D. 10% Dimethyl disulfide
11.17
AU
5.00
10.00
15.00
20.00
25.00
30.00
Retention time

Cell Viability Results & Discussion

Primary-derived human granulosa cells were only inhibited by tyrindoleninone after 4h and only at a concentration of 1mg/ml, reducing cell numbers to 84% of control (Figure 2A). In comparison, 100% of JAr cells were killed at ≥ 0.5mg/mL of tyrindoleninone after 4h exposure (Figure 2B). Furthermore, after 24h exposure to 0.5mg/ml of tyrindoleninone JAr cells were reduced to 5% of control (Figure 3B) and primary granulosa cells only to 67% of control (Figure 3A). Conversely, tyrindolinone was found to be more cytotoxic than tyrindoleninone to both primary granulosa cells and the JAr cancer cell line. At 0.5mg/mL tyrindolinone primary granulosa cells were reduced to 29% of control after 4h exposure (Figure 2A) and 0% of control after 24h exposure (Figure 3A). 100% of JAr cells were killed at 0.1mg/mL tyrindolinone, after 4 and 24h exposure (Figure 2B & 3B). The increased biological activity of tyrindolinone over tyrindoleninone towards both primay granulosa and the JAr cancer cells appears to be attributed to the second sulphide group at the second position on the indole structure of tyrindolinone. However, these data are only preliminary results and therefore, these assays would need to repeat before any definite conclusions could be made.

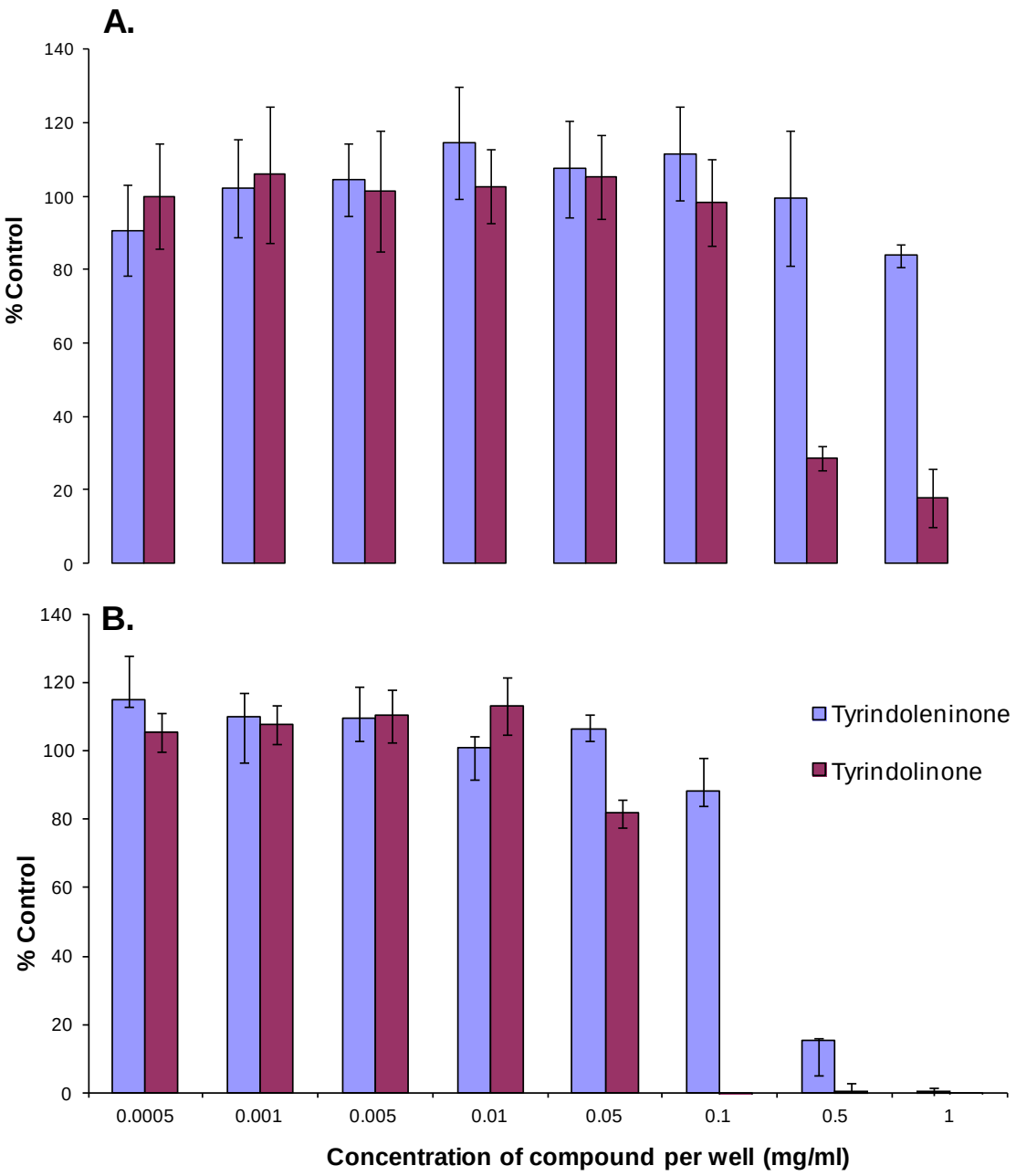


Figure 2: The effect of the Tyrindoleninone and Tyrindolinone on primary-derived human granulosa cell and JAr cell viability after 4h exposure
After a 24 or 2h attachment period 2 x 10^4 cells/well primary-derived human granulosa (A) and JAr cells (B) respectively were treated with tyrindoleninone or tyrindolinone (0.0005-1mg/mL) for 4h. Cell viability was determined by the uptake of crystal violet (0.5%) stain in the nuclei of live cells. Results are the mean of three replicate wells ± 1 SEM and represented as a percentage of the 1% DMSO control.

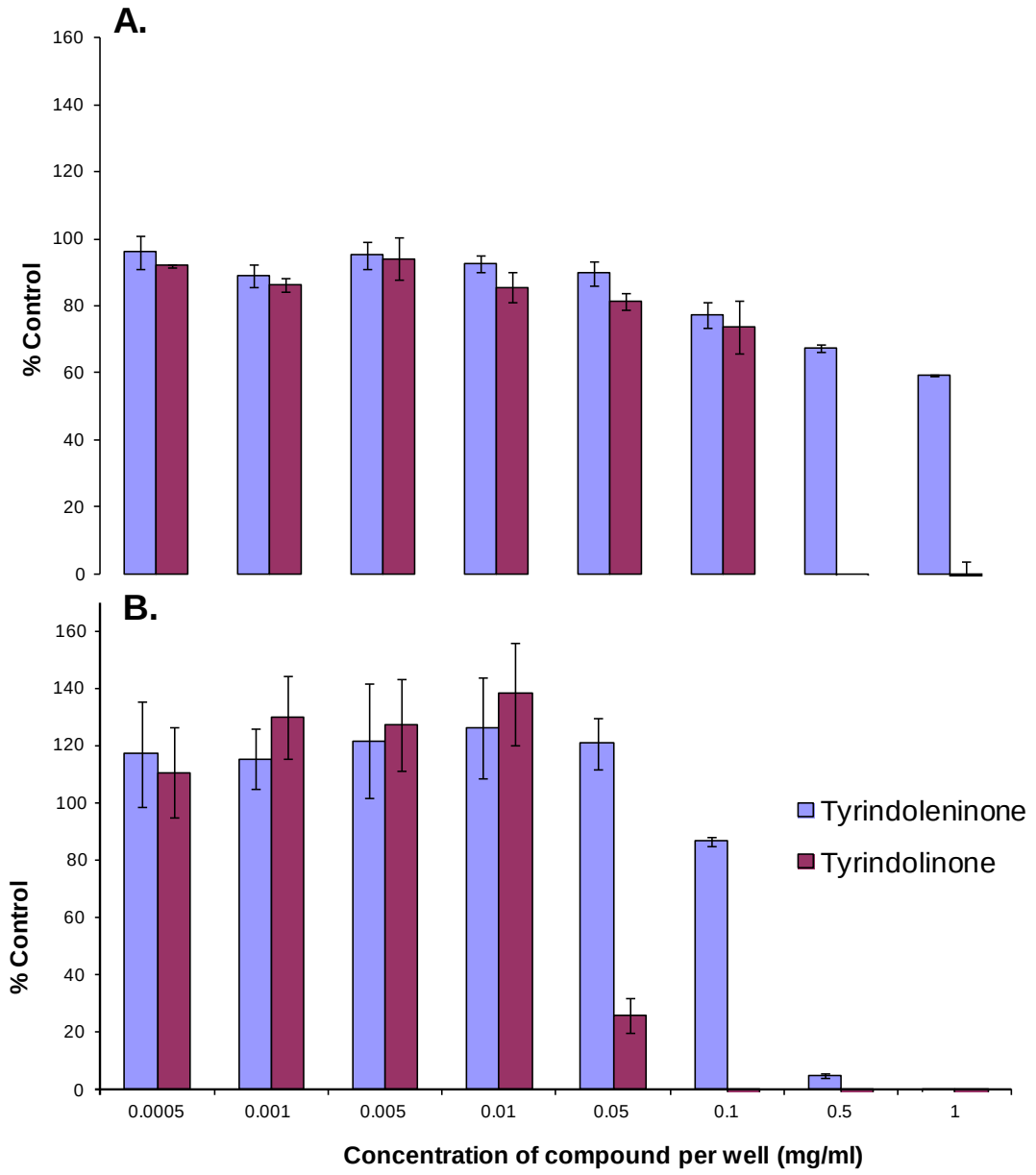


Figure 3: The effect of the Tyrindoleninone and Tyrindolinone on primary-derived human granulosa cell and JAr cell viability after 24h exposure
After a 24 or 2h attachment period 2 x 10^4 cells/well primary-derived human granulosa (A) and JAr cells (B) respectively were treated with tyrindoleninone or tyrindolinone (0.0005-1mg/mL) for 24h. Cell viability was determined by the uptake of crystal violet (0.5%) stain in the nuclei of live cells. Results are the mean of three replicate wells ± 1 SEM and represented as a percentage of the 1% DMSO control

Appendix IV: Stability of *Dicathais orbita* Compounds in Medium

Introduction/Aim

This experiment was performed to determine if the bioactive compounds from a crude *Dicathais orbita* extract, degrade at 37^{o}C + 5% CO_2 under sterile conditions in a cell culture incubator.

Method

As complete medium could not be added to the HPLC column, and the main constituent of cell culture medium is water, this experiment was performed in sterile water instead of cell culture media. A crude sample of *D. orbita* was extracted from the egg masses (section 2.2.4.4.1), diluted in DMSO (100%) and then diluted in water to a final concentration of 1mg/ml, (section 2.2.4.7). Plates were incubated for 1 or 24h and then samples from plates (10µl) were added directly to C_{18} column connected to a liquid chromatography mass spectrometer. Sterile water and 1% DMSO control in sterile water were also analysed by LC/MS.

Results and Discussion

LC/MS analysis of the crude sample (Figure 1) solvent extracted from the egg masses of D. orbita confirmed four main compounds. The dominate compound was 6-bromoisatin at 6.37mins which was confirmed by the m/z 224, 226 (Figure 2A). The second peak at 9.45min (*m/z* 302, 304; Figure 2B) was confirmed as tyrindolinone, and two smaller peaks at 11.17min (*m/z* 255, 257) and 5.52min (*m/z* 336, 338) were tyrindoleninone and tyrindoxyl sulphate respectively.

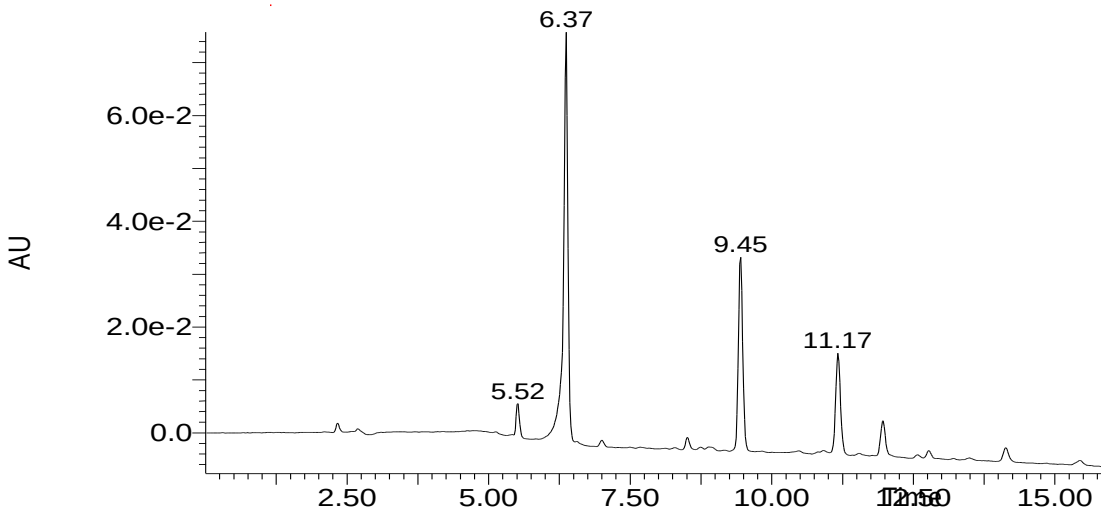


Figure 1: Liquid chromatography mass spectrometry analysis of the crude extract from the egg masses of the *D. orbita*. The chromatogram from the Diode array at 300/600nm shows a mixture of brominated indole compounds, major peak at t_R 6.37 minutes corresponds to the molecular mass of 6-bromoisatin. Smaller peaks at t_R 9.45, 11.17 and 5.52minutes correspond to the molecular mass of tyrindolinone, tyrindoleninone and tyrindoxyl sulphate respectively.

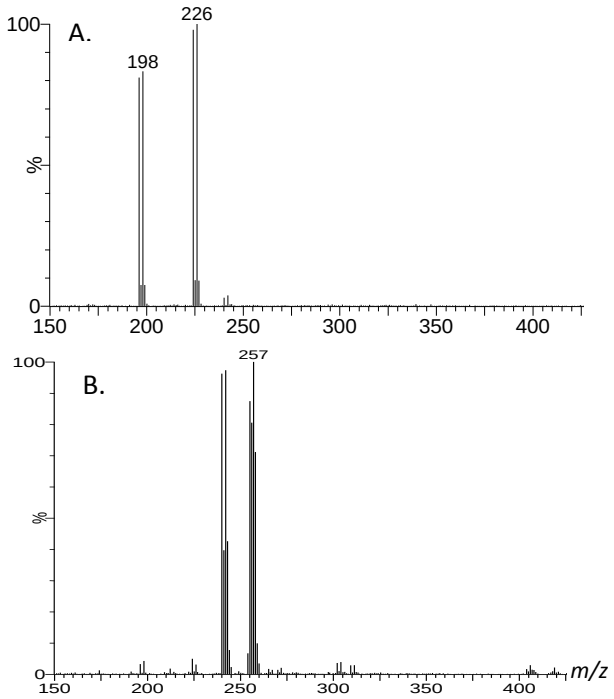


Figure 2: ESI mass spectrometry analysis of the two main peaks in the crude *D. orbita* sample 9; Figure 1 above). The major peak at t_R 6.37 minutes (A) corresponds to the molecular mass of 6-bromoisatin (*m/z* 224, 226). The second peak at t_R 9.45 minutes (B) corresponds to the molecular mass of tyrindolinone (*m/z* 302,304).

As expected the LC/MS results of the controls sterile water and 1% DMSO (Figure 3A & B) after 1h in a humidifed incubator at 37^{o}C did not reveal any brominated compounds. In contrast, four brominated indole peaks were identified in the LC/MS of the 1mg/mL crude extract (Figure 3C). The dominant peak at 6.4 minutes corresponded to 6-bromisatin confirmed by the m/z 224, 226. The smaller peaks at 12.00 minutes (*m/z* 463, 465, 467), 9.48 minutes (*m/z* 302, 304) and 5.52 minutes (*m/z* 336, 338) were tyriverdin, tyrindolinone and tyrindoxyl sulphate respectively. By 4h the dominant peak in the sample was once again 6-bromisatin by the m/z 224, 226. Two smaller peaks at 12.02 minutes (*m/z* 463, 465, 467) and 5.53 (*m/z* 336, 338) were confirmed as tyriverdin and tyrindoxyl sulphate respectively.

After 24h exposure, the dominant compound (Figure 4; 6.42 minutes) detected in the tissue culture plates by LC/MS was 6-bromoisatin (Figure 4 insert; m/z 224, 226). A smaller peak at 12.02 minutes was confirmed to be tyriverdin (*m/z* 463, 465, 467). This experiment has demonstrated that although tyrindoleneinone was in the crude sample (Figure 1) it is obviously degraded rapidly after only 1h at 37^{o}C. In contrast, 6-bromoisatin was detected even after 24h exposure. Therefore, *in vivo* tyrindoleninone would be readily oxidised to 6-bromoisatin.

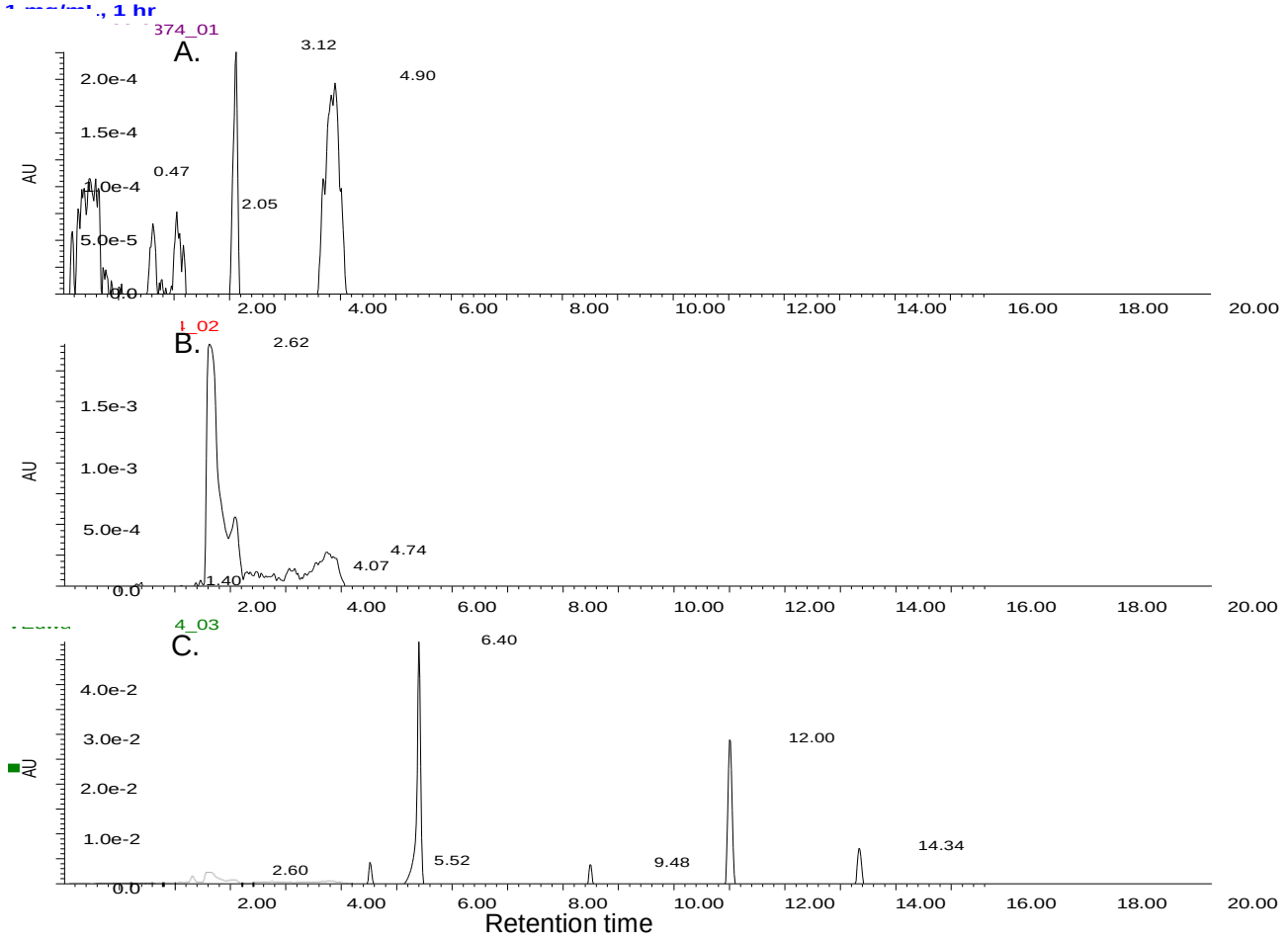


Figure 3: Chromatograms of liquid chromatography mass spectrometry analysis of (A) sterile water; (B) 1% DMSO control; (C) 1mg/ml crude *D. orbita* extract after 1h at 37°C + 5%CO_2. Four main brominated indole compounds were identified after 1h, the major peak at t_R 6.4 minutes corresponds to the molecular mass of 6-bromoisatin. Smaller peaks at t_R 12.00, 9.48 and 5.52 minutes correspond to the molecular mass of tyriverdin, tyrindolinone and tyrindoxyl sulphate respectively.

Appendix V: ELISA and RIA Standard Curves

Principle of Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA is a technique used to quantitate the amount of unknown antigen/hormone in a given sample (Brown, *et al.*, 2005). The unknown antigen competes for binding to a primary antibody, with a labelled antigen (of known quantity; Brown, *et al*., 2005). The primary antibody is coated to a microtitre plate and any excess is rinsed away (Brown, *et al.*, 2005). Then standards (0-4ng/ml), controls and samples (unlabelled antigen/hormone) are added, followed by a hormone specific enzyme conjugate (HRP-labelled), and the unlabelled and labelled antigen competes for binding to the antibody (Brown, *et al*., 2005). The plate is then washed and any unbound antigens are washed away. A coloured substrate is then added which binds to the enzyme conjugate only (i.e. the more colour the less hormone or there is an inverse relationship between the coloured substrate and hormone in samples, Brown, *et al*., 2005). The plate is read on a spectrophotometer to determine the optical densities of standards and samples at 405nm.

A standard curve (Figure 2) is then plotted against the maximum or total binding, and a slope of the curve can then be generated which is used to determine the concentration of antigen/hormone in the unknown samples. The ELISA assay is a fast, safe (no radioactive material) and reliable assay with the added advantage that a large number of samples can be tested in a given assay.

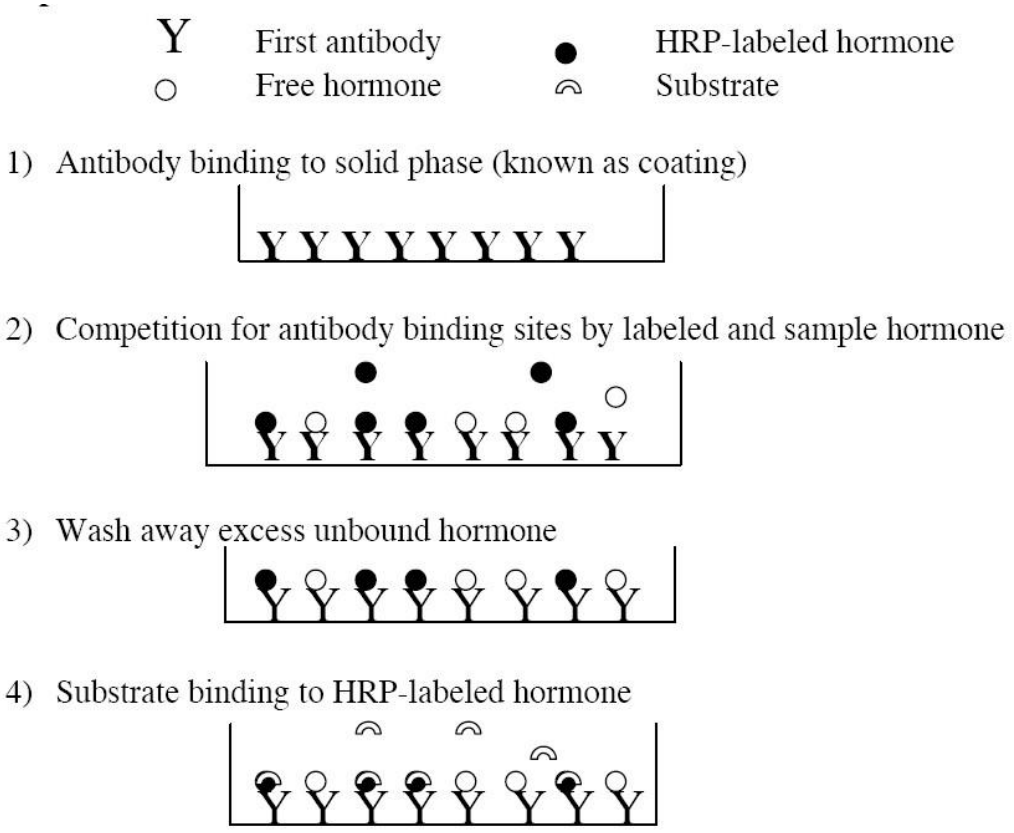


Figure 1: Principles of the single antibody ELISA (Brown, *et al.*, 2005).

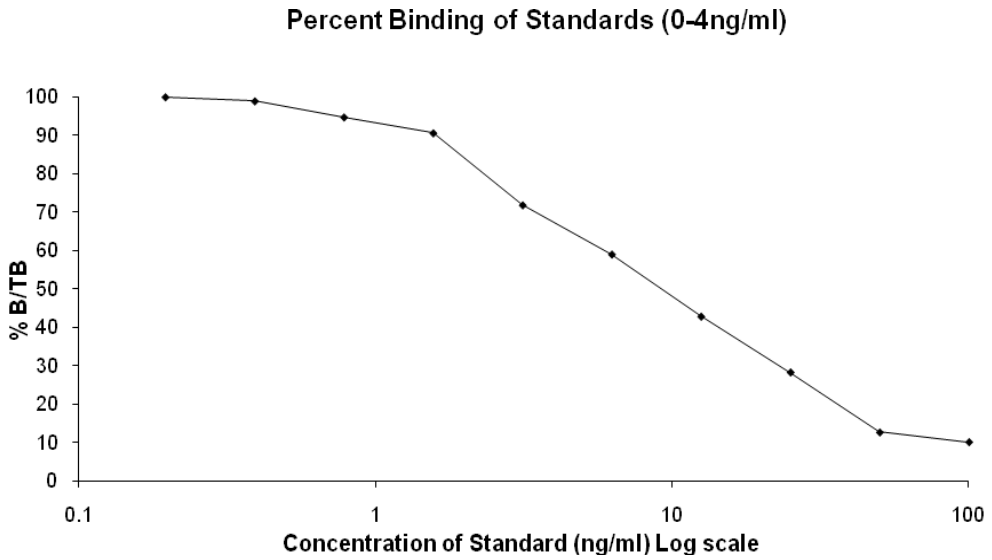


Figure 2: An example of an ELISA standard curve.

Principles of the Radioimmunoassay (RIA)

The RIA, like the ELISA is also a competitive antigen/antibody binding assay. A fixed concentration of radiolabelled antigen tracer is added to create a limited (50%) number of binding sites. Thus, when an unlabelled antigen is added with the radioactive labelled tracer there is a competition for binding to a primary antibody.

The experiment first involves adding standards, controls and unlabelled antigen (samples), followed by the labelled radioactive tracer to assay tubes. The primary antibody is then added and the unlabelled and labelled antigens compete for binding to the antibody (Figure 1). To separate the bound and free antigens a seconday antibody is added which binds to the antibody-antigen complex; this large complex is precipitated out and the bound radioactive portion is measured on a gamma machine. Like the ELISA the percent of antigen/hormone in unlabeled sample is directly proportional to the bound radioactive tracer. The standard curve is then plotted of the standard concentrations against percent binding and the concentration of antigen/hormone in samples can be calculated (Figure 4). The main disadvantage or drawbacks of the RIA in comparison to the ELISA is the radioactive material required can be a health hazard, and the expensive equipment (Gamma machines) required to detect the activity of the radioactive material.

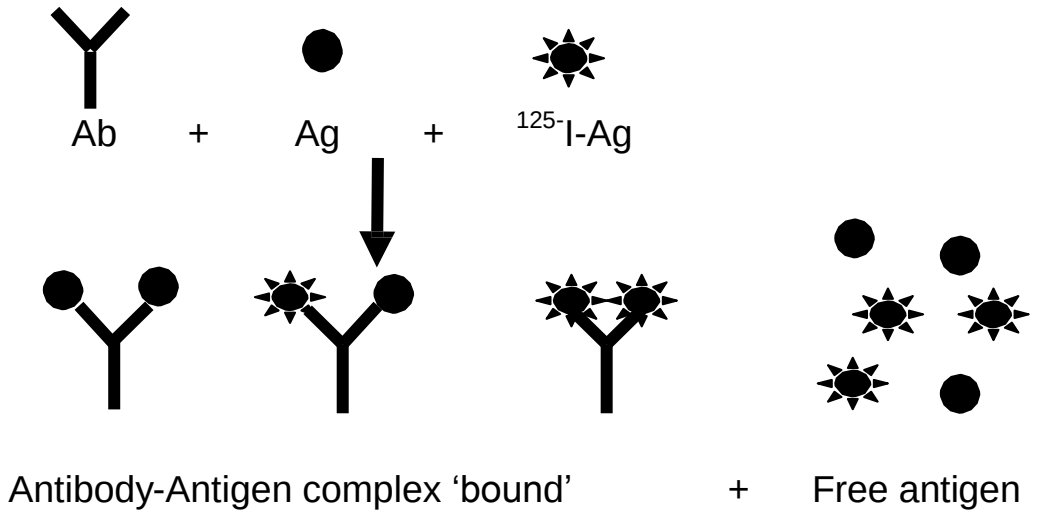


Figure 3: The principle of the radioimmunoassay (RIA). The unknown concentration of antigen (Ag) competes with the known concentration of radioactive antigen (^{125}I-Ag) for binding to the primary antibody (Ab). A secondardy antibody is added which binds to the large complex which is precipitated and any free antigen is rinsed away (Menadue, 2007).

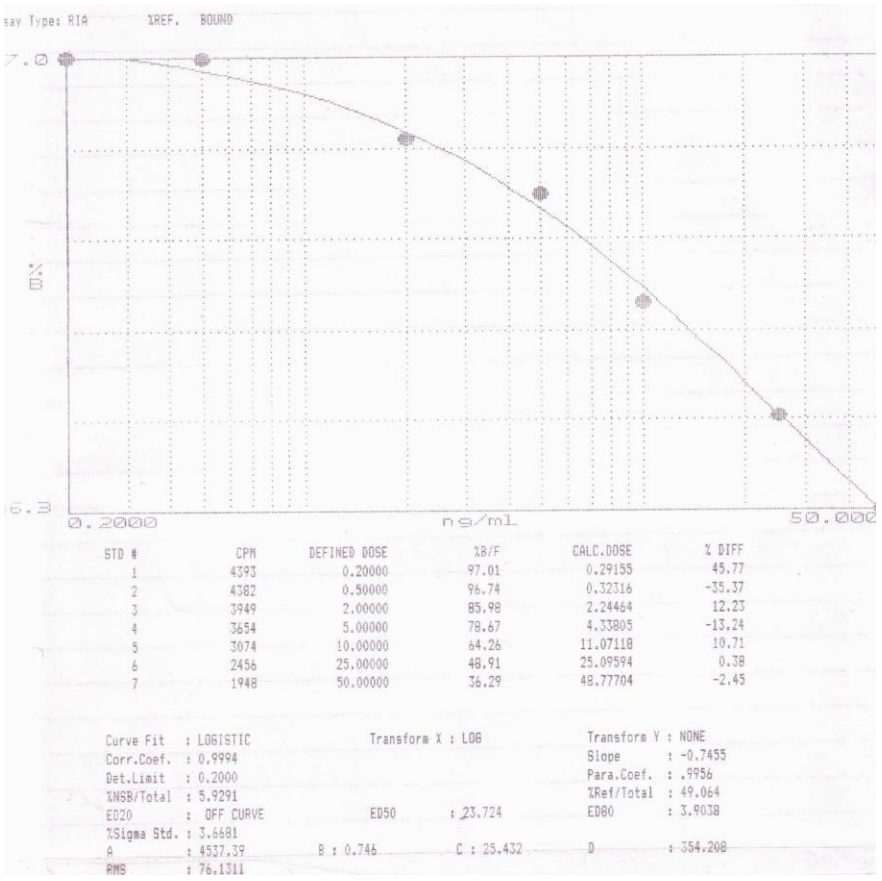


Figure 4: Example of an RIA standard curve

APPENDIX VI: Chapter 4 Supplementary Data

Introduction

In Chapter 4 the effects of the synthetic brominated indole compounds on primary-derived human granulosa cells were examined. The granulosa cells isolated from the follicular fluid donated by women undergoing IVF or ICSI, were segregated into two distinct groups and classified as either, fertile or infertile based on the womans infertility factor (Chapter 2 section 2.2.1.1 and Appendix II). Although each case was treated separately, the end results were pooled into either the fertile (n=7) or infertile (n=7) group (Chapter 4). Review of the results obtained in Chapter 4 suggested that it would be useful to split the data into each individual infertility factor. Therefore, to determine if the synthetic compounds had specific effects on different infertility groups, the data was analysed as follows.

Methods

Primary-derived human granulosa cells were isolated from the follicular aspirates donated by 14 women undergoing IVF or ICSI treatments at the Flinders Medical centre with their informed consent (number 260/067; Appendix II). Donated aspirates were divided into two groups;fertile (cases 1, 5, 6, 7, 8, 9 & 10) and infertile (cases 2 & 19 ovarian cyst; cases 3 & 17 idiopathic; case 4 endometriosis and cases 18 and 19 PCOS) under the criteria previously specified (section 2.2.1.1; Appendix II

Results and Discussion

Controls

Cell viability decreased in the media control and the ovarian cyst group at 48 and 72h and in the idiopathic group at 72h (Figure 1A & D), relative to the 24h incubation, whereas there was no decrease in cells in the endometrosis group after 72h (Figure 1C). In comparison, cell numbers were higher after 48 and 72h compared to 24h in the PCOS group with the media control, indicating the cells were still proliferating (Figure 1B). The PCOS group produced 10x more progesterone after 48 and 72h than either the endometriosis or idiopathic groups (Figure B, C &D) and 5x more than the ovarian cyst group (Figure 2A) with media only. At all incubation periods, the PCOS group produced more estradiol than the other three groups (Figure 3) with media only.

The DMSO control decreased cell numbers in the ovarian cyst group at 48 and 72h and in the idiopathic and endometriosis groups at 72h (Figure 1A, C & D) relative to the media control. In comparison cell numbers increased after 48 and 72h with 1% DMSO in the PCOS group in a similar pattern to the media control suggesting the cells had not differentiated (Figure 1B) and were not affected by the DMSO. In comparison to the media control, 1% DMSO inhibited progesterone synthesis by the ovarian group after 72h (Figure 2A) and in the PCOS group after 24 and 48h, but not at 72h (Figure 2B). There was a slight inhibition of estradiol synthesis by granulosa from the ovarian cyst group and PCOS group after 48h, when treated with 1% DMSO (Figure 3A & B). The 1% DMSO greatly increased estradiol synthesis by the endometriosis group after 24h (Figure 3C) however, the replicates were inconsistent.

Furthermore, as this data was based on cells isolated from one woman statistical significance could not be determined.

The no serum control did not affect cell viability in the PCOS group, however the cells did not proliferate at 48 and 72h (Figure 1B), as they did with the media and DMSO. In contrast, there was a time-dependent decrease in cell numbers in the three other infertile groups when treated for 24, 48 and 72h in the absence of serum (Figure 1A, C & D) relative to the respective media controls. There was an inhibition of progesterone by the PCOS group when treated with no serum (Figure 2B), suggesting that these cells require precursors (such as lipids) from the serum for both progesterone synthesis and cell maintainence. The three other infertile groups (ovarian cyst, idiopathic and endometriosis) produced negligible amounts of progesterone so there no distinct pattern or decrease in progesterone synthesis in the absence of serum (Figure 2A, C & D). Estradiol synthesis was inhibited in all groups in the absence of serum after 24h. Estradiol synthesis was also inhibited in the ovarian cyst and PCOS group at 48h and in the ovarian cyst group after 72h, in the absence of serum (Figure 3). However, at 72h granulosa cells from the PCOS group synthesised a similar amount of estradiol in the presence or absence of serum.

Indirubin

Indirubin increased cell numbers in the ovarian cyst group after 72h treatment at 0.01, 0.1, 1 and 10µg/ml (Figure 1A). After 48h cell viability was reduced in the PCOS group at all concentrations of indirubin and at 0.01 and 0.1µg/ml after 72h (Figure 1B). However, at the highest concentration of indirubin (100µg/ml) there

was a slight increase in cells in the PCOS group after 72h (Figure 1B). Indirubin also stimulated cell proliferation after 72h at 0.01, 1, 10 and 100µg/ml (Figure 1D). Progesterone synthesis by granulosa cells from the ovarian cyst group was stimulated at all concentrations at 24 and at 100µg/ml at 48h of treatment with indirubin (Figure 2A). In comparion, indirubin inhibited progesterone synthesis by the PCOS group after 24, 48 and 72h at all concentrations (Figure 2B). Progesterone synthesis was stimulated after 24h in the endometriosis group at all concentration of indirubin and after 72h at 100µg/ml (Figure 2C). There was no effect on progesterone synthesis by the idiopathic group when treated with indirubin at any incubation period (Figure 2D).

Indirubin had no effect on estradiol synthesis in the ovarian cyst group (Figure 3A). In comparison, there was a slight inhibition of estradiol by the PCOS group after 48h treatment with indirubin (Figure 3B). Indirubin inhibited estradiol synthesis by the endometriosis group after 24h at all concentrations (but only relative to the outlying DMSO control) and after 72h at 0.1, 1, 10 and 100µg/ml (Figure 3C). Estradiol synthesis was slightly stimulated by the idiopathic group after 72h at 0.01, 10 and 100µg/ml of indirubin (Figure 3D).

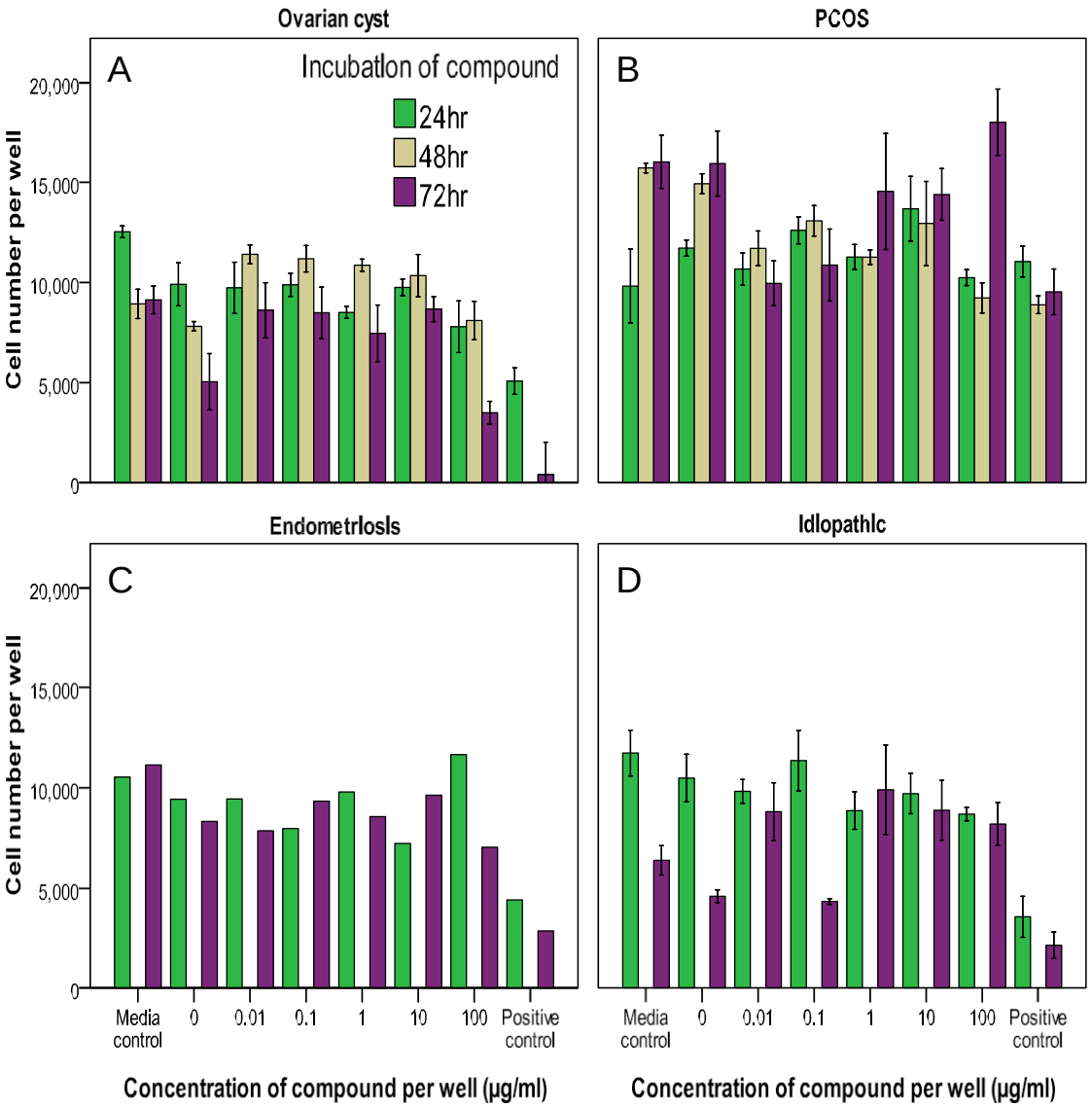


Figure 1: The effect of indirubin on primary-derived human granulosa cell viability from cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with indirubin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm.

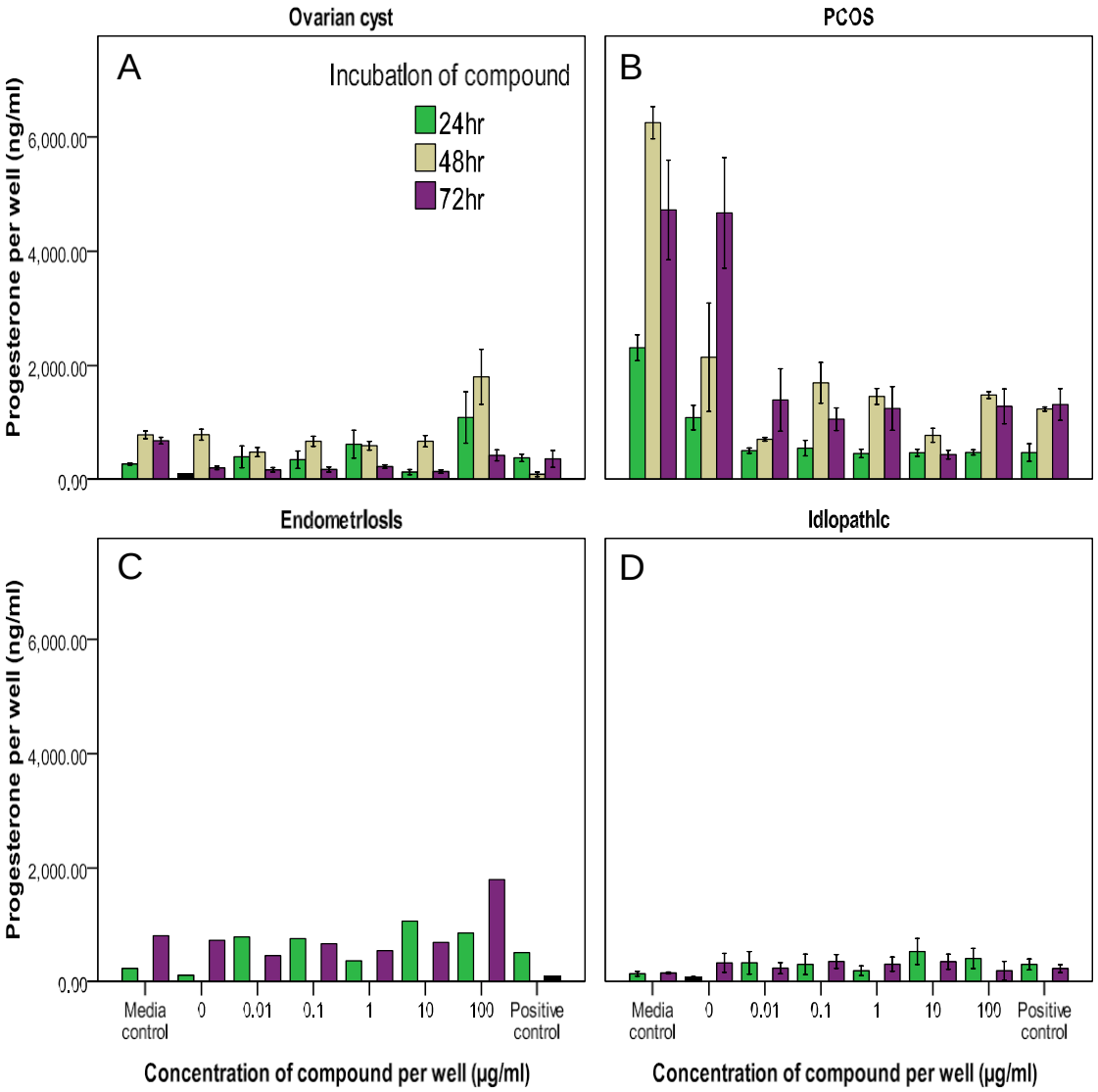


Figure 2: The effect of indirubin on progesterone synthesis by primary-derived human granulosa cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with indirubin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Progesterone synthesis was determined by the radioimmunoassay.

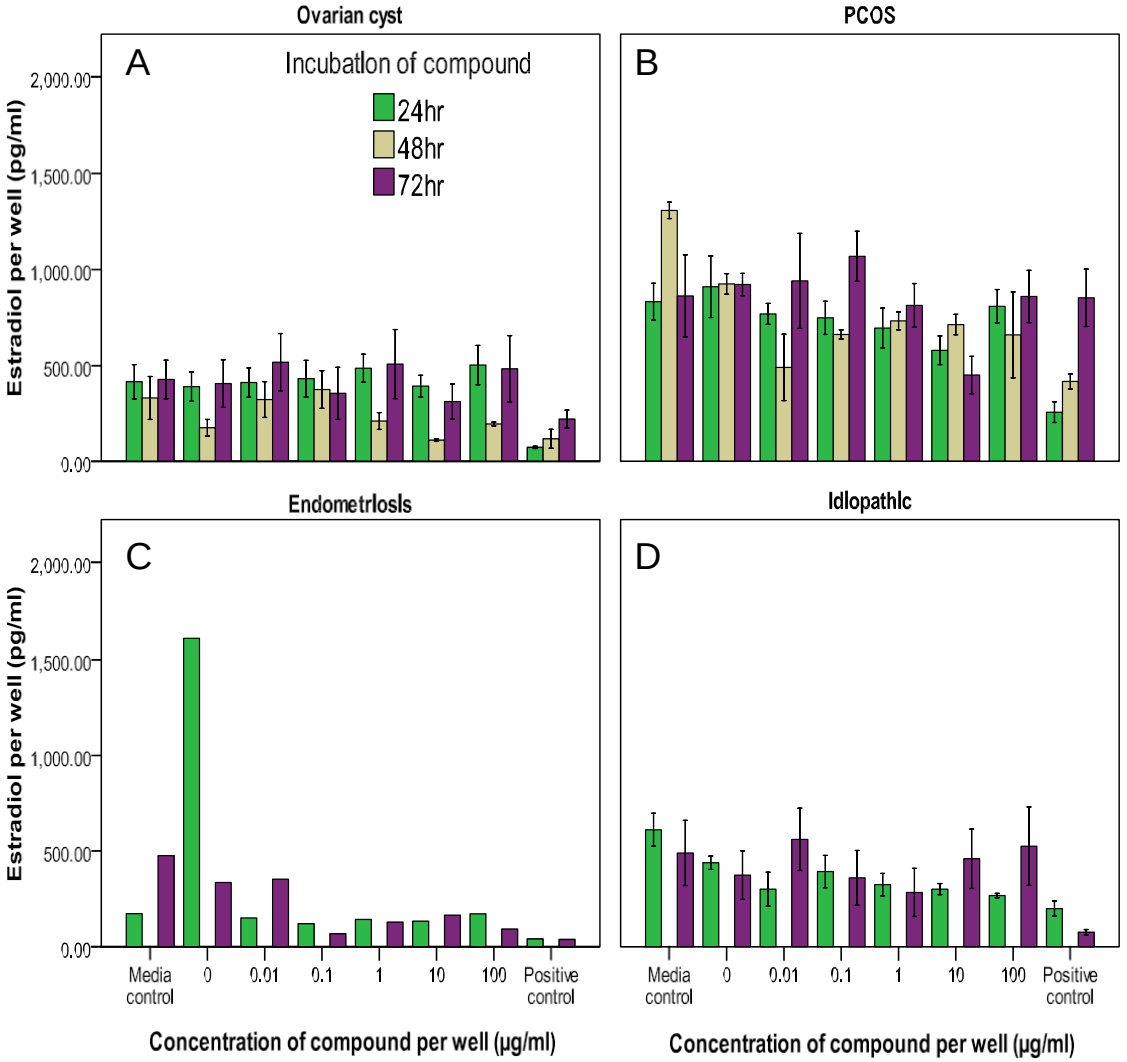


Figure 3: The effect of indirubin on estradiol synthesis by primary-derived human granulosa cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with indirubin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Estradiol synthesis was determined by the radioimmunoassay.

5-Bromoisatin

5-bromoisatin stimulated cell proliferation in the ovarian cyst group after 24 and 48h at 0.01, 0.1 and 1µg/ml (Figure 4A). In the PCOS group 5-bromoisatin was cytotoxic at 10 and 100µg/ml at all incubation periods (Figure 4B). There was an increase in cell numbers from the endometriosis group at 0.01µg/ml of 5-bromoisatin after 72h however, at 100µg/ml cell numbers decrease considerably at 24 and 72h (Figure 4C). 5-bromoisatin (100µg/ml) also decreased cell numbers in the idiopathic group at 24 and 72h treatment (Figure 4D).

Progesterone synthesis was inhibited by the ovarian cyst, endometrosis and idiopathic group at 100µg/ml of 5-bromoisatin and all incubation periods (Figure 5A, C & D). 5-bromoisatin stimulated progesterone synthesis in granulosa cells from the PCOS group at 0.01µg/ml after 48h, but inhibited it at 0.1, 1, 10 and 100µg/ml after 48 and 72h treatment and at 1, 10 and 100µg/ml after 24h treatment (Figure 5B). Progesterone synthesis by granulosa cells from the endometriosis and idiopathic groups was also stimulated by ≤ 10µg/ml of 5-bromoisatin after 24h (Figure 5C & D).

5-bromoisatin stimulated estradiol synthesis slightly in the ovarian cyst group at 0.01µg/ml after 72h, but inhibited it at 100µg/ml at all incubation periods tested (Figure 6A). There was a dose-dependent decrease in estradiol synthesis by the PCOS group when treated with 5-bromoisatin after 24 and 48h. In comparison, estradiol was only inhibited at 100µg/ml of 5-bromoisatin after 72h in the PCOS group (Figure 6B). Estradiol synthesis was inhibited in the endometriosis group at 24h and all concentrations of 5-bromoisatin and at 100µg/ml after 72h (Figure 6C).

5-bromoisatin also inhibited estradiol synthesis by the idiopathic group after 24 and 72h at 10 and 100µg/ml (Figure 6D).

In conclusion, the effects of the brominated synthetic compounds, indirubin and 5-bromoisatin, were generally fertility, concentration and exposure specific. Indirubin stimulated progesterone in granulosa cells from the ovarian cyst and endometriosis groups however it inhibited progesterone in cells from the PCOS group, and had no effect on cells from the idiopathic group. In comparison, indirubin stimulated estradiol in granulosa cells from the idiopathic group at longer exposure periods, but inhibited it in cells from the PCOS group, and had no effect on granulosa cells from the ovarian cyst group. Progesterone synthesis was also upregulated in granulosa cells from the endometriosis, PCOS and idiopathic groups when exposed to 5-bromoisatin at low concentrations and shorter exposure periods. In comparison, estradiol synthesis by granulosa cells from all groups except the ovarian cyts group, was seen to decrease in the presence of 5-bromoisatin. Therefore, these results suggest that the synthetic compounds, indirubin and 5-bromoisatin regulate steroidogenesis or, effect hormone synthesis in a variety of ways which are infertility specific. It is therefore probably that with further research they may have applications in specific gynaecological disorders.

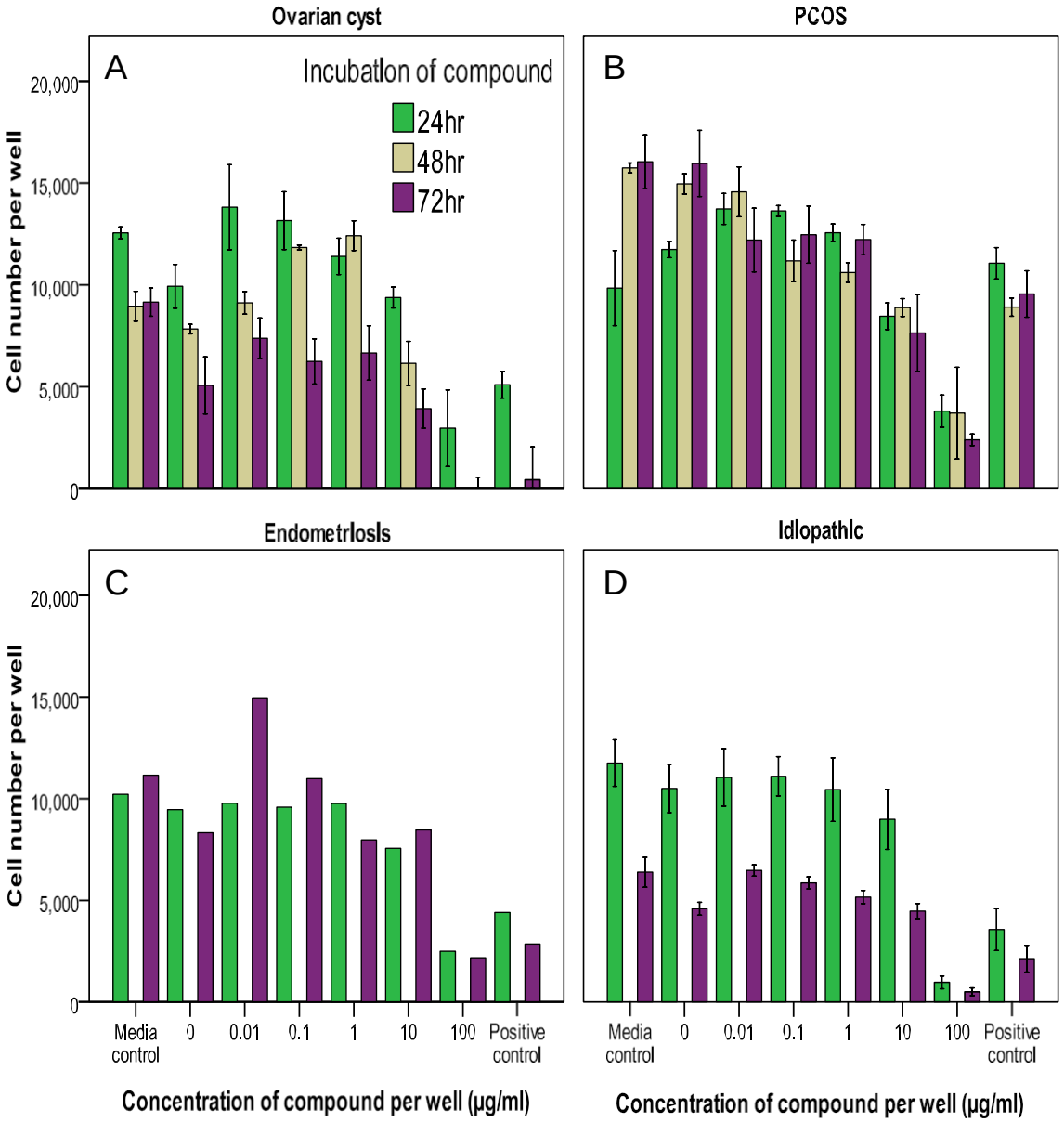


Figure 4: The effect of 5-bromoisatin on primary-derived human granulosa cell viability from cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with 5-bromoisatin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm.

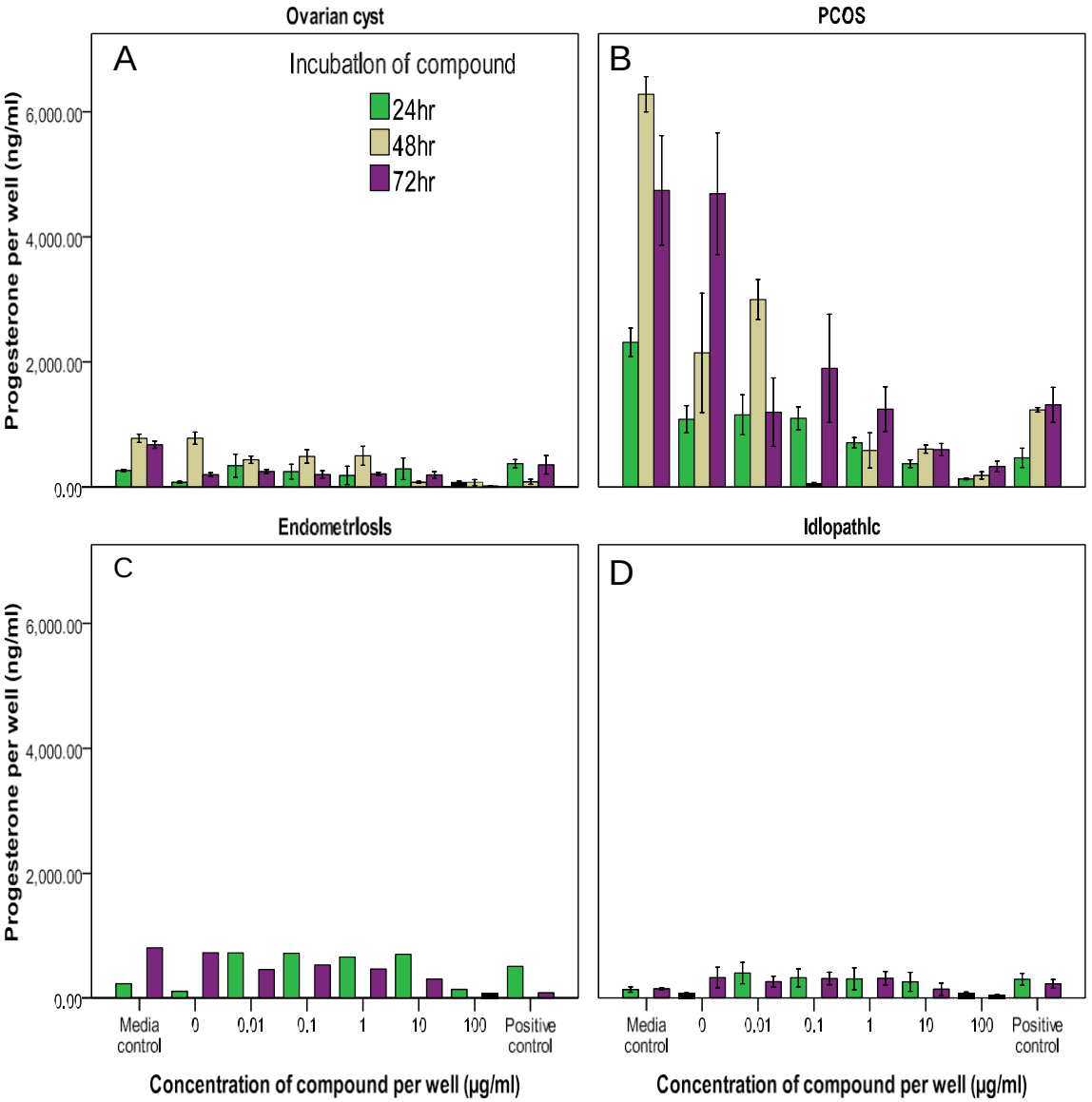


Figure 5: The effect of 5-bromoisatin on progesterone synthesis by primary-derived human granulosa cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with 5-bromoisatin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Progesterone synthesis was determined by the radioimmunoassay. (Ovarian cyst n=2; PCOS n= 2; endometriosis n=1 and idiopathic n=2).

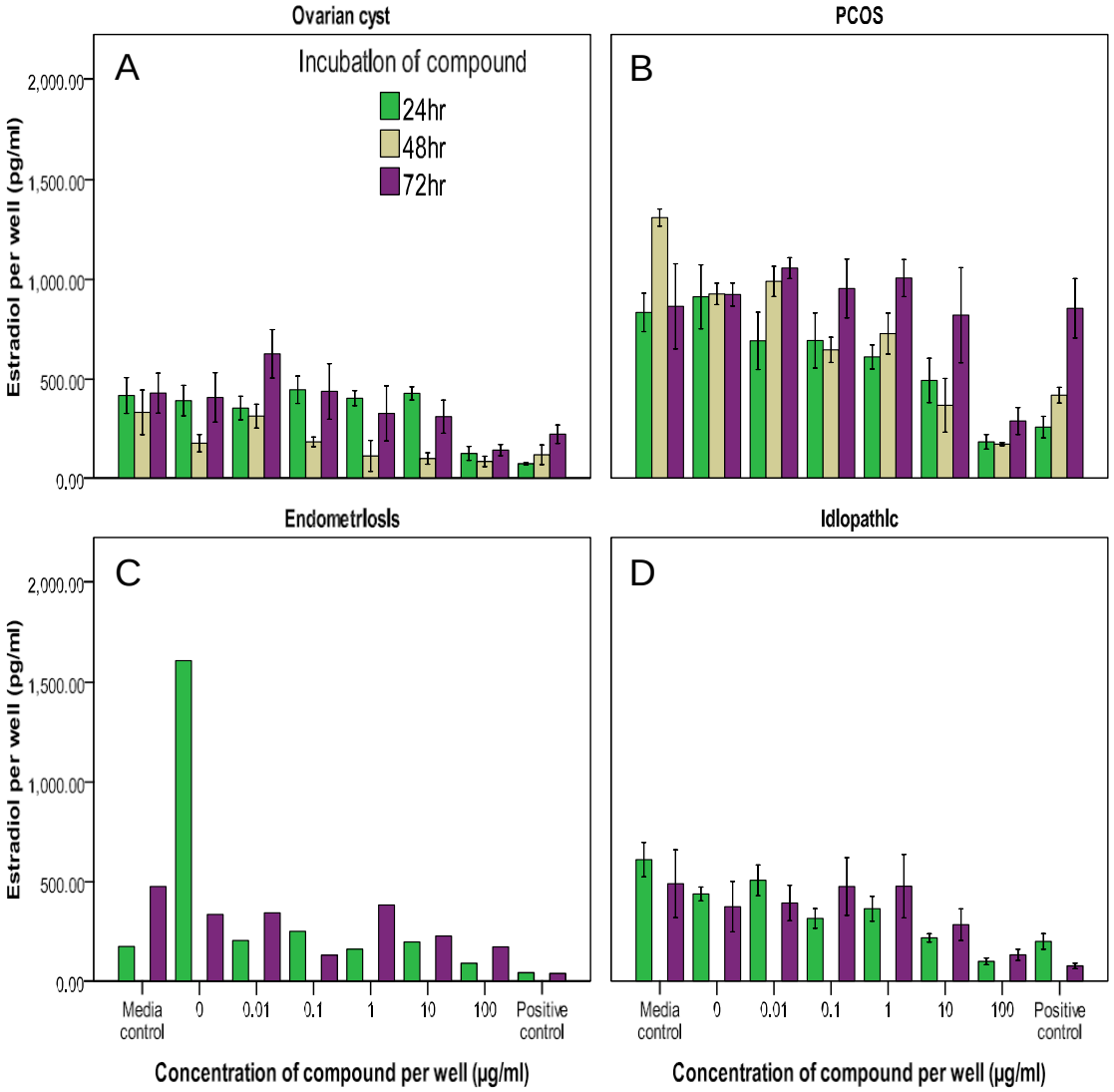


Figure 6: The effect of 5-bromoisatin on estradiol synthesis by primary-derived human granulosa cells donated by women with an infertility factor caused by ovarian cysts (A; n=2), PCOS (B; n=2), endometriosis (C; n=1) and idiopathic infertility (D; n=2). After an initial 24h cell attachment period granulosa cells (10,000cells/well) were treated with 5-bromoisatin (0.01-100µg/ml) for 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment in the absence of foetal bovine serum (FBS). Estradiol synthesis was determined by the radioimmunoassay.

APPENDIX VII: Chapter 7 Supplementary Data

Introduction

Chapter 7 examined the effects of the semi-purified *D. orbita* fractions on primary-derived human granulosa cells. For the purpose of the thesis the granulosa cells isolated from the follicular fluid, donated by women undergoing IVF or ICSI, were segregated into two distinct groups and classified either, fertile or infertile based on the womans infertility factor (Chapter 2 section 2.2.1.1 and Appendix II). Although, each case was treated separately, the end results were pooled and analysed for fertile and infertile groups as presented in Chapter 7. Based on the results in Chapter 7, the question was raised as to whether the results of the infertile women should not have been pooled together, as this may have obscured trends due to variation according to fertility. Therefore, to determine the effects of the 'semi-purified *D. orbita* fractions on cells from specific infertility factors, the data from Chapter 7 was divided into the separate cohorts of infertility and presented separately.

Methods

Primary-derived human granulosa cells were isolated from the follicular aspirates donated by 10 women undergoing IVF or ICSI treatments at the Flinders Medical centre with their informed consent (number 260/067; Appendix II). Donated aspirates were divided into two groups; fertile (cases 27, 28 & 31) and infertile (cases 25, 29 & 34 idiopathic; cases 30 & 37 endometriosis; cases 35 & 36) under the criteria previously specified (section 2.2.1.1; Appendix II). Granulosa cells were isolated from the follicular aspirates (section 2.1.7.3) and cell number was determined by the trypan blue exclusion assay (section 2.2.2.3). Cells were plated and treated with the semi-purified *D. orbita* fractions as described in Chapter 7.

Results and Discussion

Untreated Cells

Cell numbers increased in the endometriosis group up to 48h without treatment (media control) indicating the cells were still proliferating and had not lutenized (Figure 1B). After 72h there was a decrease in cell proliferation in the endometriosis group. In comparison, granulosa cells from both the PCOS and idiopathic groups did not proliferate in the absence of treatment compared to the control (Figure 1A & C). The 1% DMSO control had no effect on cell numbers in all three groups, however there was a slight proliferation in the endometriosis group after 48 and 72h, compared to the other two groups (Figure 1B).

After 4h the granulosa cells from the idiopathic group synthesized the lowest amount of basal progesterone (Figure 1F) and after 24 and 48h, granulosa cells from the endometriosis group synthesized the most progesterone (Figure 1E).

Granulosa cells from the PCOS group produced the least amount of estrogen overall (Figure 1G). After 4h granulosa cells from the endometriosis group produced the highest quantity of estrogen (Figure 1H). After 24h the idiopathic group produced 3 times more estradiol than the endometriosis group whereas at 48h both the endometriosis and idiopathic group produced similar amounts of basal estradiol (Figure 1H & I).

In the presence of hCG, endometriosis but not PCOS or idiopathic-derived granulosa cells proliferated at 48 and 72h, compared to the 1% DMSO control (Figure 2A, B & C). After 4h, hCG did not increase progesterone synthesis in any of the groups

(Figure 2D, E & F), although after 24 and 48h, hCG-stimulated granulosa cells from both the endometriosis and idiopathic groups synthesized similar quantites of progesterone, which were higher than basal production. The PCOS group however, produced much less progesterone in the presence of hCG than the other two groups (Figure 2D, E &F), implying that the PCOS granulosa cells had not developed LH/CG receptors.

At 4h, hCG-stimulated granulosa cells from the endometriosis group produced the least amount of estradiol (Figure 2H). At 24h granulosa cells from the idiopathic group synthesized more than double the amount of hCG-stimulated estradiol, than either the PCOS or the endometriosis groups (Figure 2I). However, at 48h there was a definite decrease in hCG-stimulated estradiol by cells from the idiopathic group in comparison to the control (Figure 2I).

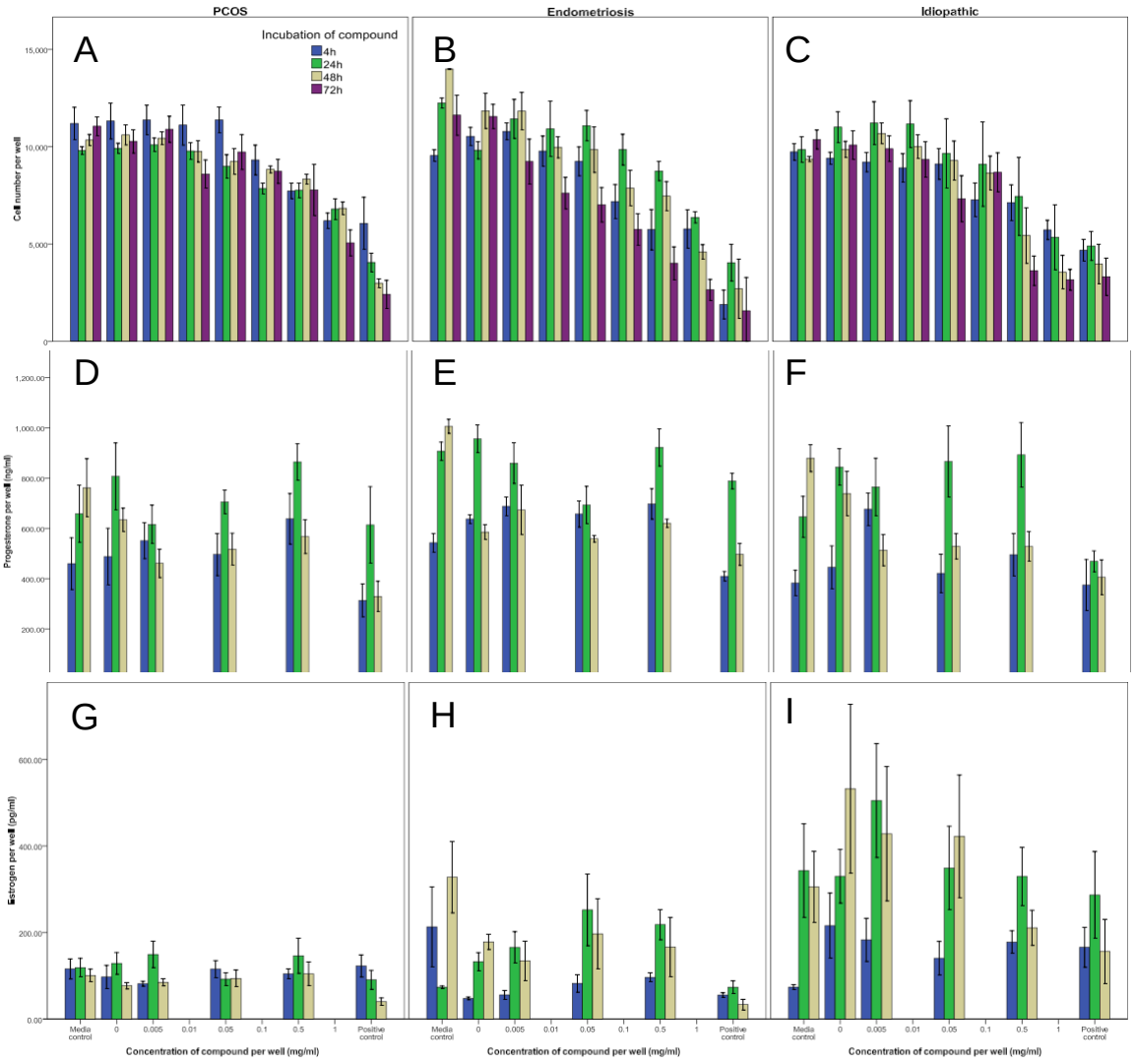
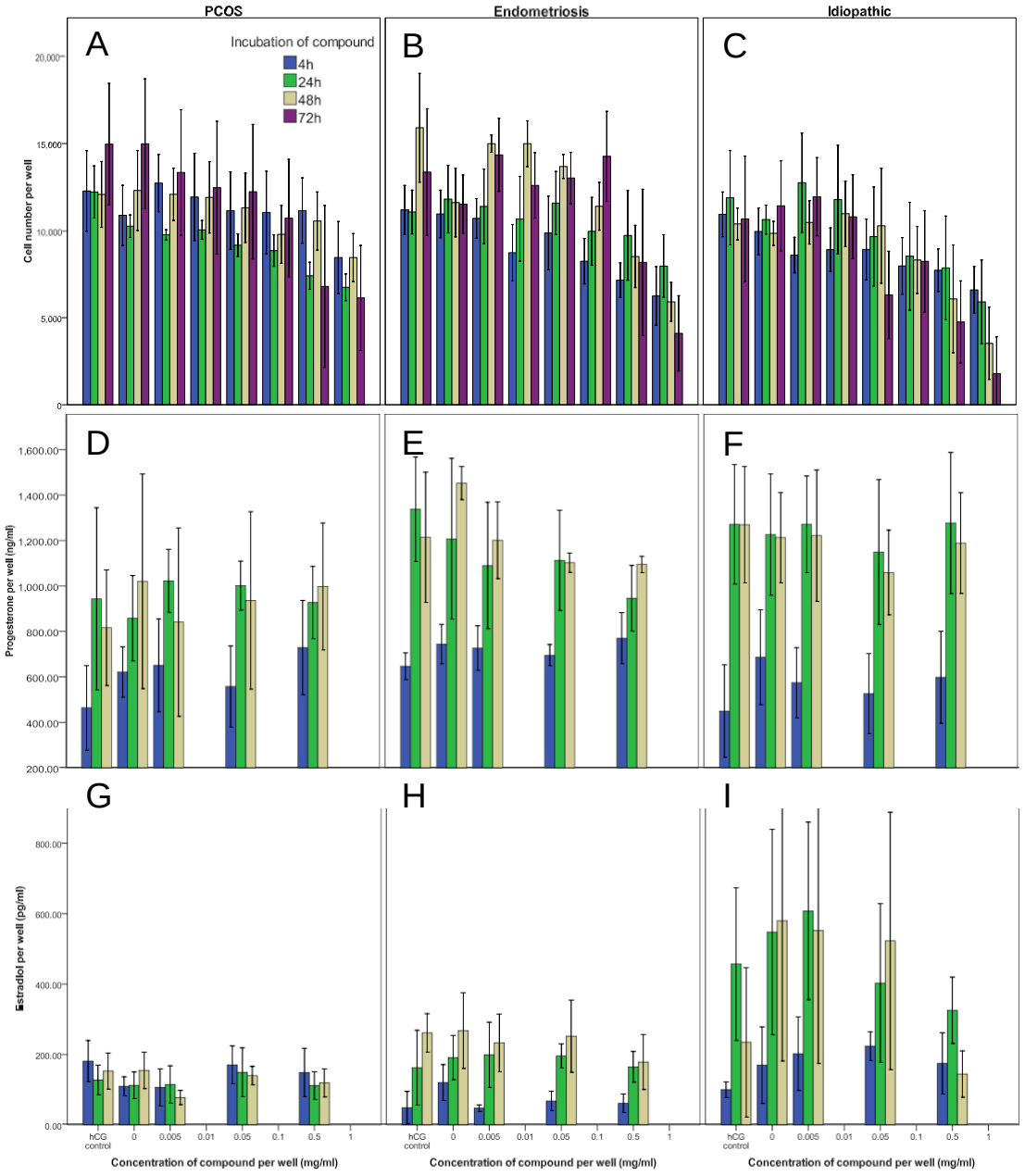


Figure 1: The effect of the semi-purified *D. orbita* fraction one on primary-derived human granulosa cell viability, progesterone and estradiol from cells donated by women with an infertility factor caused by polycystic ovarian syndrome (A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction one (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).



human granulosa cell viability, progesterone and estradiol in the presence of hCG from cells donated by women with an infertility factor caused by polycystic ovarian syndrome (A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction one (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).

Semi-purified *D. orbita* Fraction One

Cell numbers decreased in a dose-dependent and time-dependent manner in the PCOS and endometriosis cell groups when exposed to the *D. orbita* semi-purified fraction one without hCG (Figure 1A and B). In comparison, cells from the idiopathic group were only affected by fraction one at concentrations, 0.05mg/ml at 4h and at ≥ 0.1mg/ml longer than 24h exposure (Figure 1C). Fraction one only decreased basal progesterone synthesis by the granulosa cells from the idiopathic group at 48h (Figure 1F). Basal estradiol synthesis was stimulated at 0.005mg/ml (the lowest concentration tested) by cells of the idiopathic group at 24h, however, after 48h estradiol synthesis decreased (Figure 1I).

Fraction one in the presence of hCG stimulated granulosa cells from the endometriosis group after 48 at 0.005, 0.01 and 0.05mg/ml (Figure 2B). However after 0.5mg/ml there was a decrease in cell numbers in all three groups (Figure 2A, B & C). hCG-stimulated progesterone synthesis decreased slightly in granulosa cells from the endometriosis group as the concentration of fraction one increased (Figure 2E), whereas there was no effect on progesterone synthesis by granulosa cells from either the PCOS and idiopathic groups. Even though cell number decreased in both the PCOS and endometriosis group when treated with fraction one in the presence of hCG, there was no decrease in estradiol synthesis by these cells at any time or concentration tested (Figure 2G and H). There was a decrease in hCG-stimulated estradiol synthesis by the granulosa cells from the idiopathic group at the highest concentration tested (0.5mg/ml) after 24 and 48h (Figure 2I).

Semi-purified *D. orbita* Fraction Two

Granulosa cell number from the PCOS and endometriosis groups decreased when treated with semi-purified fraction two (0.1, 0.5 and 1mg/ml) without hCG at all time points tested (Figure 3A and B), whereas cells from the idiopathic group were affected by fraction two at ≥ 0.05mg/ml (Figure 3C). Progesterone synthesis by granulosa cells from the PCOS and idiopathic groups was inhibited by fraction two at 0.5mg/ml after 24 and 48h (Figure 3D and F), but at 0.05 and 0.5mg/ml in the endometriosis group (Figure 3E). Fraction two without hCG stimulated estradiol by granulosa cells in the PCOS and endometriosis group at the lowest concentration tested (0.005mg/ml) after 24 and 48h (Figure 3G and H). In contrast, estradiol synthesis was inhibited after 48h by granulosa cells of the idiopathic group at all concentrations of fraction two in the absence of hCG (Figure 3I).

Granulosa cells from the endometriosis group were stimulated slightly at 0.005 and 0.01mg/ml of fraction two in the presence of hCG after 48h exposure (Figure 4B). There was a decrease in cell numbers from the PCOS and endometriosis groups when treated with fraction two in the presence of hCG at 0.5 and 1mg/ml and in the idiopathic group at 0.05mg/ml at all time periods tested (Figure 4A, B & C). hCG-stimulated progesterone synthesis increased in cells from the PCOS group after 24h when treated with fraction two (Figure 4G). Exposure to 0.5mg/ml of fraction two decreased progesterone synthesis by granulosa cells from all three groups after 24 and 48h in the presence of hCG (Figure 4D, E & F). hCG-stimulated estradiol synthesis by granulosa cells decreased in the endometriosis and idiopathic group at 0.5mg/ml of fraction two at all time points (Figure 4I).

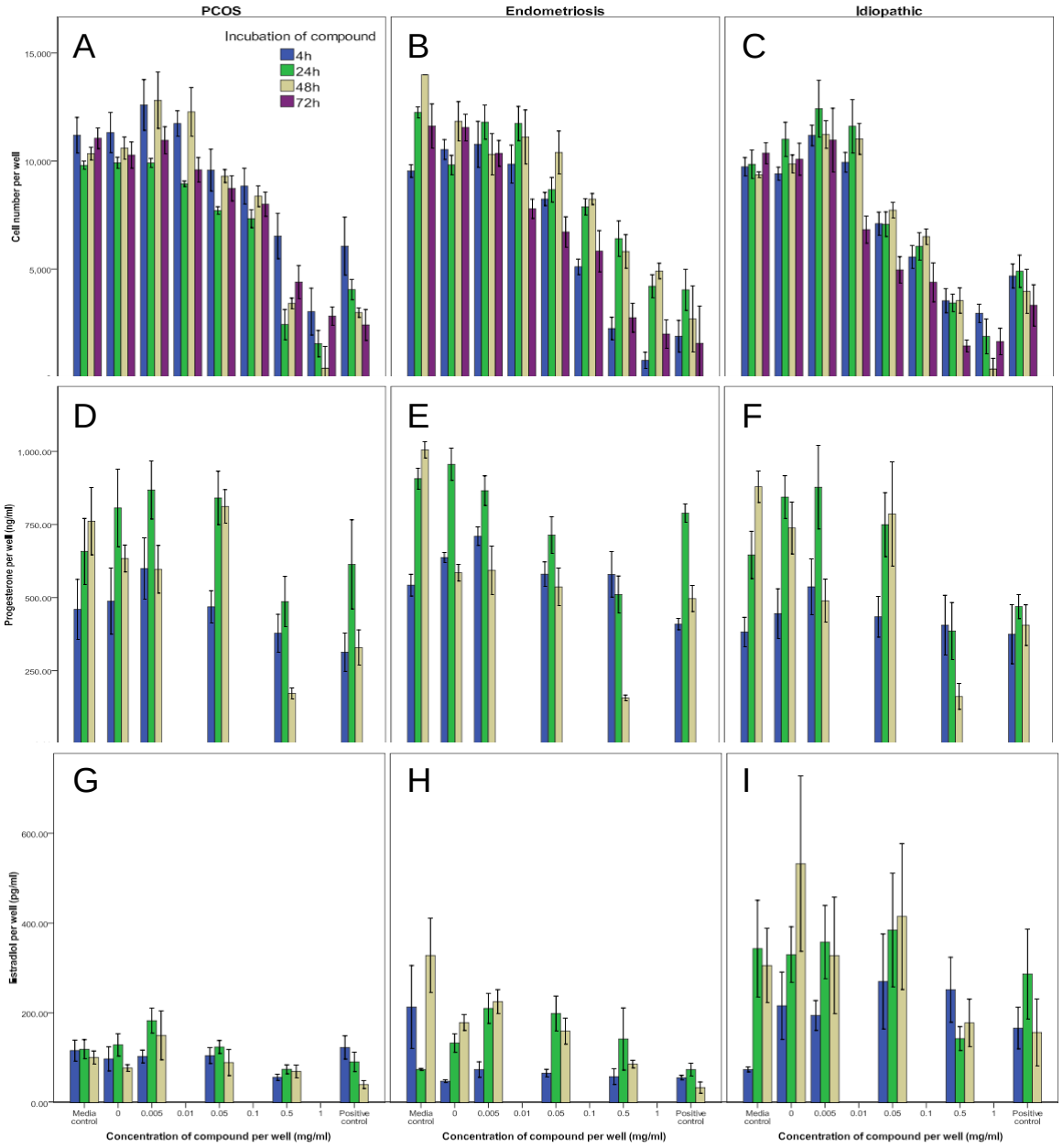


Figure 3: The effect of the semi-purified *D. orbita* fraction two on primary-derived human granulosa cell viability, progesterone and estradiol from cells donated by women with an infertility factor caused by polycystic ovarian syndrome (A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction two (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).

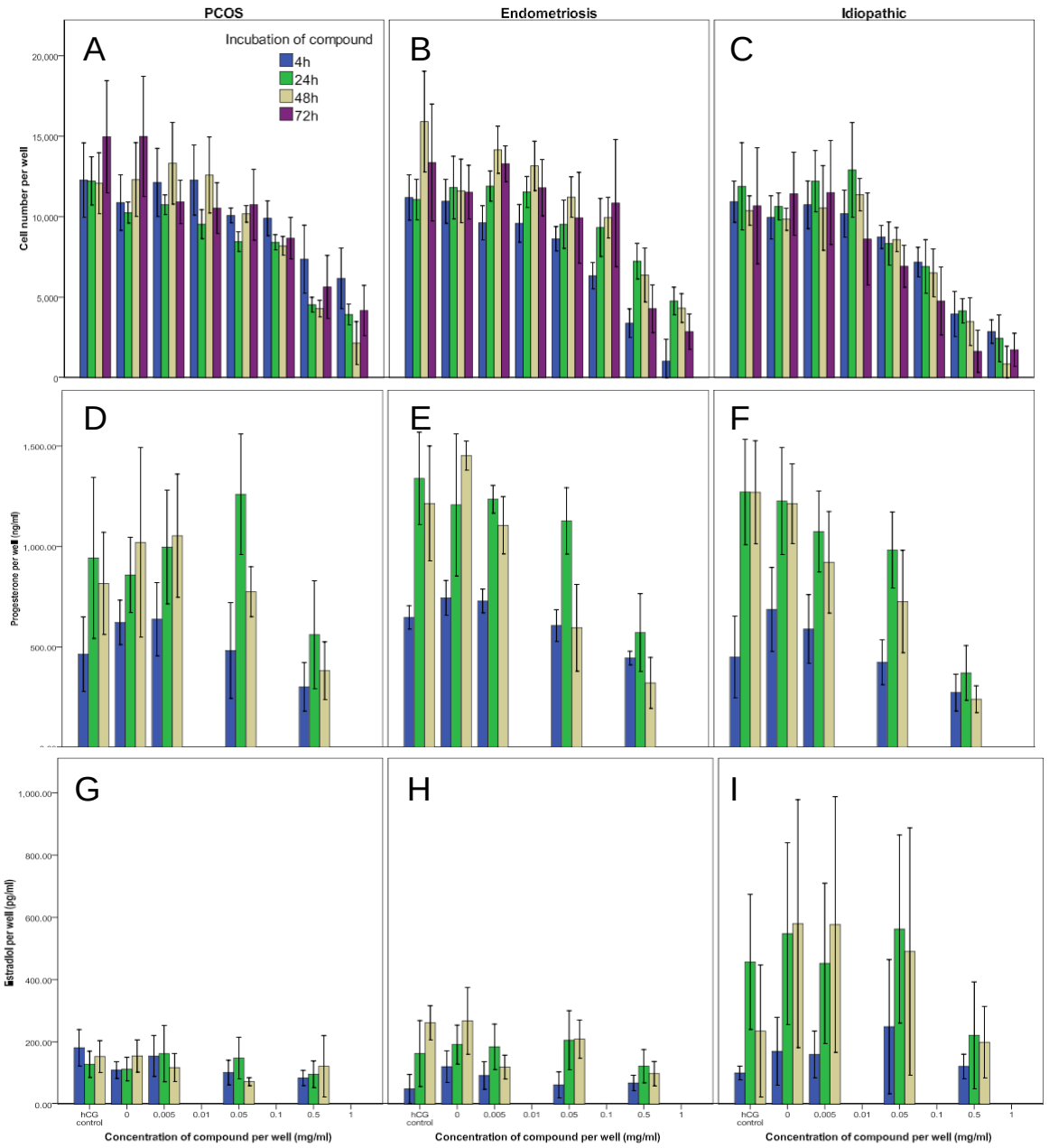


Figure 4: The effect of the semi-purified *D. orbita* fraction two on primary-derived human granulosa cell viability, progesterone and estradiol in the presence of hCG from cells donated by women with an infertility factor caused by polycystic ovarian syndrome (A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction two (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).

Semi-purified *D. orbita* Fraction Three

There was a slight increase in endometriosis-derived granulosa cells after treatment with the semi-purified *D. orbita* fraction three after 24, 48 and 72h at the lowest concentration tested (Figure 5B). Otherwise cell numbers decreased in a dose-dependent and time dependent manner in all three groups when treated with fraction three in the absence of hCG (Figure 5A, B & C). Basal progesterone was neither inhibited nor stimulated in the presence of fraction three without hCG from granulosa cells from all three infertile groups (Figure 5D, E and F). Fraction three did inhibit estradiol synthesis by granulosa cells from the endometriosis and idiopathic groups at 0.5mg/ml after 24 and 48h (Figure 5H and I), but had no effect on granulosa cells from the PCOS group (Figure 5G).

In the presence of hCG, fraction three stimulated granulosa cells from the endometriosis group after 72h at 0.005, 0.01, 0.05 and 0.1mg/ml (Figure 6B). In comparison, granulosa cell numbers from both the PCOS and idiopathic group decreased over time as the concentration of fraction three increased in the presence of hCG (Figure 6A & C). Progesterone synthesis by granulosa cells of the PCOS group increased at 0.005mg/ml after 48h in the presence of hCG (Figure 6D). Granulosa cells from the endometriosis and idiopathic groups synthesised less progesterone in the presence of fraction three with hCG after 24 and 48h (Figure 6E & F). hCG-stimulated estradiol synthesis by granulosa cells from the PCOS was not affected by the presence of fraction three (Figure 6G). In contrast, fraction three in the presence of hCG inhibited estradiol synthesis by the endometriosis and idiopathic groups after 24 and 48h, but had no effect at 4h (Figure 6H & I).

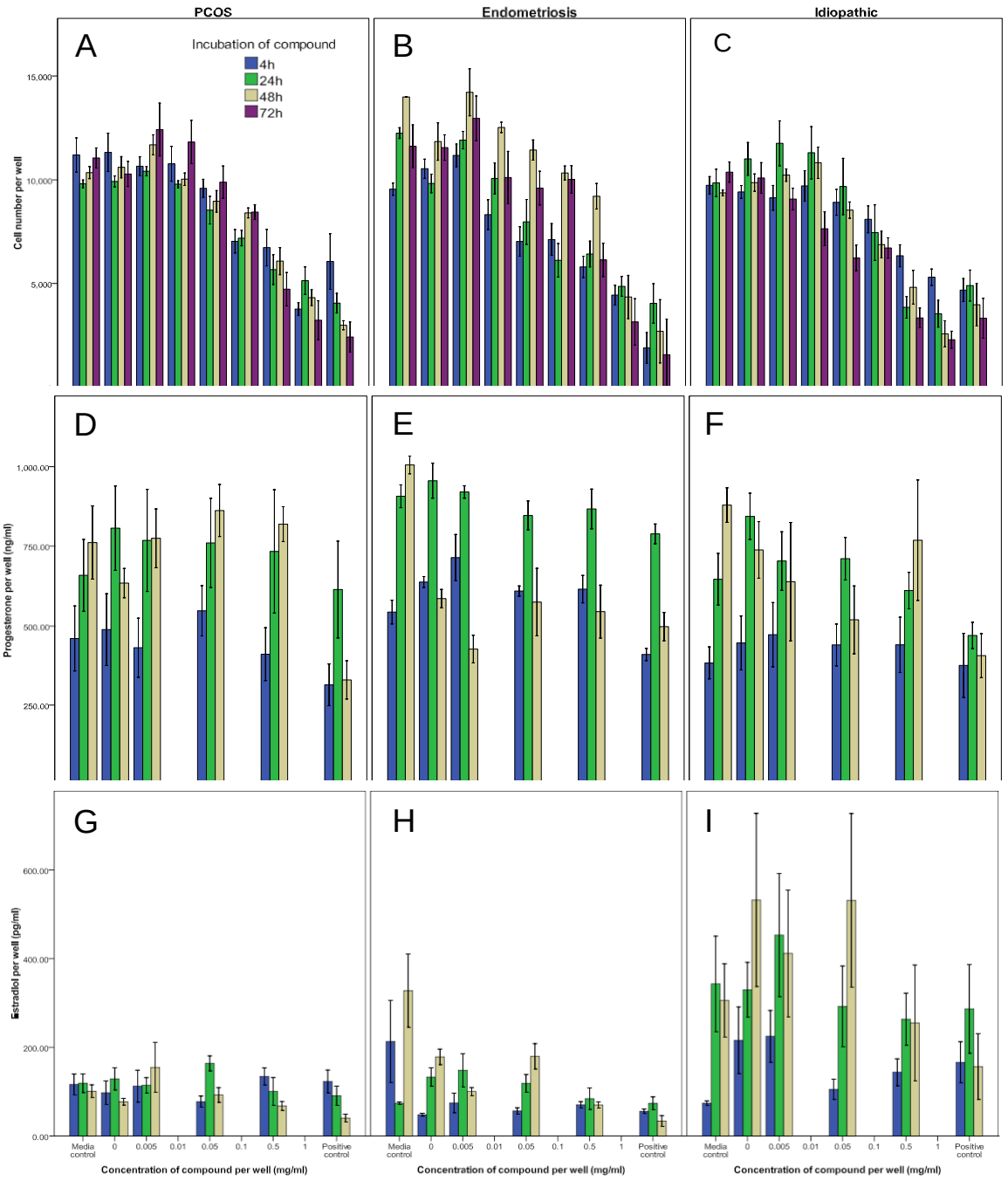


Figure 5: The effect of the semi-purified *D. orbita* fraction three on primary-derived human granulosa cell viability, progesterone and estradiol produced by cells donated by women with an infertility factor caused by polycystic ovarian syndrome(A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction three (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).

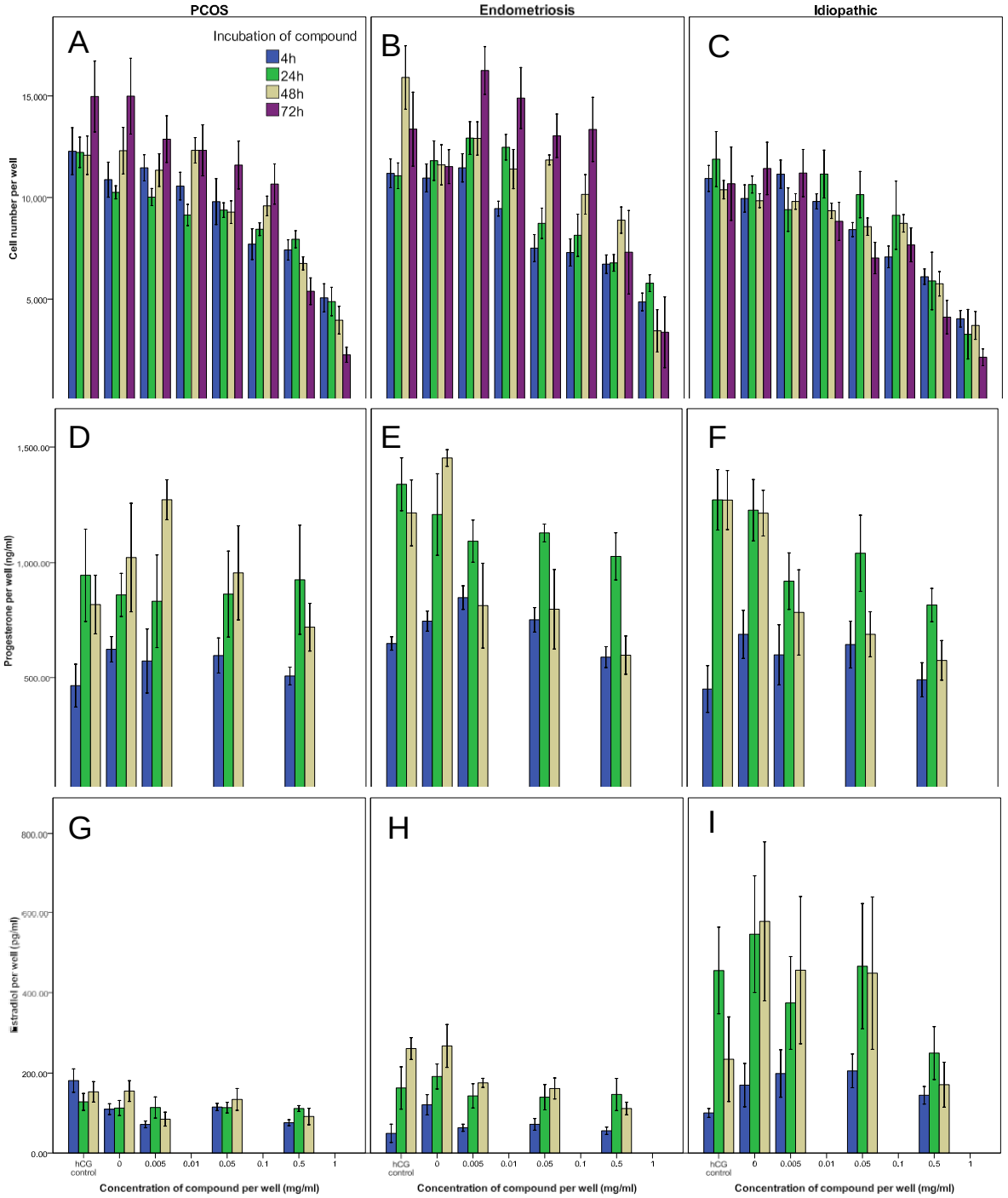


Figure 6: The effect of the semi-purified *D. orbita* fraction three on primary-derived human granulosa cell viability, progesterone and estradiol in the presence of hCG from cells donated by women with an infertility factor caused by polycystic ovarian syndrome (A, D & G), endometriosis (B, E & H); idiopathic infertility (C, F & G). After an initial 24h cell attachment period granulosa cells (10,000 cells/well) were treated with fraction three (0.005-1mg/ml) for 4, 24, 48 and 72h. The 0 concentration represent the 1% DMSO transport vehicle and the positive control treatment was medium without foetal bovine serum (FBS). Cell viability was determined by the crystal violet assay at 570nm, with reference absorbance 630nm. (PCOS n=2; endometriosis n=2 and idiopathic n=3).

Conclusion

In conclusion, the results obtained from primary-derived human granulosa cells from women with abnormal reproductive physiology (PCOS, endometriosis and idiopathic infertility factors) suggests that hCG afforded some protection against the cytotoxic effects of the three *D. orbita* fractions. Fraction one, a combination of the brominated indole compounds was the least toxic to all three groups of granulosa cells both in the presence and absence of hCG. Granulosa cells from all three groups synthesized similar quantities of basal progesterone however, when stimulated with hCG, both the endometriosis and idiopathic groups produced higher quantities of progesterone after 24 and 48h than the PCOS group. Furthermore, after 24h, granulosa cells from the idiopathic group synthesised more both basal and hCG-stimulated estradiol than either the PCOS and endometriosis groups. Finally, in the presence of all three fractions,

Appendix VIII: Statistical Analyses

Throughout this thesis a number of different statistical analyses were employed to analyse the data. Each analysis was specific for each assay depending on the experimental plan of the assay. In certain analyses including one-way and two-way analyses of variance (ANOVA) the data sometimes violated levene's homogeneity of variance. It was therefore necessary to adjust the alpha value in these cases. The following tables represent the chapters in which the data was violated, indicating exactly where data had violated homogeneity of variance and the corresponding adjusted alpha value.

Table 1: Chapter 3 statistical analyses which violated the levene's homogeneity of variance

Section	Experiment	Time point (h)	Statistical Analysis	Adjusted *p* value	Figure
3.2.1.1	MTT concentration assay	2, 4 & 6	One-way ANOVA with Dunnett post hoc	< 0.01	7
3.2.5	6'6-dibromoindirubin & progesterone	2, 4, 8 & 24 2, 4, 6, 8, 10 & 24	Two-way ANOVA with K-Matrix contrast	< 0.01	14A & B
	Indirubin on cell viability & progesterone	2. 4. 8. 10 & 24 2, 4, 6, 8, 10 & 24	Two-way ANOVA with K-Matrix contrast	< 0.01	15A & B
	5-Bomoisatin on cell viability & progesterone	4 & 24 2, 4, 6, 8, 10 & 24h	Two-way ANOVA with K-Matrix contrast	< 0.01	16A & B

Table 2: Chapter 4 statistical analyses from indirubin and 5-bromoisatin (section 4.2.3) on primary-derived human granulosa cell viability, progesterone and estradiol synthesis, showing where the time points of the data that had violated the levene's homogeneity of variance. Statistical analyses was performed using two-way ANOVA with K-Matrix contrast, the alpha value was adjusted to statistically significant if $p < 0.01$.

Cell Type	**Experiment**			**Figure #**
	Indirubin			
	Cell viability	Progesterone	Estradiol	
Fertile granulosa cells	72	24, 48 & 72	24, 48 & 72	2A, B & C
Infertile granulosa cells	48 & 72	24, 48 & 72	72	2D, E & F
	5-bromoisatin			
	Cell viability	Progesterone	Estradiol	
Fertile granulosa cells	72	24 & 48	24, 48 & 72	3A, B & C
Infertile granulosa cells	48	72	24	3D, E & F

Table 3: Chapter 5 statistical analyses from the experiment of the three *Dicathais orbita* semi-purified fractions on primary-derived human granulosa, KGN, JAr and OVCAR-3 cell viability, progesterone and estrogen synthesis (section 5.2.2.2), showing the time points of the data that violated the levene's homogeneity of variance. Statistical analyses were performed using two-way ANOVA with K-Matrix contrast, the alpha value was adjusted to statistically significant if $p < 0.01$.

Cell Type	**Experiment**			**Figure #**
	Fraction 1			
	Cell viability	Progesterone	Estradiol	
Primary granulosa	72 – hCG 24 & 72 + hCG	4, 24 & 48 – hCG 4, 24 & 48 + hCG	48 – hCG 4, 24 & 48 + hCG	3A-F
KGN	4, 24, 48 & 72- cAMP 4, 48 & 72 + cAMP	4, 24, 48 – cAMP 4, 24 & 48 + cAMP	- 4 + cAMP	5A, B, D, E & F
JAr	1, 24 & 48 – hCG 1, 4, 24 & 48 + hCG	24 & 48 – hCG 4, 24 & 48 + hCG	4 & 24 – hCG 4, 24 & 48 + hCG	7A-F
OVCAR-3	24, 48 & 72 – hCG 4, 24, 48 & 72 + hCG	24 – hCG -	4 & 24 – hCG 48 + hCG	9 A, B, C, D & F
	Fraction 2			
	Cell viability	Progesterone	Estradiol	
Primary granulosa	24, 48 & 72- hCG 24, 48 & 72 + hCG	4, 24 & 48 – hCG 4, 24 & 48 + hCG	- 4 & 48 + hCG	11A, B, D, E & F
KGN	4, 24, 48 & 72- cAMP 4, 24, 48 & 72 + cAMP	24 & 48 – cAMP 4, 24 & 48 + cAMP	4 – cAMP -	13A, B, C, D, E
JAr	1, 24 & 48 – hCG 1, 4, 24 & 48 + hCG	24 & 48 – hCG 4, 24 & 48 + hCG	24 & 48 – hCG 24 + hCG	15A-F
OVCAR-3	4, 24, 48 & 72 – hCG 48 & 72 + hCG	24 & 48 – hCG -	4, 24 & 48 – hCG 4 + hCG	17A, B, C, D & F
	Fraction 3			
	Cell viability	Progesterone	Estradiol	
Primary granulosa	72 – hCG 48 & 72h	4, 24 & 48 – hCG 4, 24 & 48 + hCG	- 4, 24 & 48 + hCG	19A, B, D, E & F
KGN	4, 48 & 72- cAMP 4, 48 & 72 + cAMP	24 & 48 – cAMP 4, 24 & 48 + cAMP	- -	21A, B, D & E
JAr	4, 24 & 48 – hCG 1, 4, 24 & 48 + hCG	4, 24 & 48 – hCG 4, 24 & 48 + hCG	4, 24 & 48 + hCG 24 + hCG	23A-F
OVCAR-3	24 & 72 – hCG 24 & 72 + hCG	4, 24 & 48 – hCG 24 + hCG	24 & 48 – hCG -	25A, B, C, D & E

Table 4: Chapter 6 statistical analyses from the experiment of the two *Dicathais orbita* semi-purified compounds, tyrindoleninone and 6-bromoisatin on primary-derived human granulosa, KGN, JAr and OVCAR-3 cells (sections 6.2.3.2 & 6.2.3.3), showing the time points of the data that violated the levene's homogeneity of variance. Statistical analyses were performed using two-way ANOVA with K-Matrix contrast, the alpha value was adjusted to statistically significant if $p < 0.01$.

Cell Type	Compound	Experiment	Time point (h)	Figure
Primary granulosa	Tyrindoleninone	Caspase-3/7	4 & 24	5A
		LDH	2 & 24	5A
		Crystal violet	24	5B
KGN	Tyrindoleninone	Caspase-3/7	4 & 24	6A
		LDH	4 & 24	6A
		Crystal violet	4 & 24	6B
JAr	Tyrindoleninone	Caspase-3/7	-	-
		LDH	4 & 24	7A
		Crystal violet	4 & 24	7B
OVCAR-3	Tyrindoleninone	Caspase-3/7	-	-
		LDH	4 & 24	8A
		Crystal violet	4 & 24	8B
Primary granulosa	6-bromoisatin	Caspase-3/7	4 & 24	9A
		LDH	4 & 24	9A
		Crystal violet	-	-
KGN	6-bromoisatin	Caspase-3/7	4 & 24	10A
		LDH	4 & 24	10A
		Crystal violet	4 & 24	10B
JAr	6-bromoisatin	Caspase-3/7	4 & 24	11A
		LDH	4 & 24	11A
		Crystal violet	4 & 24	11B
OVCAR-3	6-bromoisatin	Caspase-3/7	-	-
		LDH	4 & 24	12A
		Crystal violet	4 & 24	12B
Primary granulosa	Tyrindoleninone	TUNEL	4 & 24	13A
KGN	Tyrindoleninone	TUNEL	4 & 24	13B
Primary granulosa	6-bromoisatin	TUNEL	4 & 24	13A
KGN	6-bromoisatin	TUNEL	4 & 24	13B

Table 5: Chapter 7 statistical analyses from the experiment of the three *Dicathais orbita* semi-purified fractions on primary-derived human granulosa cells (section 7.2.2.2), showing the time points of the data that violated the levene's homogeneity of variance. Statistical analyses were performed using two-way ANOVA with K-Matrix contrast, the alpha value was adjusted to statistically significant if $p < 0.01$.

Cell Type	**Experiment**			**Figure #**
	Fraction 1			
	Cell viability	Progesterone	Estradiol	
Fertile granulosa cells	24 & 48 - hCG 4, 24 & 48 + hCG	- 24 & 48 + hCG	4 – hCG 48 + hCG	1A, C, D, E & F
Infertile granulosa cells	24 – hCG 4, 48 & 72 + hCG	- 4 + hCG	48 – hCG -	2A, C, D,& E
	Fraction 2			
	Cell viability	Progesterone	Estradiol	
Fertile granulosa cells	48 – hCG 4, 24, 48 & 72 + hCG	- 24 & 48 + hCG	- 24 & 48 + hCG	3A, D, E & F
Infertile granulosa cells	24, 48 & 72 –hCG 4, 24, 48 & 72 + hCG	48 – hCG 4, 24 & 48 + hCG	48-hCG -	4A, B, C, D & E
	Fraction 3			
	Cell viability	Progesterone	Estradiol	
Fertile granulosa cells	48 - hCG 4, 24, 48 & 72 + hCG	- 48 + hCG	48 – hCG 48 + hCG	5A, C, D, E & F
Infertile granulosa cells	24, 48 & 72 – hCG 4, 24, 48 & 72	48 – hCG 4 & 48 + hCG	- 48 + hCG	6A, B, D, E & F

both basal and hCG-stimulated progesterone synthesis was similar across all three groups, whereas both basal and hCG stimulated estradiol synthesis was much higher in the idiopathic group than either the PCOS or endometriosis groups. In conclusion, from these results, like the results of the synthetic compounds on the individual infertility cohorts, these muricid compounds had varying effects on each individual cohort of granulosa cells, showing the most promising benefit to women with unknown infertility (idiopathic group). Further research into the effects of these natural compounds on individual cohorts of granulosa cells and women might yield new treatments for infertility and gynaecological disorders.

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