EFFECTS OF CYLINDROSPERMOPSIN ON FUNCTIONS OF HUMAN GRANULOSA CELLS AND SPERMATOZOA

Dina Zebian

B.Biotech (Hons).

Department of Medical Biotechnology, Faculty of Health Sciences,

Flinders University.

A thesis submitted for the degree of Doctor of Philosophy,

June 2010

Contents

List of Figures

List of Tables

Abstract

The aim of this study was to investigate the effects of Cylindrospermopsin (CYN) on human reproductive cells. CYN is a potent alkaloid cyanotoxin that inhibits protein synthesis (PSI) and causes cytochrome P450 metabolism-dependent cytotoxicity and genotoxicity in mice and mouse cells *in vitro*. In the last two decades, evidence has suggested that CYN can have deleterious health effects in humans and animals; however, further research into CYN toxicity is essential. Currently, little is known about the effects of CYN on human reproductive cells, although preliminary data suggest that low levels of CYN may adversely affect metabolic pathways in granulosa cells and cause oxidative damage to spermatozoa membranes. Both have the potential to affect cell viability and functionality.

In vitro cultures of primary-derived granulosa cells (GC) and spermatozoa provide good reproductive models to investigate a compound's toxicity. Both are readily obtained from patients seeking assisted reproductive technology (ART). In this study, GC were isolated from women undergoing ART and cultured in defined medium. After an initial 24-h adherence period, GC were then exposed to 0 – 5 µM CYN for 24, 48 or 72 h. After each time point, cell viability was measured and conditioned medium was collected for quantification of secreted steroid hormones by radioimmunoassay. The presence of cytoplasmic cholesterol lipid substrates required for steroidogenesis, and 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme activity (which enables GC to produce progesterone from pregnenolone cholesterol substrate) were also assessed.

Exposure of GC to CYN concentrations of ≥ 1 µM for 48 h was cytotoxic. Decreased levels of cytoplasmic cholesterol lipid, 3β HSD activity and steroid hormone production 72 h after addition of ≥ 1 µM CYN was likely due to this loss of cell viability.

CYN toxicity is mediated indirectly via its CYP metabolites, but also directly by inhibition of protein synthesis (PSI). Even partial PSI may decrease GC steroid hormone production in cells, at doses without measurable loss of viability. To test

this, *de novo* protein synthesis in GC exposed to CYN was measured using [^{3}H]-Leucine. The hypothesis was confirmed with observations of dose-dependent decreases in progesterone production, and protein synthesis, after 6 h exposure to CYN concentrations up to 5 µM.

Mature, human spermatozoa lack CYP activity and do not rely on *de novo* protein synthesis, the two main mechanisms by which CYN confers its toxicity. However, spermatozoa cell membranes are densely packed with lipids, which are rapidly oxidised by reactive oxygen species when antioxidant defence mechanisms have been depleted. Since CYN has been shown to reduce antioxidant defence mechanisms, lipid peroxidation in spermatozoa is a potential secondary toxicity that could still affect fertility. The effects of CYN on both fresh and cryopreserved human spermatozoa were investigated through the use of four different viability assays: the MTT, MTS, Eosin Y exclusion, and ATP assays. Neither the MTT nor MTS assay proved capable of measuring spermatozoa viability in control experiments under the conditions used in this study. CYN concentrations up to 3 µM were not cytotoxic to cryopreserved spermatozoa after 72 h as determined by Eosin Y exclusion. There were significant decreases in ATP levels in fresh spermatozoa, but only after 24 h exposure to 30 µM CYN.

The current guideline for safe levels of CYN in drinking water is below 1 µg.L^{-1}. Short-term exposure of mature human spermatozoa and human granulosa cells to such levels of CYN is unlikely to affect the viability or functionality of the cells. However, the effects of long-term exposure to CYN need to be further investigated. The ability of CYN to decrease steroid hormone production and protein synthesis in GC may indicate a potential to disrupt normal reproductive cell functionality and development.

.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that 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.

Signed…….

Date…….

Acknowledgements

The work presented in this thesis was supported by the funding of SA Water, the Water Quality Research Australia, Flinders Research Centre for Coastal & Catchment Environments (FR3cE) and the Department of Medical Biotechnology, Flinders University. Purified Cylindrospermopsin was generously provided by Dr Andrew Humpage. I would like to thank my supervisors Prof Chris Franco, Dr Fiona Young, Dr Andrew Humpage and Dr Suzanne Froscio for their collective support and guidance throughout this research.

To the staff of the Department of Obstetrics and Gynaecology and Flinders Reproductive Medicine Unit for generously giving so much of their time to consent patients, technical expertise and their friendly company over the years. To all the patients and participants that donated their cells for research, without them this research literally could not have been possible.

Thank you to Ms Margaret Menadue for her assistance and for the kind use of her equipment.

To my colleagues in the Department of Medical Biotechnology, to name everyone is a thesis in itself, but to my closest friends and colleagues, Vicki Edwards, Daniel Inglis, Rhys Hamon and Dr Lisa Schmidt your friendship, encouragement, "chats" and travel expeditions will forever highlight the wonderful years we have shared. Thank you to Dr Ken Lange, Dr Peter Speck, Dr Emma Parkinson-Lawrence and Dr Benjamin Blyth for their words of encouragement, support and taking the time to read, provide feedback and edit the thesis.

To Ms Elektra Spathopolous, Executive Director for the Australian Institute of Policy and Science for her patience, understanding and support- Thank you.

I would like to thank my immediate family Mum, Dad, Serge and Tania and my Aunt- Soumaya Jaber for their understanding, patience, support, laughter, stories, encouragement and prayers, I wouldn't have made it without you all.

Ultimately the words of Bette Midler “Wind Beneath My Wings” go to my mother Hanaa Zebian. Mum, it has been your support, faith and love over the last 25 years that has shaped me and made me who I am today. You gave me my ‘wings’ my success is because of you- you have forever inspired me to achieve everything that my heart desires. Thank you for the never ending words of encouragement, love and support which never wavered over the years.

It is to you that I dedicate this...

Publications

Papers in Preparation

Zebian, D., Froscio, S. M., Humpage, A. R. & Young, F. M. Reproductive effects of Cylindrospermopsin on healthy and unhealthy primary-derived human granulosa cells.

Zebian, D., Froscio, S. M., Humpage, A. R. & Young, F. M. Characterisation of *in vitro* human mature spermatozoa assays to determine reproductive effects of Cylindrospermopsin.

Young, F. M., Zebian, D., Humpage, A. R. & Froscio, S. M., Effect of a blue-green algal toxin, cylindrospermopsin (CYN), on human luteinized granulosa cell (GC) protein synthesis *in vitro*.

Selected Conference Papers:

Blooming Problems In Water, And Possible Effects On Future Baby Booms. *Australian Water Association Regional Conference.* Barossa Valley, South Australia, August 2008. Oral Presentation. **Awarded best paper presented at the conference.**

CYN-FULL SPERM? *The Flinders Research Centre for Coastal and Catchment Environment & Flinders Bioknowledge Postgraduate Research Conference.* Adelaide, Australia, July 2008. Oral Presentation.

Human *In Vitro* Fertilisation (IVF) Derived Granulosa Cells: *In Vitro* Characterisation and Comparison of Two Viability Assays. *The Endocrine Society of Australia Proceedings 2007, 50th Annual Scientific Meeting.* Christchurch, New Zealand, Sept 2007. Oral Presentation.

Reproductive Effects of Cylindrospermopsin on Human Granulosa Cells. *The 7th International Conference on Toxic Cyanobacteria.* Rio de Janeiro, Brazil, August 2007. Oral Presentation. **Received an honourable mention: one of three best presentations at the conference.**

CYN, Steroids & Babies. *The Flinders Research Centre for Coastal Catchment Environment & Flinders Bioknowledge Postgraduate Research Conference.* Adelaide, Australia, July 2007. Oral Presentation.

The Effects of Cylindrospermopsin on Human Granulosa Cells and Spermatozoa. *CRC for Water Quality and Treatment Postgraduate Student Conference* Melbourne Australia, July 2006.

Abbreviations

%	Percent
µg	Micrograms
µg/mL	Micrograms per millilitre
µL	Microlitre
µM	Micromolar
3βHSD	3- beta hydroxysteroid dehydrogenase
ADP	Adenosine di-Phosphate
Ah	Aromatic hydrocarbon
ANOVA	Analysis of Variance
AP-1	Activator Protein -1
ART	Assisted Reproductive Technology
ASC	Adherent Standard Curves
ATCC	American Type Culture Collection
ATP	Adenosine tri-Phosphate
BMI	Body Mass Index
BNi	Micronuclei in Binucleated cells
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	cyclic Adenosine Monophosphate
CI	Confidence Interval
CL	Corpus Luteum
CO_2	Carbon dioxide
CPS	Counts Per Second
CYN	Cylindrospermopsin
CYP	Cytochrome P450 enzymes
D	Day (s)
Da	Daltons
DAG	Donkey anti-goat Antibody (Secondary antibody)
DHA	Dehydroepiandrosterone
DMEM/F12	Dulbeccos Minimum Essential Medium (MEM) nutrient mixture
DMSO	Di- methyl sulphoxide
DNA	Deoxyribose nucleic acid
DOB	Date of Birth
E_2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked-immunosorbent assay
EP	Ectopic Pregnancy
ER	Estrogen Receptor
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β

FCS	Fetal Calf Serum
FIF	Female Infertility Factor
FSH	Follicle Stimulating Hormone
G	Grams
GC	Granulosa Cell
GJ	Gap Junctions
GnRH	Gonadotropin Releasing Hormone
GSH	Glutathione
H	Hour (s)
Ham's F12	F-12 Nutrient Mixture (Ham)
HSA	Human Serum Albumin
hCG	human Chorionic Gonadotropin
HCl	Hydrochloric Acid
HDF	Human Dermal Fibroblasts
HPLC	High Performance Liquid Chromatography
ICSI	Intra Cytoplasmic Sperm Injection
IGF	Insulin-like Growth Factor
IL	Interleukin
IMM	Inner Mitochondrial Membrane
IP	Intraperitoneal
IS	Intense Stained Cells
ITS	Insulin/Transferrin/Selenium
IU	International Units
IU/L	International Units per Litre
IVF	*In Vitro* Fertilisation
kDa	Kilo Daltons
Kg	Kilograms
KGN	Granulosa Cell Tumour Cell line
L	Litre
LC_{50}	Concentration lethal to half of population
LD_{50}	Lethal Dose to half of population
LDH	Lactate dehydrogenase
LH	Luteinising Hormone
LOEAL	Lowest observable adverse effect level
LS	Light Stained Cells
MQ water	Milli Q water
Mg	Milligrams
mg/kg	Milligrams per kilogram
mg/mL	Milligrams per millilitre
MIF	Male Infertility Factor
Min	Minute
MIS	Mitotic Inhibitory Signal
mIU/mL	Milli International Units per millilitre
mL	Millilitre
MLTC-1	Mouse Leydig Tumour Cell- 1 line

Mm	Millimeter
mRNA	messenger Ribose nucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-[4,5-dimethyl(thiazole-2-yl-3,5-diphenyl] tetrazolium bromide
N	Number of participants/experiments
NAD	Nicotinamide adenine dinucleotide
NBT	Nitro Blue Tetrazolium
nm	Nanometer
NOAEL	No observable adverse effect level
NRS	Normal Rabbit Serum
NSB	Non-specific Binding
OD	Optical Density
OHSS	Ovarian Hyperstimulation Syndrome
OMM	Outer Mitochondrial Membrane
ORO	Oil Red O
P_4	Progesterone
PCOS	Polycystic Ovary Syndrome
PEG	Polyethylene Glycol
pg	Pico grams
pg/mL	Pico grams per millilitre
PK	Protein Kinase
PKA	Protein Kinase A
pmol/L	Picomole per Litre
PMSF	Phenylmethylsulfonylfluoride
PR	Progesterone Receptor
PS	Protein Synthesis
PSI	Protein Synthesis Inhibition
QAHMs	Quinns Advantage Medium with Hepes
RBC	Red Blood Cells
RIA	Radioimmuno Assay
RNA	Ribose nucleic acid
ROS	Reactive Oxygen Species
SAR	Sheep anti-rabbit Antibody (Secondary Antibody)
SDR	Short-chain dehydrogenase reductase family
SDS	Sodium Dodecyl Sulphate
sec	Second (s)
SEM	Standard Error of Mean
SF-1	Steroidogenic Factor-1
SPSS	Statistical Package for the Social Sciences
StAR	Steroidogenic acute regulatory protein
TL	Tubal Ligation
TNF	Tumour Necrosis Factor
WBC	White Blood Cells
WCIF	Wright Cell Imaging Facility
WHO	World Health Organisation

$\chi 2$	Chi-square

"Amongst all theories that deal with the origin of life "liquid water is the quintessential environmental criterion for both the origin and sustenance of life" (Davis & McKay 1996).

Chapter 1. General Introduction

Assisted reproductive technologies (ART) were established to address problems associated with abnormal reproductive functioning in order to help couples conceive. There are an estimated 70 million infertile couples worldwide; with demographic factors such subsequent delays in starting a family, due to other social factors taking precedence, thought to have contributed to lower fertility rates (Ledger 2009; Ombelet et al. 2008). Furthermore, general health issues such as obesity and the rise in sexually transmitted diseases have also contributed to decreases in infertility (Ledger 2009). Assisted reproductive techniques involve protocols which down-regulate the endogenous female cycle and allow exogenously administered gonadotropins to stimulate development and production of oocytes and surrounding steroidogenic cells (granulosa and theca cells) within the ovary, referred to as superovulation or hyperstimulation (Salamonsen et al. 2001). Granulosa cells (GC) play a major role in production of steroid hormones, vital for the establishment and maintenance of pregnancy (Keren-Tal et al. 1995). GC can be used as an investigative *in vitro* reproductive model to determine possible effects of toxins and pollutants (Beckman et al. 1991; Breckwoldt et al. 1996; Chaffkin et al. 1993; Harvey & Everett 2003; Quinn et al. 2006; Rodgers et al. 1996).

Infertility can be attributable to either male factors, female factors or both. The collected oocyte is fertilised with a spermatozoon through *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). Couples undergo ICSI when there are abnormal semen parameters. Declines in semen quality are associated with a decline in male fertility (Aitken et al. 2004; Pichini et al. 1994). Alternatively, couples seeking ART due to the women's menstrual cycle abnormalities, endometriosis or physiological barriers preventing fertilisation undergo IVF (Fatum et al. 2009; Ledger 2009; Ng et al. 2000; Nuojua-Huttunen et al. 1999; Stephansson et al. 2009; Templeton et al. 1996).

Humans can be exposed to drugs, chemicals and toxic compounds (collectively called xenobiotics) in the environment through routes such as inhalation and

consumption of contaminated water (Aitken et al. 2004; Pacifici et al. 1995; Wagner et al. 1990; Yousef et al. 2007). Some environmental pollutants affect human reproduction; a specific study on male patients seeking treatment for their infertility in south eastern Spain, found that exposure to occupational toxins such as glues, solvents and silicones were major contributing factors to male infertility in patients undergoing ART (Mendiola et al. 2008).

1.1 Human Primary-derived Granulosa Cells

Human granulosa-lutein cells are commonly used as a reproductive model to investigate the effects of environmental chemicals and pollutants on viability and steroid hormone production (Heimler et al. 1998; Hong et al. 2007; Hsia et al. 2007). The ovarian follicle is divided into two compartments: the vascularised theca externa and interna, and the non-vascular compartment containing granulosa cells, oocyte and the follicular fluid (Shalgi et al. 1973). The ovarian follicle has an extensive blood supply through which many proteins, lipophilic substances and potential xenobiotics can penetrate the blood-follicle barrier and reach the developing oocyte and steroidogenic cells (Murphey 2004; Shalgi et al. 1973; Wagner et al. 1990). Human granulosa cells play a key role in successful reproduction (Hosokawa et al. 1998). The growth, development and survival of the ovarian follicle are dependent on successful passage through the sequential stages of initiation, growth, selection, ovulation and luteinisation and optimal functioning of all steroidogenic tissue (Richards 1994). Follicular growth is dependent on the proliferation and secretion of steroid hormones from granulosa cells to create the ideal environment for oocyte maturation and nourishment (Huang et al. 2004; Tapanainen et al. 1987). Throughout the follicular phase, follicle stimulating hormone (FSH) and estradiol (which acts via the estrogen receptors α and β) stimulate mitotic proliferation of granulosa cells and induction of luteinising hormone (LH) receptors (Chu et al. 2004; Drummond & Findlay 1999; Enmark & Gustafsson 1999; Mangelsdorf et al. 1995). This phase is crucial in development and growth of primordial follicles into one mature dominant follicle from which an oocyte will be ovulated (Macklon & Fauser 1998; Yong et al. 1992). The newly formed LH receptors on GC allow LH to induce ovulation whilst stimulating the GC to luteinise (now granulosa-lutein cells) and to cease proliferating (Chaffkin et

al. 1993). *In vivo,* human chorionic gonadotropin (hCG) - a placental homologue of LH - has the ability to activate LH receptors and increase progesterone output by granulosa-lutein cells (Devoto et al. 2002; Figenschau et al. 1997; Huhtaniemi 2004).

The differentiation of GC into luteinised cells is characterised by a wide range of functional changes (Ben-Ze've & Amsterdam 1987; Chaffkin et al. 1993; Delforge et al. 1972; Figenschau et al. 1997). These hallmarks include:

- the cessation of proliferation;
- increases in cyclic adenosine 3':5' monophosphate AMP (cAMP) production;
- expression of steroidogenic enzymes and hormone receptors;
- progesterone production;
- cytodifferentiation;
- morphological changes of GC in the presence of an extracellular matrix;
- cellular aggregation; and,
- the formation of gap junctions

Figure 1-1, illustrates the difference between proliferative granulosa cells (high nuclear to cytoplasmic ratio), under the influence of FSH, and a luteinised granulosa cell (low nuclear to cytoplasmic ratio) which is under the influence of LH (Chaffkin et al. 1993). It is considered that a cell which is still in its proliferative state is incapable of accumulating cholesterol lipid required for steroidogenesis as it is not in terminal state of differentiation (Kinkel et al. 2004).

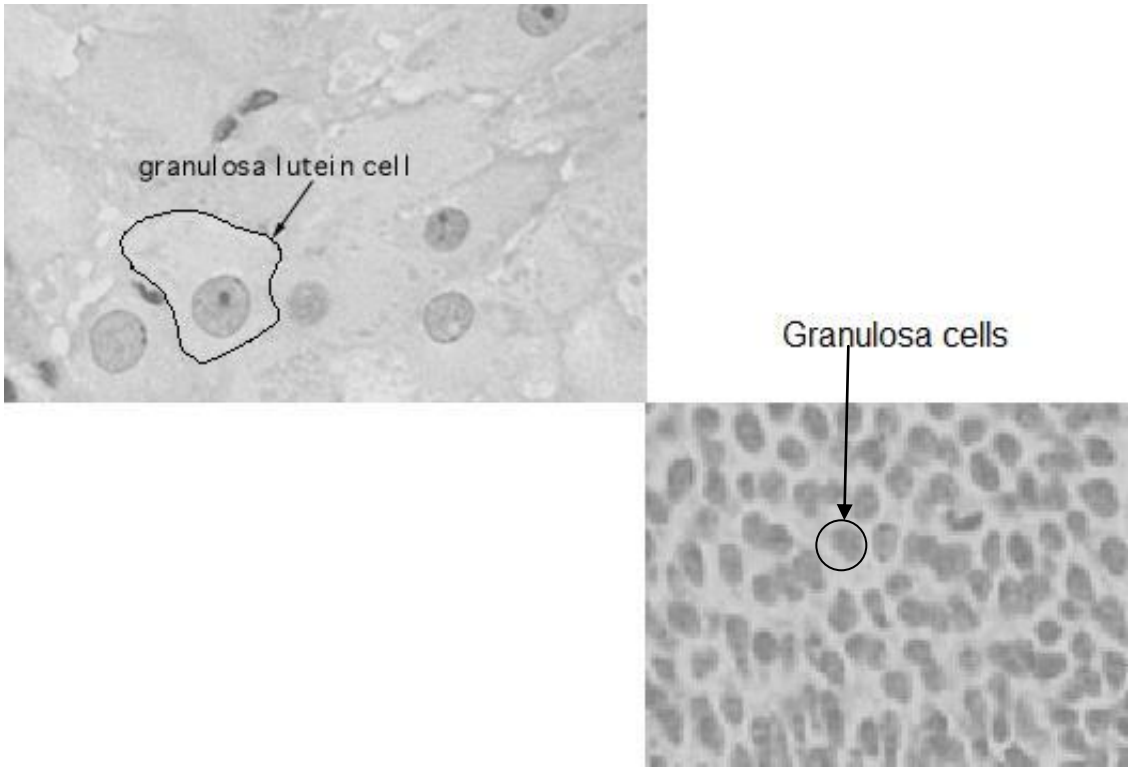


Figure 1-1: Proliferation or Differentiation of Granulosa Cells (Vaughan 2002)

A granulosa lutein cell has a high cytoplasmic to nuclear ratio- indicative of the cell no-longer in a proliferative state. In comparison a granulosa cell is smaller and has a high nuclear to cytoplasmic ratio and maintains mitotic capabilities.

1.1.1 Ovulation

The growth, differentiation, and viability of ovarian cells are regulated by endocrine, paracrine and autocrine factors (Soboloff et al. 2001; Vaughan 2002). The key gonadotropins involved in regulation of survival (viability) and functionality (steroid hormone production) of granulosa cells are FSH and LH/hCG (Richards 1994). Progesterone regulates viability, differentiation and cell proliferation with autocrine effects on gene transcription in granulosa cells via the progesterone receptor (PR) (Espey et al. 2004; Peluso 2003). As a result, gene expressions of specific enzymes are up-regulated to combat oxidative damage caused by inflammatory processes associated with ovulation. These include the glutathione-S-transferase enzymes induced by the protein kinase A (PKA) pathway (Adler et al. 1999). Progesterone and estradiol function as receptor ligands and can also induce the transcription and translation of immediate early stress response genes, which regulate the expression of other genes and granulosa cell differentiation (Enmark & Gustafsson 1999; Espey et al. 2004; Mangelsdorf et al. 1995; Peluso 2003).

1.1.2 Hormonal Regulation of Steroidogenesis

Steroidogenesis is regulated and modulated predominately via the protein kinase A (PKA) signalling pathway under the influence of tropic hormones (Stocco et al. 2005). Steroidogenesis and the production of sex-steroid hormones are essential for function of the ovary and the testes, and the establishment and maintenance of pregnancy (Keren-Tal et al. 1995). These processes are dependent on pituitary-derived FSH, and LH, which increase the concentration of cAMP (Fritz & Speroff 1982). These two hormones act via the cAMP secondary messenger signalling system which regulates transcription of hormone synthesis genes (Devoto et al. 2002; Fritz & Speroff 1982).

However, other pathways exist which act independently of, or work in synergy with, the PKA system to regulate steroidogenesis, including the protein kinase C (PKC) pathway, arachidonic acid metabolism (involved in regulation of signalling pathways) and calcium messenger system (Stocco et al. 2005). The rate limiting factor to steroidogenesis is the steroidogenic acute regulatory protein (StAR) and its

ability to translocate cholesterol from the outer to the inner mitochondrial membrane (Stocco & Clark 1996). StAR belongs to the mitochondrial phosphoproteins which are regulated by FSH and LH. Changes in StAR reflect changes in steroid hormone biosynthesis (Kohen et al. 2003). The main signalling transduction pathway of steroidogenesis involves the binding of the gonadotropins to the ovary activating G-stimulatory proteins which activate adenylyl cyclase increasing cAMP levels which activate PKA and other protein kinases stimulating steroidogenesis (Leung & Armstrong 1980; Leung & Steele 1992; Richards 1994; Yong et al. 1992).

Estradiol synthesis by granulosa cells is primarily stimulated by FSH (Devoto et al. 2002). The androgens are the precursors for estradiol biosynthesis. In the early follicular phase, FSH stimulates increased estradiol production by inducing expression of the aromatase enzyme (Leung & Armstrong 1980). Leung & Armstrong had proposed the two – cell two-gonadotropin theory of ovarian steroidogenesis whereby interaction exists between granulosa and theca cells, with GC unable to synthesise the androgen substrates needed to produce estradiol on their own. Granulosa cells lack the 17_{α}-hydroxylase and $C_{17,\ 20}$-lyase enzymes necessary to produce androgens from C_{21} precursors, Figure 1-2 (Havelock et al. 2004; Leung & Armstrong 1980). Androstenedione produced by the theca cells, can diffuse through the basement membrane which separates the theca and granulosa cells, into the avascular compartment. Here, it can be aromatised inside GC into estradiol by one of the key steroidogenic enzymes, cytochrome P450 aromatase (CYP19), under the influence of FSH (Havelock et al. 2004). It is the amount of C_{19} androgens synthesised from cholesterol in theca cells (stimulated by LH) that determines how much estradiol can be produced by granulosa cells. As the developing follicle draws nearer to ovulation, LH levels increase as estradiol levels drop (Leung and Armstrong, 1980), due to decreased androgen availability and LH also exerting an inhibitory effect on aromatase activity (Devoto et al. 2002; Havelock et al. 2004).

Progesterone production is increased when granulosa cells enter the luteal phase, the continued communication and interaction between now theca-lutein and

granulosa-lutein cells increases expression of steroidogenic enzymes cytochrome P450 side chain cleavage enzyme (CYP11A1) and 3β-hydroxysteroid dehydrogenase (3βHSD), the latter critical to the production of progesterone (Devoto et al. 2002; Havelock et al. 2004).

1.1.3 Steroidogenesis

Differentiated granulosa-lutein cells are abundant with cytoplasmic lipids which store cholesterol substrates required for steroid hormone production (Ho et al. 2004; Schmidt et al. 1984). The steroid hormones of interest are the estrogens, progestins and androgens, all of which are structurally similar and stem from a common pathway (Miller 1988). There are thus numerous steps where xenobiotic toxicity could disrupt steroidogenesis. The key enzymes involved in steroid hormone biosynthesis are steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (3βHSD) and aromatase (CYP19), Figure 1-2 (Harvey & Everett 2003; Ho et al. 2004; Payne & Hales 2004).

The human cytochrome P450 (CYP) family is comprised of 57 genes that code for enzymes involved in drug and xenobiotic metabolism, cholesterol metabolism and steroid synthesis (Nebert & Russell 2002). The CYP's involved in the metabolism of xenobiotics are mainly CYP1, CYP2 and CYP3, which can produce reactive intermediates which are more toxic than the parent compounds (Nebert & Russell 2002; Runnegar et al. 1995). StAR is the rate limiting step in steroidogenesis where it translocates cholesterol from outer mitochondrial membrane (OMM) to CYP11A1 in the inner mitochondrial membrane (IMM) initiating steroidogenesis (Stocco & Strauss 1998). 3βHSD enzyme, type II isoform found within the ovary, is essential for progesterone production. It is membrane-bound and distributed to both mitochondrial and microsomal membranes (Kerin et al. 1976; Payne & Hales 2004; Sanderson 2006; Wattenberg 1958). The steroidogenic pathway has multiple molecular points which can be targeted by endocrine disruptors, altering enzyme activity in the ovaries and testis, Figure 1-2 (Harvey & Everett 2003; Hoyer 2001). Human granulosa cell *in vitro* assays can be utilised to investigate xenobiotics

acting as steroid receptor antagonists and/or agonists, by inhibiting or activating steroidogenic enzymes (Enmark & Gustafsson 1999; Fan et al. 2007; Harvey & Everett 2003; Hughes et al. 1990; Sanderson 2006).

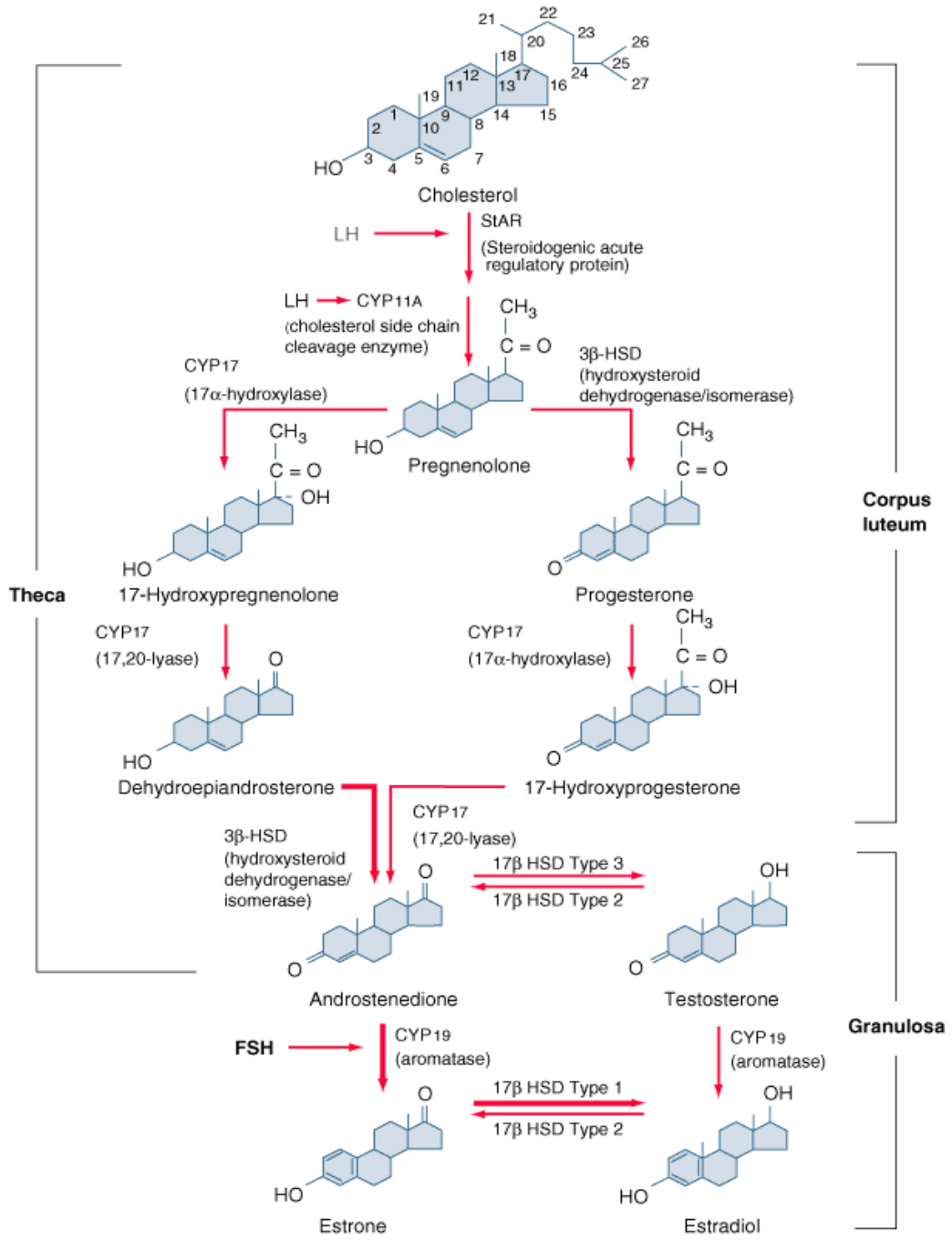


Figure 1-2: Steroidogenic Pathway in granulosa-lutein and theca-lutein cells (Kasper et al. 2006)

Key steroidogenic enzymes highlighted are CYP11A1; 3-βhydroxysteroid dehydrogenase and CYP19 (aromatase). Pregnenolone, C-21 cholesterol, is synthesised by the mitochondrial CYP11A1 and is the immediate precursor for all steroid hormones (Breckwoldt et al. 1996; Doody et al. 1990; Harvey & Everett 2003; Miller 1988; Payne & Hales 2004). Luteinising hormone/hCG regulates the early steps in steroidogenesis; cholesterol is transported into the mitochondria by the steroidogenic acute regulatory protein (StAR) and is converted to pregnenolone (Devoto et al. 2002; Kasper et al. 2006; Saxena et al. 2004; Yong et al. 1992). Diagram adapted and modified from (Kasper et al. 2006).

1.2 Human Mature Spermatozoa

The testes produce spermatozoa and steroid hormones required for normal reproductive function. Many studies have focussed on the long term developmental effects of xenobiotics and chemicals on male testis, reproductive tract and spermatozoa (Mendiola et al. 2008). Semen quality is often assessed as the biological end point for toxicology studies (Fraser et al. 2006; Mendiola et al. 2008; Pacifici et al. 1995; Pichini et al. 1994; Sanderson 2006; Wagner et al. 1990; Yousef et al. 2007). The simplest index of spermatozoa viability is motility, and it also determines clinical fertilisation capability (Morris et al. 2007). Sertoli cells provide the ideal environment for spermatogenesis and maturation of spermatozoa. Sertoli cells also create the blood-testes barrier facilitating intracellular transport of nutrients (Amann 1981; Griswold 1998; Hecht 1998). The process of spermatogenesis involves the repackaging of nuclear proteins to increase genomic stability. Genomic transcription in spermatozoa does not resume until after fertilisation (Grootegoed et al. 2000; Hecht 1998). Spermatogonia (original germ cell) contain highly vacuolated mitochondria capable of undergoing protein synthesis, whereas mature spermatozoa contain only mitochondrial ribosomes and no cytoplasmic ribosomes, and are thus only capable of mitochondrial gene expression (Hecht 1998; Revelli et al. 1994). These changes reflect changing metabolic needs throughout spermatogenesis (Hecht 1998). Mature spermatozoa are comprised of four essential parts; the head, an acrosome, the midpiece and the tail, Figure 1-3. The head contains the complete genetic information, the acrosome contains the enzymes required to penetrate the zona pellucida and fertilise the oocyte, and the tail provides forward progressive motility required to reach the oocyte (De Kretser et al. 1998; Hecht 1998; Liu & Baker 1992). Microtubules form the spermatozoon tail, an extension of the axoneme in the neck of the spermatozoon which renders the spermatozoon motile (De Kretser et al. 1998). The axenomes are densely packed with mitochondria. Abnormalities which affect ATPase activity or production of ATP energy molecules will directly affect spermatozoa motility (De Kretser et al. 1998; Grootegoed et al. 2000).

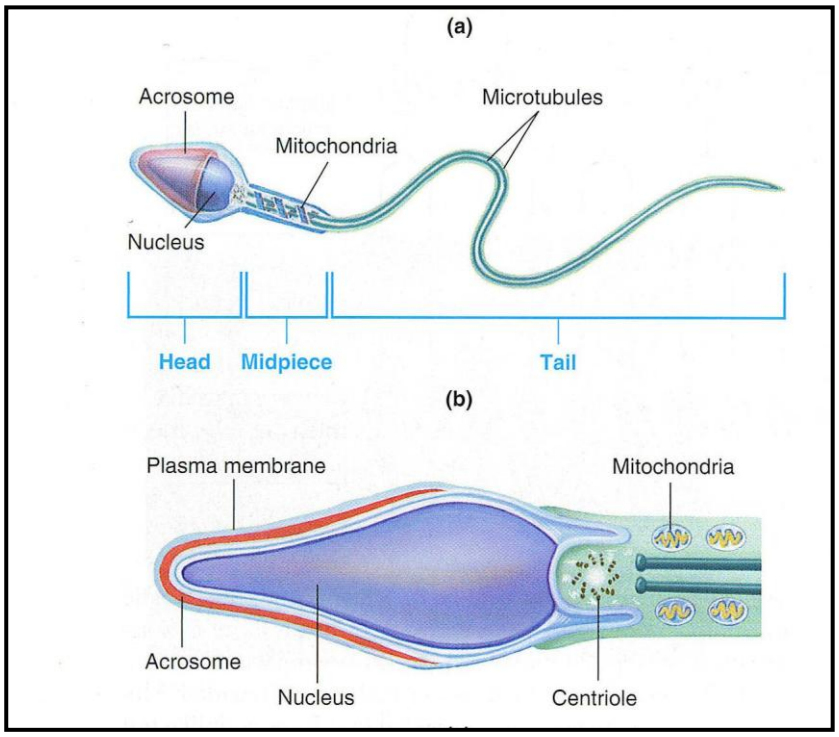


Figure 1-3: Human Spermatozoa

The anatomy of a mature human spermatozoon (a) represents schematic representation of the side view of spermatozoon; (b) details a cross-section view of the spermatozoon head portion. Figure extracted from Sherwood (2001).

1.2.1 ATP Production by Human Spermatozoa

ATP is the predominant energy store in mammalians cells, including spermatozoa (Orlando et al. 1982; Peterson & Freund 1970). The ratio of ADP and ATP nucleotides reflects the physiological state of spermatozoa and directly relates to their motility (Orlando et al. 1982). Glycolysis has been found to be more efficient than oxidative respiration in maintaining ATP levels in spermatozoa due to the limited oxygen consumption by spermatozoa (Peterson & Freund 1970).

The glycolylic rate of spermatozoa is 0.5 µmoles of glucose per 10^8 sperm.h^{-1} producing 2 µmoles of ATP for each glucose molecule metabolised (Peterson & Freund 1970). Spermatozoa with ATP concentrations less than 40 pmole per 10^6 sperm have limited success in IVF, thus ATP concentration is an indicator of viability, functionality and fertilisation capability (Vigue et al. 1992).

1.2.2 Effects of Reactive Oxygen Species on Human Spermatozoa

The primary susceptibility of spermatozoa is membrane integrity compromise via oxidation (Aitken et al. 2004; Pacifici et al. 1995; Wagner et al. 1990; Yousef et al. 2007). Oxygen is essential for energy production, however reactive oxygen species (ROS) are also unavoidably generated (Griveau & Lannou 1997). Seminal plasma is one of the most powerful antioxidant fluids; deficiencies in seminal fluid are often associated with oxidative stress that results in male infertility (Griveau & Lannou 1997; Sanocka et al. 1997). Spermatozoon membranes are comprised of high concentrations of unsaturated fatty acids which increase their susceptibility to oxidative damage (Aitken et al. 1993; Griveau & Lannou 1997; Said et al. 2005; Said et al. 2005). ROS damage decreases fertilisation capability by inducing lipid oxidation of spermatozoa membranes, and with increased permeability there is an increased chance of DNA damage and decreased motility due to a loss of intracellular ATP (Aitken et al. 1993; Griveau & Lannou 1997; Said et al. 2005; Said et al. 2005; Yousef et al. 2007).

1.3 Environmental Toxins in Australian Waters

Due to recent lower-than-average rainfalls in Australia, potable water shortages have been observed in Queensland and southern Australia (Climate Analysis Section 2006; McKay & Moeller 2001). As a result of water shortages, and the additional effect of increased temperatures, water restrictions and water recycling programs have been implemented to manage the increasing demand for water (Stenekes et al. 2006). As new water sources are tapped, there is heightened need to ensure that the drinking water supply is safe to consume, free of contaminants such as microbes (pathogenic bacteria and viruses), aquatic biota (such as blue green algae) and inorganic and organic chemicals. Under favourable conditions, cyanobacteria are the predominant phytoplankton in Australian reservoirs, lakes and rivers (Hoeger et al. 2004). As a result, toxins produced by cyanobacteria could pose a serious health risk if they enter the drinking water supply (McKay & Moeller 2001).

1.4 Cyanobacterial Blooms and Associated Toxins

Cyanobacteria, commonly known as blue-green algae, belong to an ancient group of photosynthetic autotrophic prokaryotes thought to be responsible for the accumulation of oxygen in the earth's atmosphere nearly 2 billion years ago (Ressom et al. 1994). Bloom formation and the dominance of one cyanobacterial species in a water body depends on several factors: nutrient availability (correct ratio of phosphorus and nitrogen), water column stability, temperature, pH and carbon dioxide (CO_2) levels (Dignum et al. 2005; Fabbro & Duivenvoorden 1996; Mur et al. 1999; Reynolds & Walsby 1975; Steinberg & Hartmann 1988).

Not all cyanobacteria are toxic to water supplies, and even within a single toxic species there can be differing toxicity between strains (Sivonen & Jones 1999). In Australia, 42% of cyanobacteria samples tested in mouse bioassays were hepatotoxic or neurotoxic, and up to 84% of cyanobacteria samples tested by high-performance liquid chromatography (HPLC) were found to contain neurotoxins (Baker & Humpage 1994; Negri et al. 1997; Sivonen & Jones 1999). The increasing threat of cyanobacterial toxins entering drinking water supplies makes preventative measures and effective removal techniques essential (Griffiths & Saker 2003;

Hoeger et al. 2005). Copper sulphate is the most common preventative treatment method for control of cyanobacterial blooms; however, it is the least effective for preventing contamination of the water supply as it results in cell lysis and release of intracellular toxin (Falconer 1998). Effective methods for the removal of cyanobacterial cells without causing cell lysis and release of associated cyanotoxins is continuously being researched (Hrudey et al. 1999).

1.5 Cyanotoxins

Cyanotoxins can be classified into three groups based on their chemical structures: the cyclic peptides, alkaloids and lipopolysaccharides; and each differs in its mechanism of toxicity (Hoeger et al. 2004; Sivonen & Jones 1999). Of specific interest is the tricyclic alkaloid hepatotoxin known as cylindrospermopsin (CYN, Figure 1-4) which was classified as the highest priority for cyanotoxin investigations at the U.S. Environmental Protection Agency Unregulated Contaminant Monitoring Regulation (UCMR) meeting on cyanobacterial toxins.

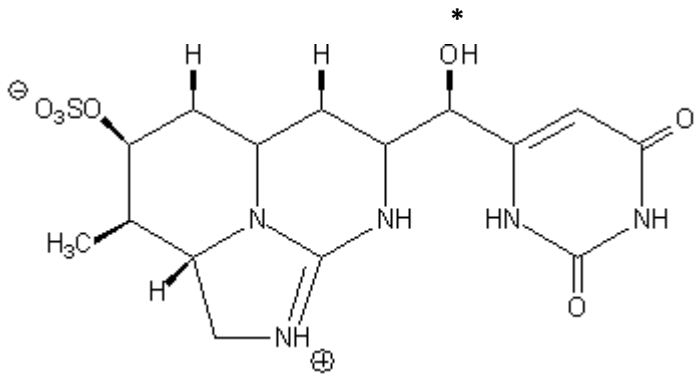


Figure 1-4: Structure of Cylindrospermopsin

* Denotes that the OH can be an epimer thus producing 7-epicylindrospermopsin or be substituted by H to produce deoxycylindrospermopsin.

1.6 Cylindrospermopsin (CYN)

Cylindrospermopsin, $C_{15}H_{21}N_5O_7S$, has a low molecular weight of 415 Daltons (Figure 1-4) and its zwitterionic properties enable it to be highly soluble in water (Chong et al. 2002; Hawkins et al. 1985; Ohtani et al. 1992; Sivonen & Jones 1999). The pure form of CYN is stable in sunlight, the dark, UV light, pH 4, 7 and 10 and cannot be degraded by boiling (Chiswell et al. 1999; Duy et al. 2000; Moore et al. 1998; Sivonen & Jones 1999). A total of six species have been identified worldwide which produce CYN. In Australia, *Cylindrospermopsis raciborskii* (originally identified by Woloszynska 1912) is the species posing the greatest threat of CYN contamination, and is considered toxic in Australian waters (Falconer & Humpage 2006; Hawkins et al. 1985; Ohtani et al. 1992; Seifert et al. 2007). *C. raciborskii* can contain between 1.5 – 5.5 $mg.g^{-1}$ of CYN in freeze-dried cell (Sivonen & Jones 1999).

The elimination of CYN via various methods has been investigated, with chlorination, ozone and activated carbon found to be effective in the elimination of CYN (Rodríguez et al. 2007). When directly comparing the effectiveness of chlorine treatment and ozone treatment, ozone had a higher rate of oxidation and elimination of CYN than chlorine (Rodríguez et al. 2007). Chlorine, although effective in the removal of 99% of CYN (pH 6-9 at 0.5 $mg.L^{-1}$), can produce harmful trichloromethanes if concentrations of chlorine exceeds 1 $mg.mL^{-1}$, and is less effective if organic matter is present (Rodríguez et al. 2007; Senogles et al. 2000). If not detected early and eliminated from the contaminated water, CYN is not only stable but can remain unnoticed, potentially causing harm if ingested.

1.7 Cylindrospermopsin Toxicity

The no observable adverse effect level (NOAEL) for CYN is 30 $\mu g.kg^{-1}.day^{-1}$ determined from *in vivo* male mice toxicity studies obtained from purified and cell-extract CYN derived from *C. raciborskii* (Humpage & Falconer 2003). However, the lowest observable adverse effect level (LOAEL) is reported as 20 $\mu g.kg^{-1}.day^{-1}$, and was derived from studies involving long-term chronic exposure of female and male mice to CYN derived from *Aphanizomenon ovalisporum* (Sukenik et al. 2006),

which could explain the discrepancy. The dose-response and threshold doses observed upon toxin exposure, depends upon the biological endpoint chosen. Although organ and/or body weight is the usual index of an adverse physiological response to toxin exposure, if an increase in protein levels in urine is to be considered an indicator of a toxic response then the NOAEL for CYN determined by Humpage and Falconer (2003) would be 60 $\mu g.kg^{-1}.day^{-1}$. The recommended guideline for safe levels of CYN in drinking water has been set for 1 $\mu g.L^{-1}$, based on the NOAEL of 30 $\mu g.kg^{-1}.day^{-1}$ (Humpage & Falconer 2003). However this is yet to be adopted in Australia by the National Health and Medical Research Council (NHMRC). CYN concentrations of up to 100 $\mu g.L^{-1}$ can be detected in bulk water of Queensland, Australia and Florida, USA (Burns et al. 2000), and levels of up to 1 – 10 $mg.L^{-1}$ have been recorded in the waters of Germany and Brazil (Carmichael et al. 2001). Thus, it is vital that research into the toxic effects of CYN is undertaken, with particular regard for low dose, chronic effects on the sensitive human reproductive system.

1.7.1 Incidence and Location of CYN

In recent years, the occurrence of C. *raciborskii* in human drinking water storages has increased significantly in various climates such as tropical and subtropical regions, temperate climates of Australasia, the US, Europe and cooler regions of Africa (Baker & Humpage 1994; Falconer et al. 1999; Falconer & Humpage 2006; Griffiths & Saker 2003; Padisak 1997). In the last two decades, research into the toxicity of CYN has found deleterious health effects in humans and animals (Hawkins et al. 1985; Ohtani et al. 1992). The "mystery disease" of Palm Island in 1979 was the first in Australia to be associated with toxicity arising from *C. raciborskii* algal blooms. Cylindrospermopsin was the probable causative agent of the hospitalisation of 138 children and 10 adults who suffered from hepatoenteritis, acute liver enlargement, constipation, vomiting and headaches (Byth 1980; Falconer 2001; Hawkins et al. 1985). A follow-up review of medical records from the children poisoned from the 1979 outbreak found an increased rate of gastrointestinal cancers in the period of 1982-1999 compared to an unexposed similar population; however no significance was found due to the low number of individuals in the exposed population (Falconer & Humpage 2006).

A second human poisoning arising from algal blooms was recorded in 1997 in Caruaru, Brazil, where 76 of the 131 patients undertaking treatment at the dialysis centre developed acute liver failure and died. The phytoplankton found in the clinic's water source was examined and revealed that it was contaminated with the cyclic peptide microcystin and CYN (Carmichael et al. 2001).

1.7.2 *In vivo* Toxicity of CYN

Cylindrospermopsin exhibits its toxicity firstly in the liver, where the effects occur in four phases: protein synthesis inhibition, membrane proliferation, fat droplet accumulation and cell death. This profile is comparable to the effects of cycloheximide, a potent protein synthesis inhibitor (Terao et al. 1994). In addition to the hepatotoxic effects in mice, histopathological analysis of tissues after acute CYN exposure found necrosis in kidney tissues, atrophy in the thymus, damage to heart tissue and ulcers in the gastrointestinal tract (Hawkins et al. 1985; Seawright et al. 1999; Terao et al. 1994). Several *in vivo* studies have been carried out to determine acute toxicity thresholds, using the standard LD_{50} endpoint. For freeze-dried cell suspension of cultured *C. raciborskii* an acute dose of 168 $mg.kg^{-1}$ was lethal for mice, 6-9 hours after administration, with an LD_{50} of 64 ± 5 $mg.kg^{-1}$ at 24 h (Hawkins et al. 1985). A subsequent study by Hawkins et al. (1997) using a sonicated suspension of *C. raciborskii* obtained from an ornamental lake resulted in an LD_{50} of 52 $mg.kg^{-1}$ at 24 h and 32 $mg.kg^{-1}$ at 7 d. In comparison, injection of purified CYN has a LD_{50} of only 2.1 $mg.kg^{-1}$ after 24 h and 0.2 $mg.kg^{-1}$ after 5 days (Ohtani et al. 1992).

The variation in toxicity thresholds between studies likely reflects variation between mouse strains, the degree of purification of CYN and the presence of additional, potentially toxic components within *C. raciborskii* extracts yet to be identified (Baker & Humpage 1994; Falconer 2001; Fastner et al. 2003; Hawkins et al. 1997). Many of the *in vivo* studies have used aqueous cyanobacterial extracts or purified CYN administered via intraperitoneal injection; however, the most likely route of exposure to CYN is via oral uptake (Seawright et al. 1999).

An oral exposure study investigated the effects of subacute exposure with increasing daily CYN concentrations of 10 – 55 $\mu g.kg^{-1}.day^{-1}$ in the drinking water

of female and male mice over a 42-week period (Sukenik et al. 2006). The CYN source was *Aphanizomenon ovalisporum*, and analyses were carried out on body and organ weights, serum and haematological samples at 20 and 42 weeks. The results were largely consistent with those of Humpage and Falconer (2003). Specifically, exposure to low concentrations of CYN was found to have no significant effect on body weight at a final daily intake of 50 $\mu g.kg^{-1}.day^{-1}$ after 42 weeks.

Toxicokinetic studies were carried out to determine the distribution and excretion pattern of CYN. An IP dose of 0.2 $mg.kg^{-1}$ of ^{14}C-labelled CYN was administered to Quackenbush mice, the results showing that CYN was predominately excreted into the urine (48.2%) with approximately 20.6% accumulating in the liver within 12 h (Norris et al. 2001).

Glutathione enzyme (GSH) and cytochrome P450 (CYP) systems are cellular defence mechanisms involved in the elimination of xenobiotics. Norris et al. (2002) investigated the toxic effects of CYN on hepatic cells pre-treated to lower GSH concentrations and found that 0.2 $mg.kg^{-1}$ significantly decreased hepatic GSH concentrations (Norris et al. 2002). However, as this dose is not lethal to mice, it is thought that decreases in hepatic GSH are not a primary mechanism of CYN toxicity (Humpage et al. 2005; Norris et al. 2002). In the same study, pre-treatment with CYP inhibitors attenuated toxicity in Quackenbush mice resulting in a 100% survival rate in comparison to controls, implicating CYP metabolism of CYN as an important mechanism of toxicity (Norris et al. 2002).

1.7.3 *In vitro* Toxicity of CYN

It was first noted by Terao et al. (1994) that CYN diminished the total amount of liver CYP enzymes which caused severe hepatotoxicity. CYN uptake *in vitro* was rapid and caused a complete and irreversible block of protein synthesis after 4 h exposure to CYN concentrations above 0.5 µM (Froscio et al. 2003). Although CYN blocked protein synthesis it did not immediately cause cytotoxicity as some mammalian cells survive short term without protein synthesis *in vitro*. When mouse hepatocytes were pre-treated with CYP inhibitors, such as proadifen, cytoxicity was diminished with no effect on protein synthesis inhibition. Thus CYP metabolites

induce cytotoxicity but not protein synthesis inhibition which was most likely mediated by the parent compound (Froscio et al. 2003). The CYP isoforms involved in the bioactivation of CYN and the generation of cytotoxic and genotoxic effects have yet to be identified (Froscio et al. 2003). Hence the two proposed mechanisms of action of CYN are the production of metabolites via CYP oxidation and the second is direct effect on protein synthesis inhibition bypassing production of CYN metabolites (Froscio et al. 2001; Terao et al. 1994).

1.7.4 The Effects of CYN on Cell Viability *in vitro*

The effects on the viability of cells after exposure to toxic chemicals can be investigated using a variety of methods. Cytotoxicity is a result of one or a combination of effects on a cell. These include DNA damage, protein synthesis inhibition, destruction of cell membrane, and inhibition of enzymatic reactions (Ishiyama et al. 1996). Cytotoxicity from exposure to xenobiotics such as CYN can be evaluated by various assays including the lactate dehydrogenase (LDH), MTT, MTS, ATP, trypan blue exclusion and crystal violet assays, and these are commonly used to determine viability of granulosa cells (Bain et al. 2007; Chaffkin et al. 1993; Ferran et al. 1998; Fessard & Bernard 2003; Froscio et al. 2003; Guet et al. 1999; Knaggs et al. 1998; Lambert et al. 2000; Runnegar et al. 1994; Soboloff et al. 2001; Young et al. 2005).

Figure 1-1 summarises the cytotoxic effects of CYN in various cell types and at various concentrations *in vitro* (Bain et al. 2007; Chong et al. 2002; Froscio et al. 2003; Runnegar et al. 1994). The outcome of cell viability after CYN exposure may be dependent on the sensitivity of the viability assay, mechanism of uptake of CYN in different cell types and concentration of CYN. Recent studies also suggest sensitivity to the cytotoxic effect of CYN differs between cell lines and primary-derived cells (Bain et al. 2007; Chong et al. 2002; Froscio et al. 2009). Toxicity to reproductive cells such as granulosa cells is not necessarily reflected in assessments of viable cell numbers but rather manifests as decreases in steroid hormone production (Endo et al. 1993; Hong et al. 2007; Young et al. 2005).

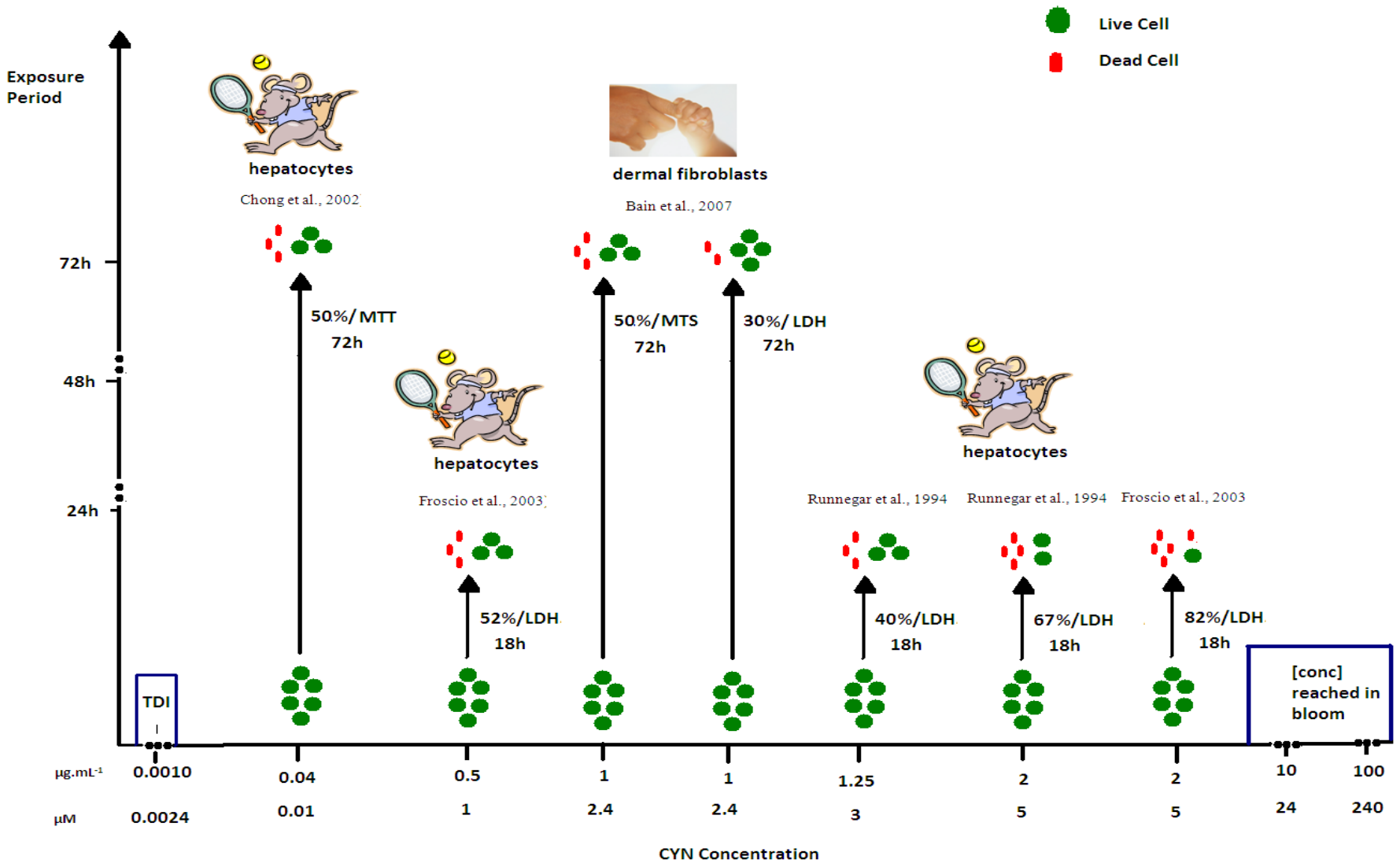

Live Cell
Dead Cell
Exposure Period
72h
48h
24h
hepatocytes
Chong et al., 2002
50%/ MTT
72h
dermal fibroblasts
Bain et al., 2007
50%/MTS
72h
30%/ LDH
72h
hepatocytes
Froscio et al., 2003
52%/LDH
18h
hepatocytes
Runnegar et al., 1994
40%/LDH
18h
Runnegar et al., 1994
67%/LDH
18h
Froscio et al., 2003
82%/LDH
18h
TDI
[conc] reached in bloom
µg.mL-1
0.0010
0.04
0.5
1
1
1.25
2
2
10
100
µM
0.0024
0.01
1
2.4
2.4
3
5
5
24
240
CYN Concentration

Figure 1-5: Review of CYN toxicity *in vitro*

Live cells are indicated by green circle, dead cells are indicated by red circles. The y-axis shows increasing exposure period (h) and the x-axis indicates increasing CYN concentration. The picture indicates organism from which cells were derived from mouse and human. Each arrow indicates an assay investigating toxicity of CYN at a specific concentration (µg.mL^{-1} or µM). MTT= MTT assay, MTS= MTS assay both of which are tetrazolium assays whereby viability assessment is based on functional mitochondrial dehydrogenase activity. LDH= Lactate dehydrogenase, which is released into culture medium due to disruption of cell membrane. Blue boxes indicate concentrations (conc) of CYN that can be reached and current guideline drinking water safety level. In summary CYN concentration ranges of 1µM – 5 µM were toxic to primary derived rat and mouse hepatocytes causing 40-67% cell lysis and complete cell death after 18h, respectively, as measured by LDH assay (Froscio et al. 2003; Runnegar et al. 1994). Moreover, cytotoxicity in primary human dermal fibroblast (HDF) cells and rat hepatocytes occurred after 72 h exposure to 2.4 µM, as measured by the MTS (IC_{50}) and LDH (30% cytotoxicity) assays, respectively (Bain et al. 2007; Chong et al. 2002).

1.7.5 Other Effects of CYN

The indication that there was an increase in gastrointestinal cancers throughout the period of 1982-1999, following the cyanobacterial poisoning of 1979 in Palm Island (Section 1.7.1), suggests that CYN may have carcinogenetic effects. However further research is required as the increase was not statistically significant (Chong et al. 2002; Froscio et al. 2003; Humpage et al. 2000; Humpage et al. 2005). Both *in vitro* and *in vivo* research have been carried out to investigate the carcinogenic and genotoxic effects of CYN. Initial *in vitro* studies using the WIL2NS lymphoblastoid cell-line found that CYN was genotoxic, inducing both chromosome fragmentation and whole chromosome loss (Humpage et al. 2000). This was detected by the comet assay, and with a significant increase in micronucleus formation observed at CYN concentrations of 6 and 10 $\mu g.mL^{-1}$. CYN is also considered to be a mutagen, causing the formation of DNA adducts *in vivo* (Shaw et al. 2000). Falconer and Humpage (2001) showed that CYN was potentially carcinogenic by inducing tumours in male Swiss Albino mice, and *in vivo* genotoxic effects where significant DNA strand breakage was detected in DNA isolated from mice livers after 0.2 $mg.kg^{-1}$ exposure for 6, 12, 24 and 48 h (Falconer & Humpage 2001; Shen et al. 2002). The direct genotoxic effects of CYN may vary between cell types and species, since then a study carried out by Fessard and Bernard (2003) using the comet assay did not detect direct genotoxic effects of CYN on Chinese Hamster Ovary K1 cells *in vitro*. They found that cell growth was inhibited, with cell blebbing and rounding occurring, but without direct damage to DNA (Fessard & Bernard 2003). CYN-metabolites may also be genotoxic, as genotoxic effects of CYN were completely inhibited in primary-mouse hepatocytes co-treated with CYP inhibitors, omeprazole (CYP2C19) and 2-diethylaminoethyl-2,2-diphenylvalerate (SKF525A, a CYP3A enzyme) (Franklin & Hathaway 2008; Humpage et al. 2005). Thus, the various profiles of CYP enzymes (section 1.1.3) present in different cell types may generate metabolites with varying genotoxic potencies. The study by Humpage et al. (2005) also found that the DNA damage caused by CYN at 0.05 µM was 10-fold below the effective concentration (EC_{50}) inducing cytotoxicity. A marker for lipid peroxidation, malondialdehyde (MDA), was not affected after CYN exposure (Humpage et al. 2005).

1.8 Cylindrospermopsin- A Possible Reproductive Toxin?

Recent *in vivo* studies implicate CYN as a developmental toxicant. For example, Rogers et al. (2007) evaluated the *in uteri* toxicity of purified toxin in mice. Exposure to 32 $\mu g.kg^{-1}$ CYN (via IP injection) at gestational days (GD) 8-12 was lethal to 60% of dams, although in the surviving dams, no adverse effects on litter size or fetal weight were found; at the higher dose of 50 $\mu g.kg^{-1,}$ litter size was decreased. The same concentrations administered at GD 13-17 *in uteri*, had lower maternal toxicity in comparison to GD 8-12 but increased number of dead pups, premature births, and significant decreases in pup weight with reduced postnatal growth (Rogers et al. 2007). The increased pup toxicity during the later gestational period may be associated with the inability of CYN to cross the placenta and thus metabolites were unable to affect the developing embryo during organogenesis. Although CYN did not cross the placenta, its delay and absence of toxicity in the early gestational period may be due to an absence of the necessary enzymes for metabolic activation of CYN or the delayed development of the enzymes later in gestation required to cross the placenta (Rogers et al. 2007). Premature births are highly uncommon among the murine species, previous recorded incidences of premature births were due to altered estradiol and progesterone levels by xenobiotics such as ticonazole (Rogers et al. 2007) and thus CYN may have affected steroid hormone production *in vivo* which resulted in the premature births detected by Rogers et al. (2007).

More recently, Young et al. (2008) investigated the effects of CYN on the viability and steroid hormone production by human primary-derived granulosa cells *in vitro*. Viability was assessed by the MTT assay and basal and human chorionic gonadotropin (hCG)-stimulated estradiol and progesterone production was quantified by radioimmunoassay. Cytotoxicity was observed after 24 h exposure to 1 $\mu g.mL^{-1}$ (2.4 µM) CYN. Basal progesterone production decreased after 24 h exposure to 0.0625 $\mu g.mL^{-1}$ (0.3 µM) CYN, while hCG-stimulated progesterone production decreased after 6 h exposure to 1 $\mu g.mL^{-1}$ (2.4 µM) CYN.

A key finding was that decreases in progesterone production per well did not occur in hCG non-responsive cells. This suggests that a heterogeneous population of cells

existed which were not only hCG non-responsive but the same cells did not respond to CYN, whereby cytotoxicity or steroid hormone production was not affected after CYN exposure. It is the 24 – 72 h period post-isolation that the majority of granulosa cells differentiate and desensitise from *in vivo* gonadotropin stimulation. Thus, the 18 h post-isolation granulosa cells used by Young et al. may not have been responsive to further hCG stimulation (Lambert et al. 2000; Landefeld et al. 1997). It is also possible that those cells which did not respond to hCG were non-steroidogenic cells, such as macrophages and leukocytes (Baranao et al. 1995; Beckman et al. 1991; Lobb & Younglai 2006; Loukides et al. 1990). The granulosa cells used in the study by Young et al. (2008) were obtained from women seeking ART for their infertility. Information on each woman's individual infertility and reproductive histories in this study were not disclosed. It is unclear if the ART-sourced granulosa cells recapitulate the responses of normal, healthy cells, since donors with abnormal fertility are likely to be over-represented, and all donors underwent ART hyperstimulation. It is possible that women seeking ART with endocrine-related infertility may have unhealthy granulosa cells which respond differently to *in vitro* gonadotropin stimulation and exposure to toxins, such as CYN (Young et al. 2008). Hence, there is a need to determine if sensitivity to the effects of CYN differs between granulosa cells representative of the normal, healthy, fertile general population and those classified as unhealthy (section 2.2.1 Characterisation of Primary-Derived Human Granulosa Cells).

1.9 Environmental Toxins and their Effects on Steroid Hormone Production

Reproductive toxicology has been a major concern for over two decades, with various xenobiotics acting as reproductive toxicants (Mattison 1983). Xenobiotics are generally defined as a foreign compound to the human body and can sometimes be synonymous to environmental pollutants. A toxin is defined as a poisonous substance produced by a live organism (MediLexicon 2011), such as CYN. These toxins, if adversely affecting the reproductive system of the host organism are reproductive toxicants. The toxicity of a toxin is dependent and varies with species, gender and it is heavily dependent on how the toxin is metabolised, absorbed, detoxified and cleared within an organism (Mattison 1983). If the metabolites

produced are not cleared from the body then damage will occur. A toxin can have direct or indirect effects, the latter requiring metabolism of the toxin in order to act as a reproductive toxicant (Mattison 1983). It is now increasingly common to find environmental pollutants that can alter endocrine homeostasis in granulosa cells by disrupting the production of steroid hormones causing development and reproductive defects. Such compounds are referred to as endocrine disruptors (Heimler et al. 1998), and include pollutants like dioxin, which induces programmed cell death (apoptosis) and depletes substrate availability for steroidogenesis in human granulosa cells in both a dose- and time-dependent manner (Heimler et al. 1998). Other endocrine disruptors include:

- Bisphenol A (BPA), a plasticizer and well known endocrine disruptor inhibits aromatase (CYP19) activity and estradiol secretion by impairing normal FSH-stimulated aromatase mRNA and protein expression in granulosa cells (Kwintkiewicz et al. 2009);

- Roundup (a herbicide), with the chemical name of isopropylamine salt of Glyphosate, inhibits steroidogenesis by targeting StAR expression post-transcription. The StAR protein is the rate limiting step in the steroidogenic pathway. The active ingredient of Roundup – glyphosate was not the inhibitory component, thus more research is required to identify the inhibitory component (Walsh et al. 2000); and,

- Amino-glutethimide, a complete steroidogenesis (CYP11A1) inhibitor; pharmacotherapy for steroid-dependent cancers such as breast cancer and adrenal function suppressant in Cushing's syndrome (Gross et al. 2007; Harvey & Everett 2003; Havelock et al. 2004).

In luteinised rat granulosa cells, Adlay, a Chinese herbal medicine (100 µg.mL^{-1}), decreased progesterone and estradiol production by inhibiting major signalling pathways (cAMP-PKA signal transduction pathway), intracellular messengers (cAMP accumulation), steroidogenic enzyme activity (CYP11A1 and 3βHSD enzyme) and protein expression of steroidogenic enzymes (CYP11A1 and StAR) *in vitro* (Hsia et al. 2007).

1.9.1 Do Parallel Mechanisms of CYN Toxicity Exist in Human Reproductive Cells?

CYN's toxic mechanisms are also found in the human reproductive systems. These mechanisms can affect viability of steroidogenic cells, granulosa cells and mature spermatozoa (Hsia et al. 2007).

1.9.1.1 Cylindrospermopsin Uptake processes *in vitro*

Since CYN is a water-soluble toxin, cellular uptake mechanisms are required for it to penetrate the lipid bilayer and enter cells. However less efficient mechanisms have been observed in rat hepatocytes where the primary uptake mechanism involves the bile acid transport system, with passive diffusion of CYN into the cell due to its low molecular weight (415Da) a slower, secondary process (Chong et al. 2002; Froscio et al. 2009; Froscio et al. 2003). Hence the effects of CYN *in vitro* may be dependent on the uptake mechanism, which may influence the response induced in a particular cell type (Chong et al. 2002).

1.9.1.2 Apoptosis in Granulosa Cells

The gonadotropins FSH and LH are pro-survival signals for granulosa cells, moderately increasing cAMP levels, followed by a desensitisation period and increasing progesterone production up to four–fold (Aharoni et al. 1995; Amsterdam et al. 1999). Yet human dermal fibroblasts exposed to CYN show increased cAMP levels and induction of apoptosis (Bain 2007). Similarly, pre-ovulatory granulosa cells stimulated *in vitro* with increased cAMP levels to very high levels undergo apoptosis mediated by p53 (Aharoni et al. 1995; Amsterdam et al. 1999). Granulosa cell differentiation (i.e. increased steroidogenic output) is a response to moderately increased cAMP levels; however, very high levels of cAMP seem to induce apoptosis. This also occurs *in vivo* and is dependent on the duration

of the cAMP signal (Aharoni et al. 1995; Amsterdam et al. 1999; Keren-Tal et al. 1995). The results presented by Aharoni et al. (1995) suggest that stimulants and/or exogenous compounds which increase cAMP levels, excluding gonadotropin releasing hormone (GnRH), bypass G_s –coupled receptor processes. From this, it can be speculated that certain checkpoints or regulatory points are bypassed, from which the signal can be directed to either apoptosis or prolonged cell survival. This was evident in a study carried out by Keren-Tal et al. (1995) which found that granulosa cells can maintain a repertoire of signalling pathways directed to either cell death or survival in the development of a mature follicle.

In vivo, ovulation is considered to be analogous to an inflammatory response (Espey et al. 2004). This is indicated by increased vascularisation of the follicle (hyperaemic) which induces large amounts of prostaglandins (PGs) and hyaluronan (HA) rich-matrix that are usually found at sites of inflamed tissue (Richards et al. 2008). As a result, key immediate early-response genes for steroidogenesis and angiogenesis are activated (Espey et al. 2004; Richards et al. 2008). It is these key immediate early-response genes and respective nuclear transcription factors which have also been identified in pathways associated with CYN toxicity. Cells respond to stress and DNA damage by activating transcription factors such as tumour suppressor protein 53 (p53) which regulates gene expression associated with growth arrest, programmed cell death (apoptosis) and DNA repair. After 24 h exposure to 1µg mL^{-1} CYN (2.4 µM), human dermal fibroblasts responded with increased expression of apoptotic genes, most likely attributed to activation of anti-proliferative effects of transcription factors p53 and NF-κB (Bain 2007; Bain et al. 2007; Chu et al. 2004). The DNA damage response pathways were also up-regulated and mediated by the immediate response genes *JUN*, *FOS*, and *MYC* (Bain 2007).

1.9.1.3 Signalling Pathways in Granulosa Cells and CYN's Up-Regulation of Gene Expression

TNF-α induces apoptosis in granulosa cells, whilst also modulating steroid hormone production in intact human follicles in response to gonadotropins (FSH/hCG), via cAMP-dependent PKA pathways such as MAPK, regulating StAR expression

(Amsterdam & Sasson 2004; Baud & Karin 2001; Espey et al. 2004; Peluso 2003; Saliba & Henrot 2001; Stylianou & Saklatvala 1998; Yu et al. 2005). CYN also induces an inflammatory stress response by activating the MAPK pathway which activates transcription of Activator Protein-1 and NF-κB via TNF-α (Baud & Karin 2001; Hess et al. 2004). Even if increased cAMP levels commit granulosa cells to apoptosis, it is not necessarily reflected in an immediate cessation of steroidogenesis (Aharoni et al. 1995; Amsterdam et al. 1999). Granulosa cells are capable of steroid hormone production in the early stages of apoptosis unless major levels of intracellular disorganisation of key organelles (mitochondria, smooth endoplasmic reticulum and golgi body) and presence of lipid droplet has occurred (Aharoni et al. 1995; Amsterdam et al. 1999).

1.9.1.4 Possible Oxidative Effects of CYN

In all mammalian cells, glutathione (GSH) quenches ROS generated in tissues exposed to high levels of exogenous toxins such as the liver, kidneys, lung and intestines (DeLeve & Kaplowitz 1991). GSH levels were significantly reduced in mouse hepatocytes exposed to a single-IP dose of 0.2 $mg.kg^{-1}$ CYN *in vivo* (Norris et al. 2002). The mechanisms by which GSH levels fall can be associated with oxidative stress (Deneke & Fanburg 1989). Although Bain et al. (2007) showed that CYN exerted stress in cells and induced the tumour suppressor gene p53, the type of stress could not be determined in the study. CYN was found not to decrease GSH levels due to oxidative stress, but by inhibiting GSH synthesis (Runnegar et al. 1995). When mouse hepatocytes were exposed to CYN concentrations of 1 µM, GSH levels were depleted after 18 h and after only 10 h at a higher concentration of 5 µM; both occurred prior to CYN-associated cytotoxicity and were not correlated with increased lipid peroxidation (Humpage et al. 2005; Runnegar et al. 1995).

The two mechanisms by which CYN confers its toxicity, directly via protein synthesis inhibition and indirectly via CYP-metabolites are not relevant for mature spermatozoa. Hence, it is unlikely that CYN will directly compromise the membrane integrity of mature human spermatozoa *in vitro*, but lipid peroxidation may occur due to decreased defence mechanisms as spermatozoa develop and mature *in vivo* (Aitken et al. 2004).

1.10 The Effects of Environmental Toxins on Human Reproductive Cells- A Need for Investigation

1.10.1 Human Granulosa cells Derived from Assisted Reproductive Technology

Human granulosa cells can be used to investigate effects of xenobiotics on viability and steroidogenesis *in vitro* (Enmark & Gustafsson 1999; Fan et al. 2007; Harvey & Everett 2003; Hughes et al. 1990; Sanderson 2006). Many xenobiotics, such as polychlorinated biphenyls (PCB's), fungicides and herbicides have been found to interfere with the estradiol producing enzyme, aromatase, resulting in a dose-dependent decrease in estradiol production by granulosa cells (Sanderson 2006). Thus, it is necessary to determine if and how toxins interfere with steroidogenesis and viability, specifically focussing on cyanobacterial toxins such as CYN, as similar mechanisms of toxicity are present in reproductive cells. (Fan et al. 2007; Nishi et al. 2001; Ohno et al. 2004).

1.10.2 Human Spermatozoa

There are various studies that have investigated the effects of naturally occurring environmental xenobiotics and chemicals on human spermatozoa (Fraser et al. 2006; Pacifici et al. 1995; Pichini et al. 1994; Wagner et al. 1990; Yousef et al. 2007). Declines in semen quality are associated with a decline in fertility (Aitken et al. 2004; Pichini et al. 1994). The effects of xenobiotics on viability and motility of spermatozoa needs to be investigated as xenobiotics can potentially be transported into seminal plasma by ion trapping process dependent on lipid solubility and pH of plasma and seminal fluid (Aitken et al. 2004; Pacifici et al. 1995; Pichini et al. 1994; Wagner et al. 1990; Yousef et al. 2007). Interestingly, spermatozoa that survive direct exposure to xenobiotics and chemicals in seminal fluid still have to combat those present in cervical mucus of the female, further emphasising the importance of reproductive toxicological studies (Aitken et al. 2004; Wagner et al. 1990).

1.11 Research Aims

This project aims to investigate the effects of the blue green algal toxin cylindrospermopsin on human reproductive cells from patients undergoing Assisted Reproductive Technologies (ART).

1.12 Scope

The scope of this research specifically investigates the effects of CYN on human derived spermatozoa and primary-derived human granulosa cells from ART patients. Furthermore granulosa cell functionality and (progesterone and estradiol production) protein synthesis will also be investigated.

1.13 Hypotheses

1.13.1 Human Spermatozoa

Cylindrospermopsin will not affect the viability of human mature spermatozoa. Spermatozoa which have yet to fertilise the oocyte are transcription inactive and lack CYP enzymes. Thus the main mechanisms by which CYN confers its toxicity are absent. However, CYN may potentially generate free radicals that may target spermatozoon membranes, which are densely packed with lipids, and hence spermatozoon viability is at risk and needs to be investigated.

1.13.2 Human Primary-Derived Granulosa Cells

Cylindrospermopsin will be cytotoxic to human primary-derived granulosa cells as they contain the enzymes belonging to the CYP family and, thus have the ability to metabolise CYN and generate toxic metabolites.

Cylindrospermopsin will inhibit protein synthesis in granulosa cells as previous studies have shown that CYN causes protein synthesis inhibition.

Cylindrospermopsin will decrease progesterone and estradiol production by human granulosa cells potentially interfering with CYP enzymes in the steroidogenic pathways such as the CYP11A1 enzyme.

1.13.3 Experimental Approach

Presented below is a flow diagram representing experimental approaches used to address the aims of this project.

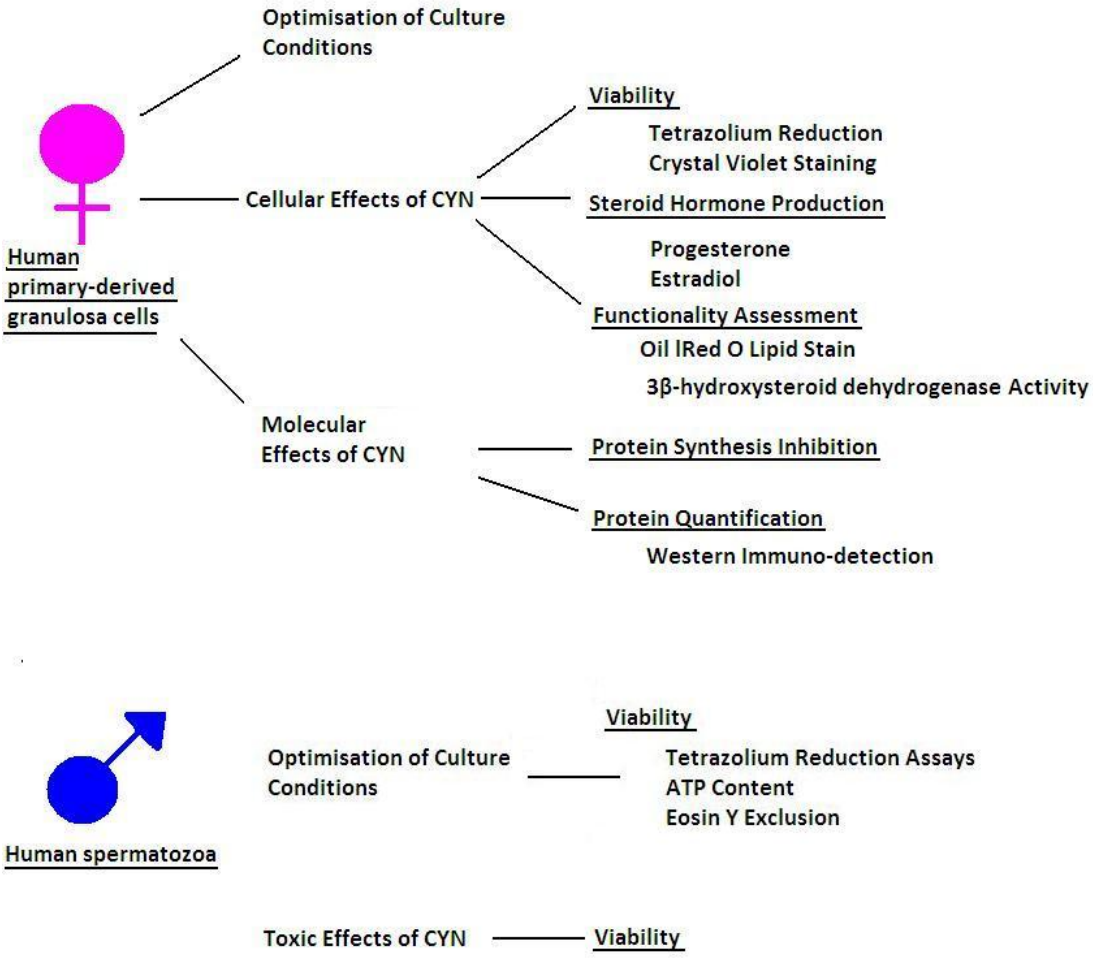


Figure 1-6: Flow-diagram of Experimental Approach

Chapter 2. General Material and Methods

2.1 General Chemicals and Reagents

Chemicals and reagents that were used in cell culture experiments were of cell culture grade and are listed in Chapter 8 Appendices: Reagents, Stock Solutions & Media Preparation.

2.2 Assisted Reproductive Technology (ART)

During ART cycles, women are stimulated to produce multiple follicles (Section Chapter 1 General Introduction) from which granulosa cells can be collected (Beckman et al. 1991; Chaffkin et al. 1993; Quinn et al. 2006). An advantage of using ART derived- granulosa cells was that it allowed access to the clinical ART case notes (listed in Table 2-1). The information collected, based on their infertility factors, can indicate the likelihood of producing healthy granulosa cells with normal functionality (Table 2-1). The granulosa cells isolated for use in research were segregated into two subgroups referred to within this research as ‘healthy and ‘unhealthy’. This segregation was based on the woman’s infertility factor and from the type of ART treatement.

Parameter	Fertility Indicator of:	Status of Granulosa Cells
Age	Influencing Factor	Healthy (< 35 years)/ Unhealthy (>36 years)
ART Procedure		
Intra-cytoplasmic Sperm Injectin (ICSI)	MIF	Healthy
In Vitro Fertilisation (IVF)	FIF	Unhealthy
Infertility Issue		
Ectopic Pregnancy (EP)	FIF	Healthy
Endometriosis	FIF	Unhealthy
Irregular Menstrual Cycle/Amenorrhoea	FIF	Unhealthy
Polycystic Ovary Syndrome (PCOS)	FIF	Unhealthy
Tubal Ligation (TL)	FIF	Healthy
Spermatozoa	MIF	Healthy
Previous Pregnancy		
Yes	Influencing Factor	Healthy
No	Influencing Factor	Unhealthy
Years of Infertility		
< 1 year		Unknown
2-5 years	Influencing Factor	Unhealthy
> 6 years	Influencing Factor	Unhealthy
Demographic Information		
Smoker	Influencing Factor	Unknown
Body Mass Index (kg/m^2)	Influencing Factor	Unknown
Regularity of Menstrual Cycle		
Regular Cycle (26-28d)	-	Healthy
Long Cycle(>33d)	-	Healthy
Short Cycle(<28)	-	Healthy
Irregular Cycle	FIF	Unhealthy
Number of Previous Treatment Cycles	Influencing Factor	Unhealthy
Stimulation Protocol		
Long Protocol	-	-
Short Protocol	-	-
ART Data		
Number of Follicular Fluid Tubes	Responsiveness	-
Number of Mature Follicles (>15mm)	Responsiveness	Healthy >3 follicles/ Unhealthy<3 follicles
Number of Oocytes Aspirated	Responsiveness	-
Number of Embryos Transferred	Responsiveness	-
Pregnancy Outcome	Responsiveness	Healthy= Positive pregnancy
Granulosa Cells Isolated (cells/mL)	Responsiveness	-

Table 2-1: Demographic and Ovarian Response Data

Lists demographic and clinical factors that indicate infertility due to male infertility factor (MIF) or female infertility factor (FIF), these factors can also influence fertility outcome and the responsiveness to ART regime. The factors indicate the status of isolated granulosa cells as being healthy, unhealthy or unknown.

2.2.1 Characterisation of Primary-Derived Human Granulosa Cells

The overall aim of using primary-derived granulosa cells in this research was to determine if CYN can act as a reproductive toxicant. As described in section 1.1, granulosa cells are a good reproductive model to investigate the effects of toxins *in vitro*. Natural IVF cycles are those that allow the normal reproductive cycle to mature an oocyte which is then retrieved. However most often this oocyte is missed and is a limitation of this type of ART, thus the development of hyperstimulated-IVF protocols whereby the ovary produces multiple mature oocytes thus increasing the chances of producing a mature healthy oocyte.

However the affects of the hyperstimulation protocol on the health of status of the oocyte have had minimal investigation. The current theory is that granulosa cells function differently to their normal counterparts due to the hyperstimulation *in vivo* (Lobb & Younglai 2001). Granulosa cells obtained from hyperstimulation regimes produce five-fold less progesterone than their normal counterparts and hence cannot attain their full steroidogenic output (Lobb & Younglai 2001). With this in mind the aim of this research was to determine the toxicity of CYN on reproductive cells and CYN's effects on viability and steroidogenic output. If the cells are adversely affected, then further investigations as to whether this is attributed to the mechanisms also present in other cell types (refer to section 1.7) will be carried out. Furthermore many patients seeking fertility treatment (attributed to endocrine, biological age and physical barriers) may have reproductive cells that function differently to the general population that have been able to conceive naturally (Alviggi et al. 2009).

The cumulus-oocyte cell complex contains a heterogeneous population of cells which vary in steroidogenic capabilities, fertilisation and cleavage potential (Bar-Ami et al. 1989). Progesterone production by granulosa cells isolated from large mature pre-ovulatory follicles is dependent on the maturation of the corresponding cumulus-oocyte complex (Bar-Ami et al. 1993). The maturation and steroidogenic capability of granulosa cells is dependent on the *in vivo* priming regime, of which FSH stimulated (the GC used in this work were from FSH primed ART-cycles, refer to section 2.3) have higher estradiol and progesterone output (Lobb et al.

1998). It was not the scope of this research to assess the cumulus-oocyte complex as they were collected by the technical staff for use in ART-cycle, as routinely done and further described in section 2.3 (Bar-Ami et al. 1993; Lobb et al. 1998).

The response to the hyper-stimulation regime can be influenced by demographic factors such as duration of clinically diagnosed infertility, age of woman (above or below 35 years of age), current or prior smoker and body mass index; and these data can be used as predictors of ovarian response. The ovarian response can be determined from the clinical data, the indicators being basal FSH levels and the number of mature oocytes generated, Table 2-1. The two treatment regime options for ART are IVF or ICSI. Female infertility factors (FIF) are attributed to the following: tubal ligation (TL), ectopic pregnancy (EP), polycystic ovary syndrome (PCOS), irregular menstrual cycle, amenorrhoea, or endometriosis; and are associated with women undertaking IVF cycles, Table 2-1 (Esterhuizen et al. 2001; Ng et al. 2000; Stephansson et al. 2009). Male infertility factors (MIF) include: semen volume, semen pH, concentration of spermatozoa in each semen sample, motility of spermatozoa, morphology and white blood cell count in semen sample, as listed in, Table 2-3 and are associated with ICSI cycles for ART treatment of male infertility. Couples undergoing infertility treatment for female reproductive problems and with suboptimal spermatozoa parameters were classified as having both male and female infertility factor. Granulosa cells defined as healthy were primarily from ICSI cycles (MIF). Granulosa cells isolated from healthy women, with normal fertility (non-FIF related) were regarded as representative of those from a normal, healthy population. It is speculated that sub-fertile women with endocrine disorders will have unhealthy granulosa cells, and respond poorly to their ovarian hyper-stimulation regime. Granulosa cells which survive in culture 24 h-post isolation can be considered healthy (Lobb et al. 1998). However the definition of healthy and unhealthy granulosa cells in this study is referring to the development of granulosa cells *in vivo* whereby MIF and FIF factors have been shown to influence the development of granulosa cells and their functionality. Thus unhealthy granulosa cells which have poorly developed *in vivo* may be more sensitive to reproductive toxins than their healthy counterparts. Thus by selecting granulosa cells from ‘healthy’ and ‘unhealthy’ women and observing viability and

steroidogenic output in control treatments and CYN exposed cells it will identify differences in sensitivity, similar to a study carried out by Huang et al. (2004). The differences in sensitivity to the toxin may be due to the functionality, development of GC due to hyperstimulation or in the case of the unhealthy cohort poor development *in vivo* associated with the infertility factor.

2.2.1.1 The Study Cohort

A total of 55 women were included in this study, of which 35 cases were attributed to MIF (healthy), 5 cases were attributed to FIF (unhealthy) and 15 cases were unable to be classified (Appendix Chapter 8, Table 8-1). The combined data of all women (n=55) were: mean age 35 ± 5 years, mean body mass index 26.7 ± 5.9 (above normal range of 18-25), although with normal response to the hyper-stimulation regime, Table 2-2. When granulosa cells were segregated into MIF, FIF and unknown fertility factors, the response to the hyper-stimulation regime were also within normal parameters, Table 2-2. The differences between healthy and unhealthy granulosa cells allowed the identification of extraneous variables which may influence the results. The data from women used in this study indicate that the granulosa cells were representative of a normal, healthy and fertile population. It is possible that other factors not listed in Table 2-2, could contribute to the development of unhealthy granulosa cells despite a normal and good ovarian response occurring. There were no significant differences in the demographic and ovarian data collected from patients classified as having MIF, FIF, combined or unknown infertility, (Chi-squared test, $p>0.05$).

	Infertility Factor			
	Combined	Male Factor	Female Factor	Unknown
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Age (years)	35 ± 5	35 ± 7	37 ± 4	35 ± 3
FSH Level (IU/L)	7.78 ± 3.8	7.79 ± 4.27	8.7 ± 4.36	7.4 ± 2.01
Body Mass Index (BMI kg/m^2)	26.7 ± 5.9	27.75 ± 6.12	25.88 ± 2.81	23.1 ± 6.19
Maximum Plasma E_2 (pmol/L)	8112 ± 6243	8064 ± 6833.00	7770 ± 4989.00	8338 ± 5454.00
Number of Mature Follicles (>15mm)	5 ± 3	6 ± 4	4 ± 3	5 ± 2
Number of Oocytes Aspirated	11 ± 7	11 ± 6	13 ± 10	12 ± 9
Number of Follicular Fluid Tubes	7± 4	6 ± 3	7 ± 5	7 ± 4
Number of Granulosa Cells Isolated (cells/mL)	$8.18 \times 10^5 \pm 6.61 \times 10^5$	$8.18 \times 10^5 \pm 5.35 \times 10^5$	$7.35 \times 10^5 \pm 2.56 \times 10^5$	$8.32 \times 10^5 \pm 9.85 \times 10^5$
N	55	35	5	15

Table 2-2: Summary of Demographic and Ovarian Data of ART Cases

Total ART cases N=55; Basal FSH (IU/L) blood sample taken prior to commencement of treatment cycle; BMI= body mass index (kg/m^2); E_2 = maximum plasma estradiol concentrations (pmol/L); Number of Mature Follicles aspirated per woman for each cycle; Number of Oocytes aspirated refers to the total number of oocytes collected from cycle; Number of Follicular Fluid Tubes refers to number of tubes of follicular fluid collected from each woman on day of oocyte retrieval; Number of GC isolated refers to GC yield from density gradient centrifugation; mean= ± 1 standard deviation.

.

2.2.2 Characterisation of Spermatozoa Samples Used in the Study

The optimal parameters for a fresh semen sample to be a reflection of normal functioning spermatozoa as set out by the World Health Organisation (WHO) are listed in Table 2-3. A summary of the semen analyses from the male cohort used in this study is detailed in Table 2-4. Although these parameters individually do not indicate whether successful fertilisation will take place, they provide valuable information on spermatozoa functionality, and were used as the selection criteria to assess which samples were included in experiments (Liu & Baker 1992). This body of research involved both cryopreserved and fresh spermatozoa from males seeking ART. An analysis of the semen samples prior to cryopreservation indicated normal, healthy spermatozoa which conform to the WHO (1992) specifications detailed in, Table 2-3. However, post-thawing, the samples had decreased motility, viability and were less concentrated. This was the only source of cryopreserved samples available. Viable spermatozoa were also isolated and included in the research. Cryopreserved samples from each male were stored in multiple straws. For each experiment, four straws were used; samples that showed greater than 20% motility post-thawing proceeded were used in the research as carried out in clinical practice.

Spermatozoa Parameter	Normal Criteria
Volume	>2.0mL
pH	7.2-7.8
Concentration	>20×10^6sperm/mL
Motility	>50% with forward progressive motility
Morphology	>30% with normal morphology
WBC	<1×10^6/mL

Table 2-3: Normal Spermatozoa Parameters

The criteria of a healthy semen sample set by the World Health Organisation (1992). WBC = white blood cell concentration.

Spermatoza Source	Variable	Mean ± Std Dev		WHO
		Pre-cryopreservation	Post-thaw	Reference Value
Cryopreserved	*Motility (%)*	54 ± 7	33 ± 7	>50%
	Viability (%)	n/av	34 ± 12	-
	Concentration (sperm/mL)	1.8 x 10^8 ± 1.3 x 10^8	2.8x 10^6 ± 6.6x 10^5	>20 x 10^6 sperm/mL
Fresh	*Motility (%)*	60 ± 20	n/ap	>50%
	Viability (%)	66 ± 12	n/ap	-
	Concentration (sperm/mL)	1.67 x 10^7 ± 1.3 x 10^7	n/ap	>20 x 10^6 sperm/mL

Table 2-4: Summary of Semen Analysis of Cryopreserved and Fresh Spermatozoa

Cryopreserved (n=7) and fresh (n=10) spermatozoa from semen samples were analysed for forward progressive motility (%) and presence of live spermatozoa (%) and concentration (sperm/mL). Pre-cryopreservation refers to analysis of semen prior to cryopreservation; post-thaw refers to semen sample analysis after samples were allowed to thaw. Pre- cryopreservation % viability of spermatozoa was not determined by the IVF Laboratory thus data not available (n/a). Fresh spermatozoa samples were not cryopreserved and thus "post-thaw" data was not applicable (n/a). The World Health Organisation (WHO 1992) reference guidelines of a normal healthy semen sample were included to make direct comparison to each variable.

2.3 Isolation of Human Granulosa Cells

Human granulosa cells were obtained from women (total n=55, Flinders Clinical Research Ethics approval 47/00) undergoing ART at Flinders Reproductive Medicine Unit, South Australia. The number of participants recruited in this thesis was limited as it was dependent on their suitability with respect to the selection criteria and the patients consent for the use of their cells in research. Signed consent to use cells for research was obtained from each patient prior to collecting cells. ART regimes involve the use of gonadotropins which stimulate the ovaries to allow the growth of multiple follicles; there are two protocols which can be used. The two protocols include short protocol (FLARE-up) or long protocol. The first step involves the down-regulation of the endogenous cycle by administration of gonadotropin releasing hormone (GnRH), Synarel (Serono Laboratories, Sydney, Australia). This was then followed by hyper-stimulation with recombinant FSH, Puregon (Organon, Australia) or Gonal F (Serono Laboratories, Sydney, Australia) to produce multiple follicles. What is commonly referred to as the "short" protocol involves the additional use of the oral contraceptive pill for the duration of one menstrual cycle, followed by two days without the contraceptive pill to allow onset of menstruation which is then followed by the administration of Synarel on day 2 of the new menstrual cycle and the incorporation of FSH by day 4. The concentration at which Synarel is administered is maintained throughout the regime and stopped prior to human chorionic gonadotropin administration (hCG). The short protocol allows the removal of remaining corpus luteum from previous cycles and the administration of Synarel on the "contraceptive free" days which stimulates the woman's pituitary gland to "Flare-up" FSH production. The short protocol is generally recommended for women who have previously responded poorly to the long protocol, older patients and women with raised FSH levels at the beginning of their periods. In contrast, the long protocol does not incorporate the oral contraceptive pill but involves the administration of Synarel 7 days prior to onset of menstrual cycle and the incorporation of FSH on day 4 of the menstrual cycle. In this protocol concentration of Synarel is reduced throughout the regime and stopped prior to hCG. An ultrasound of the ovaries determines whether the follicles are

mature (minimum of 2 follicles with 18 mm diameter). Human chorionic gonadotropin (hCG; 5000 IU; Profasi; Serono Laboratories) is then administered after 34-36 h, whereby the women become peri-ovulatory and their follicles are ready to be aspirated. Oocytes can then be collected from the follicular fluid and used for *in vitro* fertilisation. There were four women who underwent the short protocol, these women were seeking ART for male infertility factors and were considered to have normal, healthy granulosa cells representative of the fertile general population.

After the removal of the oocytes-cumulus complex the granulosa cells were isolated from the remaining follicular fluid (FF). This process is most common for granulosa cells to be used *in vitro* culture (Chaffkin et al. 1993; Lobb et al. 1998; Quinn et al. 2006). The follicular fluid containing the granulosa cells were transferred into 10 mL tubes (Southern Cross Science) to evenly distribute volumes and eliminate any blood clots. Follicular fluid was centrifuged at 107 × *g* for 10 min, the supernatant was discarded and the pellets were resuspended in non-supplemented Ham's F12/Dulbecco's Minimum Essential Medium (Appendix 8.2.2 Granulosa Cell Base Medium), followed by a second centrifugation at 107 × *g* for 10 min. Granulosa cells were then isolated using a Ficoll-Hypaque (equivalent to Lymphoprep, δ= 1.0076 g.mL^{-1}) density gradient, in which 2 mL of granulosa cell sample was layered on top of a 3 mL Ficoll-Hypaque layer (Chapter 8 Appendices: Reagents, Stock Solutions & Media Preparation) gradient and centrifuged at 375 × *g* for 10 min. The granulosa cells at the medium – Ficoll-Hypaque interface were collected and washed twice with wash medium, and resuspended with DMEM/F12 supplemented with 10% fetal calf serum (FCS) and human insulin (5 µg.mL^{-1}), Apo-transferrin (5µg.mL^{-1}), and sodium selenite (5ng.mL^{-1}) (ITS, each individually purchased from SIGMA, Appendix 8.2.2 Media Full Granulosa Cell Medium). The yield of viable granulosa cells isolated from the follicular fluid was determined by trypan blue exclusion assay (Section 2.8.1 Trypan Blue Exclusion Assay Method). Culture conditions for primary-derived granulosa cells were optimised (Chapter 3). The optimal culture conditions were determined to be a pre-treatment adherence period of 24 h for granulosa cells cultured at a density of 2 × 10^4 cells/well of a 96-well plate in DMEM/F12 +10% FCS in a 37°C humidified incubator with 5% CO_2.

2.3.1 Culture Conditions of Human Primary-derived Granulosa Cells

In vitro cultures of primary-derived granulosa cells provide a good investigative reproductive model, as they are readily isolated from women undergoing ART (Beckman et al. 1991; Chaffkin et al. 1993; Quinn et al. 2006). The human mature ovarian follicle contains both mural granulosa cells (GC) and cumulus granulosa cells (CC). During the maturation of a follicle, the antrum (containing the follicular fluid) expands and the mural granulosa cells are those that surround the antrum, whilst the cumulus cells surround the oocyte (Eppig 2001; Khamsi & Roberge 2001). It is the mural granulosa cells which are commonly isolated for use in research *in vitro*. The health and function of the granulosa cells reflects that of the oocyte; however the developments of these granulosa cells are under tight control by the oocyte (Jiang et al. 2010). It can be expected that 50,000 granulosa cells can be isolated per pre-ovulatory follicle (Salustri et al. 1990). Although primary-derived granulosa cells will produce large inter-individual variations *in vitro*, under optimised culture conditions they can be controlled to represent the *in vivo* physiological model (Breckwoldt et al. 1996; Chaffkin et al. 1993; Quinn et al. 2006; Sasson & Amsterdam 2002; Ubaldi 2005; Yong et al. 1992). These conditions will be used in subsequent chapters in order to investigate the toxic effects of the cyanobacterial toxin, cylindrospermopsin, *in vitro*. The key GC culture conditions conducive to viability and functionality were optimised in our laboratory, these include: granulosa cell isolation (refer to section 2.3) cell culture density and adherence period pre-treatment. The optimal adherence period for primary derived granulosa cells pre-treatment was a 24 h adherence period in 96-well plates for cytotoxicity assays. This provides primary derived granulosa cells adequate time to desensitize from *in vivo* gonadotropin stimulation and is supported by published literature (Beckman et al. 1991). Granulosa cell density plays a key role in metabolic activity of cells whilst in culture, whereby at low densities (5×10^3 cells/well) GC maintain mitotic capabilities and can proliferate, whereas at higher cell densities (5×10^4 cells/well) GC are committed to differentiation (Chaffkin et al. 1993). The 2×10^4 cells/well was the optimal density for *in vitro* culture of GC and allows a number of treatment combinations to be examined.

2.4 Isolation of Human Spermatozoa

Semen samples, both fresh (n=10) and cryopreserved (n=7) were donated by healthy males from the general community and from males undertaking IVF treatment for infertility at Flinders Reproductive Medicine Unit, Flinders Medical Centre. The ideal samples would come from males who have previously fathered a child or children. This guideline was not included in the selection process as it would have further limited the number of participants recruited. Rather the strict guidelines defined by the WHO listed in Table 2-3 were adhered to with the exception of motility. Cryopreserved samples needed to have a minimum of 20% viability, whereas fresh samples required meeting the 50% set by WHO (1992). In addition, participant's age was to fall within the 18-35 age range. For each experiment, samples were obtained from a minimum of 4 cryo-straws per male participant and thawed at room temperature for 10 min. A glass transfer pipette was used to transfer the 4 pooled cryopreserved spermatozoa samples into a 5 mL sterile plastic tube containing QAHM's medium + 5% HSA (Appendix 8.2.2 Spermatozoa QAHM's Medium) followed by centrifugation at 300 × *g* for 10 min. Cryoprotectant medium was removed and replenished with 1 mL QAHM's medium + 5% HSA. Spermatozoa were separated from the semen and motile sperm were selected using PureCeption^{TM} (SAGE BioPharma, In-Vitro Fertilisation Inc, CT, USA) discontinuous density gradient consisting of a 80% gradient overlaid with a 40% layer made in QAHM's + 5% HSA. Samples were loaded on the column and centrifuged for 20 min at 300 × *g*. The layer at the 40%:80% interface was removed and the remaining pellet was collected and resuspended in QAHM's + 5% HSA. Spermatozoa were centrifuged at 250 × *g* for 8 min to remove any residual PureCeption ^{TM}. Spermatozoa viability and motility was determined as described in Section 2.8.5 Eosin Y & Section 2.8.6 Spermatozoa Motility. A minimum of 1 × 10^5 sperm/well was used. Fresh semen was collected by masturbation into sterile containers and samples were allowed to liquefy at room temperature for 30min prior to processing as for cryopreserved samples.

2.5 Human Granulosa Cell Tumour Cell line (KGN)

The human granulosa tumour cell line (KGN) was donated by Dr Theresa Hickey, Department of Obstetrics and Gynaecology, School of Medicine, Adelaide University, Australia. The original KGN granulosa cell line was derived from a 63 year old woman with stage III ovarian cancer, histo-pathological analysis indicated granulosa cell carcinoma. The cell line exhibited stable, long-term proliferation (5 years) confirming carcinoma origin. The KGN cells have an abnormal karyotype of 45XX7q-, -22 (partial deletion of chromosome 7 and monosomy 22) associated with ovarian tumours (Nishi et al. 2001). KGN cells were cryopreserved at passage 5 in 20% FCS and 5% DMSO and maintained in DMEM/F12 +10% FCS in a 37°C humidified incubator with 5% CO_2. The doubling rate of the KGN cells was 46.4 h and they were subcultured at confluence (>80% of flask covered with cells, 1-2 weeks) with 0.25% trypsin-1 mM EDTA (Nishi et al. 2001).

2.5.1 KGN Cell Line Maintenance

The KGN cell line can be cultured *in vitro* to investigate the potential reproductive effects of toxins and characterise signalling pathways in comparison to other granulosa cell lines and primary-derived granulosa cells as they maintain most of the physiological activities of granulosa cells (Chu et al. 2004; Minehata et al. 2007; Nishi et al. 2001). The mechanism of steroidogenesis in KGN is similar to that of normal granulosa cells. Although they lack the LH-receptor, they are still capable of producing higher amounts of progesterone in response to cAMP in comparison to primary-derived GC from pre-antral follicles (Amsterdam & Sasson 2004). The KGN cells used in experiments were thawed from passage 5. The resuscitation process involved immediate removal of cells from dimethyl sulfoxide (DMSO, SIGMA), in which the cell suspension was transferred into a 10 mL tube containing equilibrated DMEM/F12 +10% FCS cell culture medium using a sterile plastic pipette. The cells were then centrifuged at 115 × *g* for 5 min. The supernatant was discarded and the cells were resuspended in 1 mL of DMEM/F12 +10% FCS. Cells were transferred to a 25 cm^2 polystyrene sterile flask fitted with a 0.22 µM filter cap (Nunc Flasks, Nunclon™ with Filter Cap, InVitro, Australia)

containing 4 mL of DMEM/F12 + 10% FCS and were grown to 80-90% confluence. Cells were subcultured by rinsing with PBS once and incubated with 2 mL of 0.25% trypsin and 1 mM EDTA solution, for 2-3 minutes at 37°C until the cells had detached from the surface of the flask. The detachment was carried out in the 37°C incubator as per instructions from Dr Theresa Hickey, Department of Obstetrics and Gynaecology, School of Medicine, Adelaide University, Australia. Fresh DMEM/F12 + 10% FCS (2 – 4 mL) was added and gently used to resuspend the cells by pipetting. The cell suspension was then transferred to a new flask at a ratio of 1 mL of cells to 5 mL of complete medium. The concentration of KGN cells in suspension (cells.mL^{-1}) was determined by Trypan Blue (Section 2.8.1 Trypan Blue Exclusion Assay Method).

2.6 Mouse Leydig Tumour Cell Line (MLTC-1)

The mouse Leydig tumour cell line originated from transplantable Leydig tumour cells carried in C57BL/6 mice. Of the two cell lines generated, the MLTC-1 cell line is capable of basal progesterone production, and increases the amount produced when stimulated with hCG (Panesar et al. 2003). The Mouse Leydig Tumour Cell Line (MLTC-1, ATCC accession number CRL-2065) have a doubling time of 35 h, they were subcultured upon reaching >80% confluence and maintained in RPMI + 10% FCS. The subculturing of MLTC-1 cells was the same as carried out for KGN cells (Section 2.5.1).

2.7 Cylindrospermopsin

Cylindrospermopsin (CYN) was extracted from freeze-dried *Cylindrospermopsis raciborskii* and purified by HPLC. CYN was kindly donated by the Australian Water Quality Centre, Bolivar South Australia (Hawkins et al. 1997; Humpage et al. 2000). A stock solution of 1 mg of CYN was reconstituted in 1 mL sterile MQ water to give a final concentration of 1 mg.mL^{-1} (2400µM). The CYN stock was initially diluted to 100 µM in granulosa culture medium or spermatozoa culture medium before each series of experiments, as detailed in Table 2-5.

2.7.1 Cylindrospermopsin Treatments

Primary-derived granulosa cells, KGN cells and spermatozoa were exposed to CYN concentrations as detailed in Table 2-5. These concentrations were made from the 100 µM stock by diluting in the medium respective to cell type and in combination with other factors which are detailed in Table 2-5. Treatments were stored at -20°C between experiments. Viability and functionality experiments involving primary-derived granulosa cells and KGN cells were exposed to 0.1 – 3.0 µM CYN. For protein synthesis assays and protein semi-quantification experiments, primary-derived granulosa cells were exposed to 3 and 5 µM CYN. For viability assays involving human spermatozoa, the sperm were exposed to 0.1, 0.3, 1, 3, 10 or 30 µM. Experimental details are further described in their respective chapters.

CYN Exposure Experiments			
Cell Type	**Experiment (Chapter)**	**Media**	**CYN Concentration**
Human Granulosa Cells	Viability & Functionality Assays (Chapter 4)	DMEM/F12 + 10%FCS+ ITS	0, 0.1, 0.3, 1.0 & 3.0µM
	Protein Synthesis Assay (Chapter 5)	DMEM/F12 + 10%FCS+ ITS	Time Course: 0, 3.0 & 5.0µM Treatment Exposure: 0, 0.1, 0.3, 1.0 & 3.0µM
	Protein Semi-Quantification (Chapter 5)	DMEM/F12 + 10%FCS+ ITS	0 & 3.0µM
	Protein Semi-Quantification (Chapter 5)	DMEM/F12 + 10%FCS+ ITS + 1000mIU/mL hCG	0 & 3.0µM
Human Spermatozoa	Viability Assays (Chapter 6)	QAHM's + 5% HSA	0, 0.1, 0.3, 1.0, 3.0, 10.0 & 30.0µM
KGN	Viability Assays (Chapter 3)	DMEM/F12 + 10%FCS+ ITS +1mM cAMP	0, 0.1, 0.3, 1.0 & 3.0µM

Table 2-5: CYN Treatment Concentrations

CYN concentrations (µM) used with each cell line made up in respective medium types. Refer to Chapter 8 Appendices: Reagents, Stock Solutions & Media Preparation for details regarding each medium type. FCS- fetal calf serum, ITS- insulin/transferrrin/selenium, hCG- human chorionic gonadotropin, cAMP- cyclic adenosine monophosphate,

2.8 Assessment of Toxicity

2.8.1 Trypan Blue Exclusion Assay Method

The Trypan Blue Exclusion Assay allows the differentiation between live and dead cells. Trypan Blue cannot penetrate the cell membrane of live cells and thus they remain golden in colour, whereas in dying or dead cells membrane integrity is lost and hence the Trypan Blue can no longer be excluded by the cell and thus they appear blue (Petty et al. 1995). Three separate 10 µL aliquots of cell suspension were each diluted 1:1 with trypan blue (See Appendix 8.2.3 Trypan Blue). Each resulting 20 µL aliquot was transferred to one chamber on a haemocytometer whereby a total of 3 large squares, comprised of 25 smaller squares, were counted per aliquot (Neubauer, Weber, England- Figure 2-1). The average viable cell concentration (cell concentration per mL, cell.mL^{-1}) of each of the 3 aliquots was calculated by multiplying the average number of live cells of the three aliquots by 10^4 (dimensions of the haemocytometer), and the dilution factor of 2.

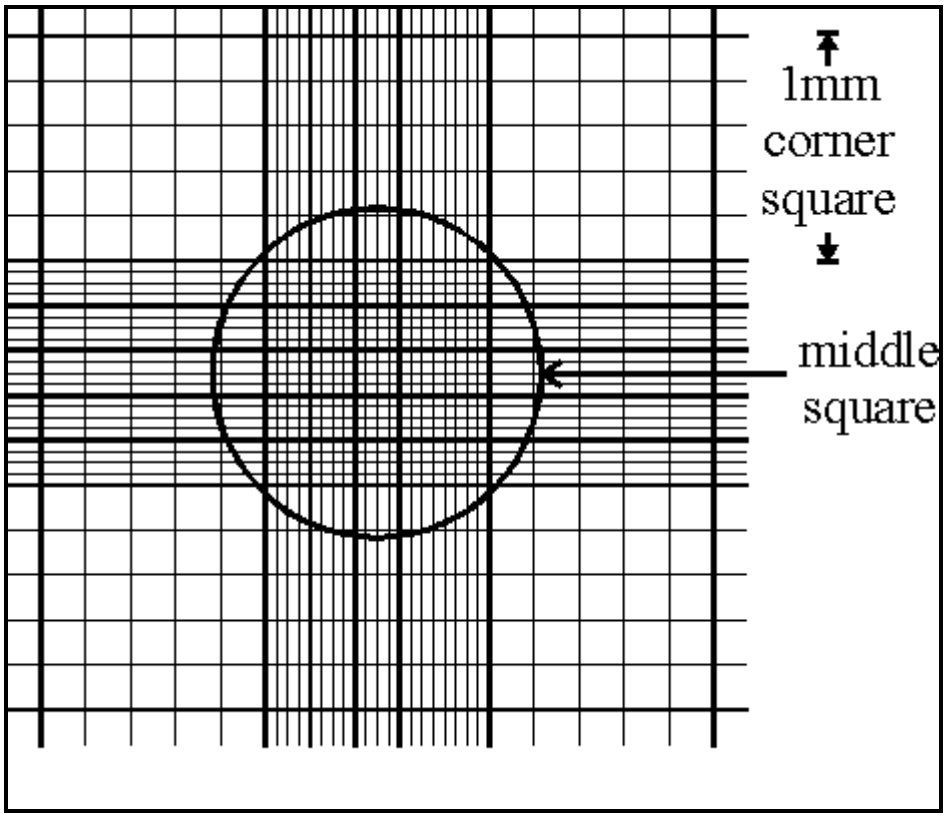


Figure 2-1: Diagram of Neubauer Haemocytometer.

The squares counted in the Trypan Blue Exclusion assay include the middle circled square and adjacent squares on either side. Each large square is divided into 25 smaller squares. Adapted from www.liv.ac.uk/ physiology/pams/haemo.gif

2.8.2 Tetrazolium Reduction Assays

The MTT and MTS assays are colorimetric assays based on the reduction of the water-soluble MTT or MTS dye to the purple formazan crystalline product (Mosmann 1983; Plumb et al. 1989). The MTT assay produces an insoluble formazan, which cannot cross the cell membrane into the extracellular environment and thus cells require treatment with a soluble solution to release and solubilise the formazan crystals in order to be quantified on a spectrophotometer at a wavelength of 570 nm. The formazan produced by the MTS assay does not require solubilisation and can be quantified directly in the culture medium at a wavelength of 490 nm (Fotakis & Timbrell 2006; Goodwin et al. 1995; O'Toole et al. 2003).

The MTT and MTS assay were used to determine the number of viable cells after treatment exposure. In order to determine number of viable cells the absorbance of formazan needs to be related to known number of viable cells in a "standard curve". The standard curve consists of a gradient of 0 to 4 $\times 10^4$ cells/well in triplicate; these were dispensed to wells of a 96-well plate (Nunc, In Vitro, Australia). The negative controls were triplicate wells which did not contain any granulosa cells. A linear relationship between absorbance of formazan and cell number needs to be established in which the absorbance of formazan produced after treatment can be used to calculate the number of viable cells

2.8.2.1 MTT Assay

The outer perimeter wells of the standard curve plates and 96-well treatment plates were not used because of evaporation of medium from these wells. The outer wells were filled with 100 µL of base medium specific to each cell type (Appendix 8.2.2 Media).

After the removal of the cells from the medium 100 µL of a 0.5 $mg.mL^{-1}$ solution of MTT (3-[4,5-dimethyl(thiazole-2-yl-3,5-diphenyl] tetrazolium bromide) was added to each of the wells, and incubated in a 37°C humidified incubator with 5% CO_2 for 24 h. The formazan was solubilised by adding 80 µL of 20% SDS in 0.02M HCl and incubating at room temperature for 1 h in the dark. After the incubation period the absorbance of formazan was read on an automatic plate reader (µQuant BioTEK

Instruments Inc, VT, USA, with KCJunior Program) at a wavelength of 570 nm with a reference wavelength of 655 nm. A standard curve was established and from it the number of viable cells in each well after treatment exposure was calculated.

A strong correlation between formazan production and number of granulosa cells was confirmed if R^2 value was > 0.98, Chapter 8 Appendices: Calculations and Conversions.

2.8.2.2 MTS Assay

The MTS assay procedure was carried out according to manufacturers' instructions (Promega 2003); briefly, 20 µL of MTS solution was added to wells of treatment and standard curve plates and incubated in a 37°C humidified incubator with 5% CO_2 for 2 h. Formazan production was measured on an automatic plate reader (µQuant BioTEK Instruments Inc, VT, USA, with KCJunior Program) at a wavelength of 490 nm. A strong correlation between formazan production and number of granulosa cells was confirmed if R^2 value was $\geq$ 0.98, refer Chapter 8 Appendices: Calculations and Conversions.

2.8.3 ATP Assay

The energy providing molecule adenosine tri-phosphate (ATP) is needed in all living cells (Petty et al. 1995; Sevin et al. 1988). The main role of ATP is for biological synthesis, transport and movement processes (Mueller et al. 2004). The ATP assay principle is based on the reaction of luciferin to oxyluciferin catalysed by luciferase enzyme in the presence of Mg^{2+} ions and ATP to yield a luminescent signal. A linear relationship between intensity of luminescent signal and ATP concentration is then established (Mueller et al. 2004; Sevin et al. 1988). Similar to the tetrazolium assays, each treatment plate was simultaneously accompanied with the setup of a standard curve in order to determine cell number post treatment or cell culture period. ATP quantification was carried out according to manufacturers' instructions, Figure 2-2 which details volumes of reagents, processing time and technique (PerkinElmer 2002). Briefly cell lysis buffer was used to lyse spermatozoa cells to release and stabilise ATP. After incubation with substrate buffer counts per second (CPS) of luminescence was determined using the

TopCount luminescence plate reader, The CPS obtained was then used to determine the number of live spermatozoa from the standard curve.

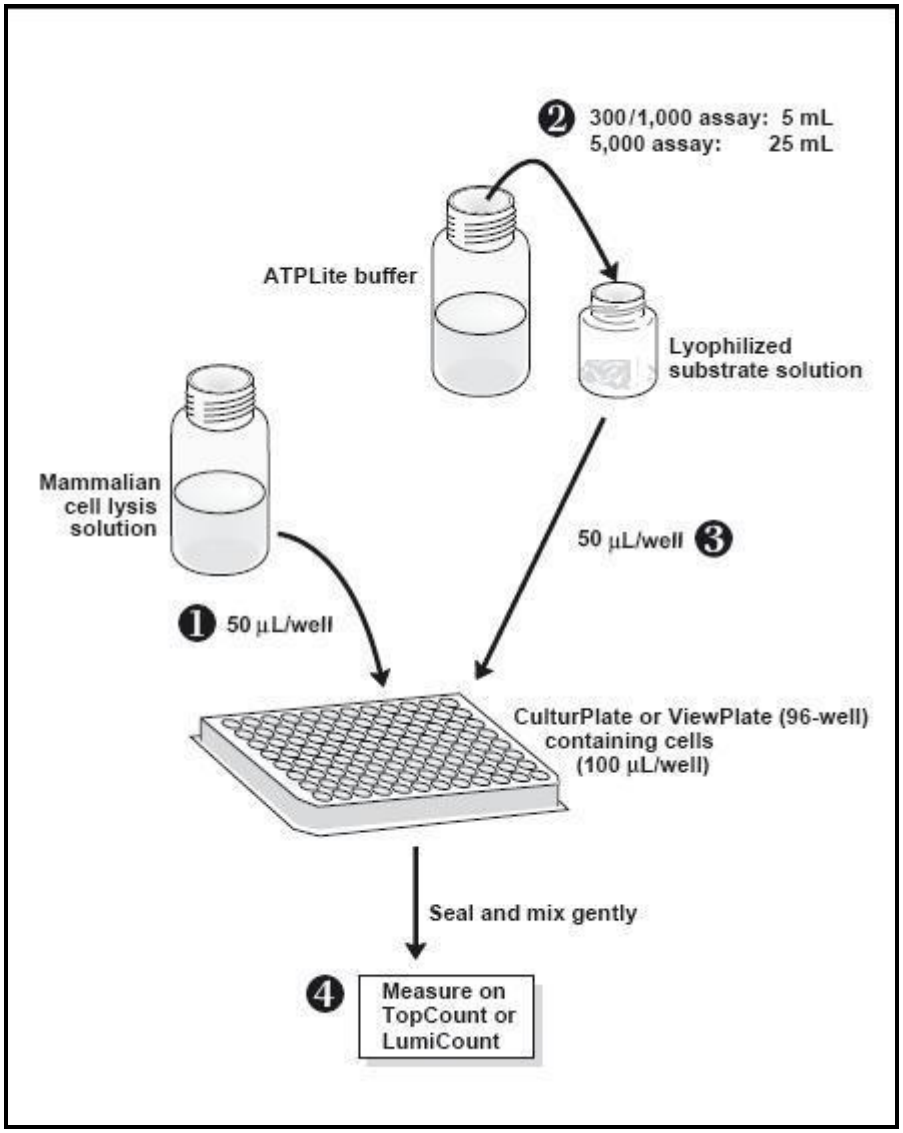


Figure 2-2: ATP Assay Flow Diagram (PerkinElmer 2002).

Refer in-text for description, briefly after treatment cells were lysed open (1) and substrate solution was added (2) & (3) allowing ATP to be quantified by measuring the luminescence emitted from wells. Figure extracted from Perkin Elmer (2002).

2.8.4 Crystal Violet Assay

One of the indicators of loss of cell viability is by the loss of membrane integrity and the detachment of cells (Gillies et al. 1986; Ishiyama et al. 1996). Crystal violet, N-hexamethylpararosaniline, is a metachromophoric basic dye that stains the membrane of attached viable cells (Gillies et al. 1986; Ishiyama et al. 1996). Similar to the tetrazolium assays each experimental treatment plate was accompanied by a standard curve in order to determine cell number post treatment or cell culture period by comparison with the standard curve. Cells were incubated with 50 µL of Crystal Violet (0.5% in 50% methanol) for 10 min at room temperature. The Crystal Violet stain was gently rinsed off cells with running distilled water, which were then air dried in a fumehood before solubilising with 50 µL of 33% acetic acid. The optical density was determined by measuring at a wavelength of 570 nm and a reference wavelength of 635 nm using an automatic plate reader (µQuant BioTEK Instruments Inc, VT, USA, with KCJunior Program). The standard curve setup was the same as that utilised in the MTT assay (Section 2.8.2 Tetrazolium Reduction Assays). A strong correlation between crystal violet stain and number of granulosa cells was confirmed if R^2 value was > 0.98 (Chapter 8 Appendices: Calculations and Conversions).

2.8.5 Eosin Y Assay

The principle of the Eosin Y assay is that live spermatozoa have structurally intact cell membranes and dead spermatozoa have disintegrating cell membranes which cannot prevent uptake of the Eosin Y stain (Björndahl et al. 2004; Eliasson & Treichl 1971; WHO 1999). A 40 µL aliquot of spermatozoa suspension was mixed with 20 µL of Eosin Y (5 $g.L^{-1}$, SIGMA) for 30 s followed by 20 µL of sperm diluent in order to immobilise spermatozoa (final dilution 1:1, Appendix 8.2.3 Stock Solutions, Sperm Diluent). Live spermatozoa with intact and functional membranes stained white and dead spermatozoa stained pink. 10 µL of Eosin Y stained spermatozoa sample was loaded on to a haemocytometer. Spermatozoa in 25 small squares were counted at × 400 magnification under a light microscope. Final live spermatozoa number was divided by 50 to get sperm concentration × 10^6 mL^{-1}. If only 5 large squares were counted then the final live spermatozoa number

was divided by 10 to give spermatozoa concentration $\times 10^6.mL^{-1}$ (Chapter 8 Appendices: Calculations and Conversions, Spermatozoa Conversion Table).

2.8.6 Spermatozoa Motility

Spermatozoa motility was assessed by placing a 10 µL drop of spermatozoa sample onto a glass slide before adding a coverslip (22 × 22 mm). Both motile (forward progressive motility) and non-motile (immobile) sperm were counted by visual counting, a minimum of 200 spermatozoa were counted in a single field of view at × 250 magnification under brightfield illumination. Samples with less than 20% motile spermatozoa were not used in experiments.

2.9 Identification of Lipids Using the Oil Red O Stain

It is considered that a granulosa cell that which has differentiated is capable of accumulating cholesterol lipids, these lipids stain red and can be detected by an Oil Red O Lipid Stain. Adherent cells were cultured in LabTekII 8-well glass chamberslides (NUNC, In vitro Australia) at a density of 4×10^4 cells /well and allowed to adhere for 24 h in DMEM/F12 + 10% FCS + ITS at 37°C with 5% CO_2. Post-culture cells were rinsed with PBS, fixed with 10% formalin for 10 min, rinsed with 60% isopropanol for 30 s, rinsed with MQ water for 1 min and stained with ORO/isopropanol for 15 min (adopted from Kinkel (2004), stain 2 from Casselman (1959)). Cells were then counter stained for 10 min with Mayer's Haematoxylin (SIGMA CA, USA), and then rinsed with MQ water. Haematoxylin staining was intensified by adding sodium phosphate solution for 5 min, followed by 2 × 30 s rinses with MQ water (Kinkel et al. 2004). Slides were allowed to air dry and cells were mounted in aqueous medium (Aquatex, Merck, Australia). Slides were visualised on Brightfield microscope at × 200 magnification for presence of red-stained lipid droplets, (section 2.11 Image Analysis of Oil Red O & 3β-Hydroxysteroid Dehydrogenase Staining).

2.10 Detection of 3β-hydroxysteroid Dehydrogenase Assay

In order for granulosa cells to produce progesterone they require 3βHSD activity (section 1.1.3 Steroidogenesis). 3βHSD is a dimeric enzyme with a bifunctional catalytic site. After binding to the substrate it undergoes a conformational change

activating the second enzyme 3-oxosteroid isomerase (Bar-Ami 1994; Simard et al. 2005). NADH acts as a cofactor which induces the conformational change in the enzyme protein that activates the isomerase reaction (Simard et al. 2005; Thomas et al. 2004). 3βHSD is located in the endoplasmic reticulum (ER) and mitochondrial membrane and catalyses sequential 3β-hydroxysteroid dehydrogenation and Δ^5 to Δ^4-isomerization, which converts DHA to progesterone (Bar-Ami 1994). Cells were cultured in LabTekII 8-well glass chamberslides (NUNC, In*vitro* Australia) at a density of 4×10^4 cells/ well. They were allowed to adhere for 24 h in DMEM/F12 + 10FCS +ITS in a 37°C humidified incubator with 5% CO_2. A 300 µL volume of 3βHSD reaction mixture (0.5 $mg.mL^{-1}$ nitroblue tetrazolium (NBT); 2 $mg.mL^{-1}$ nicotinamide adenine dinucleotide (NAD); and 0.25 $mg.mL^{-1}$ trans-dehydroandrosterone (DHA), was added as described by Fischer and Khan (1972) for 1 h at 37°C. Control wells did not contain DHA. The reaction mixture was discarded and cells were rinsed with PBS, fixed with 10% formalin for 10 min, rinsed with MQ water, counter-stained with 1% neutral red (Department of Anatomical Physiology and Pathology, Flinders Medical Centre) for 2 min at room temperature. Mayer's Haematoxylin could not be used as a counter stain as it is blue which cannot be differentiated from the blue 3βHSD formazan deposits. Alternatively neutral red was used to stain the nuclei of cells and was used as a measure of viability based on the remaining number of cells attached (Morgan et al. 1991, Fotakis and Timbrell 2006, Section 2.11 Image Analysis of Oil Red O & 3β-Hydroxysteroid Dehydrogenase Staining (Fotakis & Timbrell 2006; Morgan et al. 1991). Slides were rinsed with MQ water, followed by dehydrating through an ethanol series (2 × 70% and 1 × 100%) and 1× Histo-Clear (National Diagnostics). Slides were air dried and mounted with Clarion organic mounting medium (SIGMA, Australia). Slides were visualised on a brightfield microscope at × 200 magnification for the presence of blue spots as a positive identification of sites of 3βHSD activity.

2.11 Image Analysis of Oil Red O & 3β-Hydroxysteroid Dehydrogenase Staining

After staining for the presence of 3βHSD activity or ORO, ImageJ analysis software (National Institutes of Health, USA) was used to assess staining of cells (Chapter 8 Appendices: ImageJ Analysis). 3βHSD activity staining was differentiated into 4 groups as detailed in Table 2-6.

Similarly ORO staining was also differentiated into 2 groups. Steroidogenic cells were identified by the presence of red lipid staining within the cell. Non steroidogenic cells were identified by the absence of red staining.

The counter stains used in the assays were Mayer's Haematoxylin (ORO Lipid Stain) or Neutral Red (3βHSD assay), both of which stained the cell nuclei. This allowed the total number of attached cells to be quantified by performing the following calculations. Four images per treatment per woman per assay repeat were processed for the ORO Lipid assay and 3βHSD assay. The entire chamberslide well could not be captured in the four images. It was assumed that granulosa cells adhered randomly and formed a monolayer on the surface of the wells, thus the four photos captured were used as a representative number to estimate the total number of cells in the well. The total count in four images could be extrapolated to determine total cell number in each well post-treatment. Thus the following calculations were carried out. The area of each chamberslide well was 7×10^7 μm^2, so when incorporating the pixel dimensions of the image at ×20 magnification into the calculation, each image represented 0.34% of the chamberslide well. Four photos were taken for each well from random locations that did not overlap with each other. The four images therefore represented 1.37% of the chamberslide well area. The numbers of cells counted in the four images were then converted to total number of cells by multiplying 100/1.37.

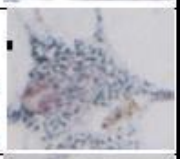
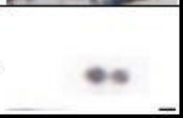
Assay	Label	Description	Image
3βHSD Activity	Negative 3βHSD large cells	Absence of Blue Formazan Crystal Deposits	
	Light stained 3βHSD large cell	Light Scatter with Even Distribution of Blue Formazan Crystal Deposits(<80% of cell)	
	Intense stained 3βHSD large cells	Very Dark Blue Formazan Crystal Deposits (>80% of cell)	
	Small Dense cells	Circular Cells, Absence of Cytoplasm,and Pseudopodia- Cell to Cell Interactions	

Table 2-6: 3βHSD Activity Assay Image Analysis of Four Different Staining Outcomes

Negative 3βHSD large cells: Light stained 3βHSD large cell: Intense stained 3βHSD large cells and small dense cells. Negative 3βHSD large cells were cells that had no blue formazan crystal deposits. Light stained 3βHSD large cells identified by the appearance of lightly scattered even distribution of formazan deposits covering <80% of cells. Intense stained 3βHSD large was classified as very dark blue formazan deposits covering >80% of cell. Small dense cells were identified as cells that were circular and lacking cytoplasm (Figenschau et al. 1997). Magnification × 400,

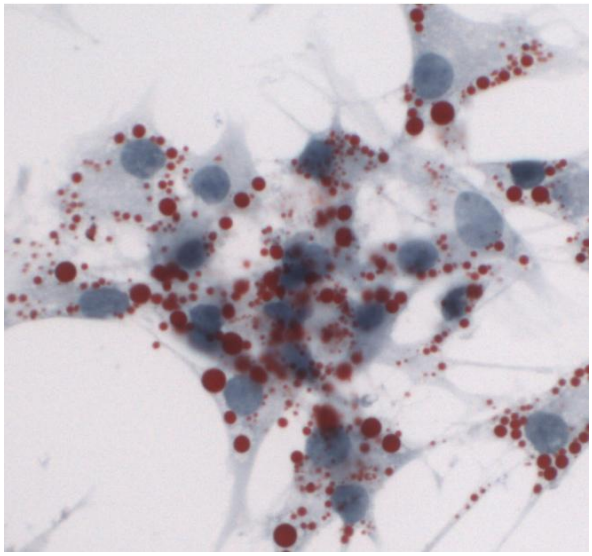


Figure 2-3: Example of ORO staining in primary-derived granulosa cells

Magnification × 400, granulosa cells stained for presence of Oil Red O Lipids, red droplets indicates presence of ORO.

2.12 Radioimmunoassay (RIA)

Radioimmunoassay's (RIA) are based on the competition between a radiolabelled hormone "tracer", and an unlabelled hormone molecule contained in the conditioned cell culture medium after granulosa cells culture.

2.12.1 Progesterone RIA

The radioactivity of a 10 µL aliquot of undiluted progesterone tracer (MP Biomedicals, Catalogue 07-170126, ^{125}I Tracer (1.1ml/vial 10 µCi) was first determined using the Packard RIASTAR gamma counter for 2 minutes. The counts per minute (cpm) were then used to determine what dilution was necessary to obtain 10,000 cpm per assay tube.

Each RIA included total count tubes (5 mL 3DT tubes) consisting only of 25 µL of progesterone tracer with 10,000 cpm per tube and Non-Specific Binding (NSB) tubes in which the anti-progesterone primary antibody was omitted. The limit of detection of the progesterone assay was 0.2 ng.mL^{-1} and the interassay coefficient of variation was 14%.

Progesterone Standard tubes ranging from 2000 ng.mL^{-1} to 0 ng.mL^{-1} were assayed in duplicate (Chapter 8 Appendices: Radioimmunoassay Reagents) and Immunoassay BioRad Controls 1, 2 and 3 (Table 8-2).

Test solutions collected from wells containing cells post-treatment were stored at -20°C and thawed overnight at 4°C before RIA. Once all tubes and necessary standards and controls were set-up (Figure 8-2), 100 µL of NaFam (Chapter 8 Appendices: Radioimmunoassay Reagents) assay buffer was added to each of the tubes except for the NSB tubes and the total count tubes which received 200 µL and 0 µL NaFam, respectively. The appropriate Progesterone Standards, Controls and Samples were added to numbered tubes; however no sample or assay buffer was added to the total count tubes. The NSB tubes and the 0 ng.mL^{-1} standard tube both contained the 0 ng.mL^{-1} Progesterone Standard.

This was followed by the addition of 100 µL of the Progesterone Tracer to each tube, freshly diluted so that each tube contained 10,000 cpm. The total count tubes

were capped and placed aside for the remainder of the assay. Progesterone Antiserum (MP Biomedicals Australia, diluted to 1:5,000 of stock solution) was added at a volume of 100 µL aliquot per tube to all of the tubes except the NSB tubes. Each tube was then shaken for 20-30 seconds and incubated for 1 h in a 37°C heated water bath. Post- incubation 100 µL of 1:100 diluted Normal Rabbit Serum (extracted from rabbit blood obtained from the Animal House at Flinders Medical Centre, Chapter 8 Appendices: Radioimmunoassay) was added. This was immediately followed by 100 µL of a 1:30 dilution of Sheep anti-Rabbit Antiserum (Chemicon, Australia, Catalogue AB 7130) and 200 µL of 14% Polyethylene glycol to all of the tubes except the total tubes. Each tube (except total tubes) was then individually vortexed for 5-10 seconds and allowed to stand at room temperature for 30 minutes.

All tubes except the total tubes were centrifuged at 2002 × *g* at 4°C for 20 minutes in a Heraeus Minifuge T Centrifuge. The supernatant of the tubes was aspirated in a way that avoided disturbance to the pellet. All tubes including the total count tubes were counted for 2 minutes in the Packard RIASTAR gamma counter.

2.12.2 17β-Estradiol RIA

The procedure of the estradiol RIA assay involved a similar setup to that of the progesterone RIA (Figure 8-4). The concentration of a 10 µL aliquot of undiluted estradiol tracer (MP Biomedicals, Catalogue 07-138 226, ^{125}I Tracer (1.1ml/vial 10 µCi) was determined using the Packard RIASTAR gamma counter for 1min. The counts per minute (cpm) were then used to determine the dilution to carry out to ensure that each tube in the RIA contained 10,000 cpm.

Each RIA consisted of total count tubes containing 25 µL of estradiol tracer (10,000 cpm per tube), Non-Specific Binding tubes without anti-estradiol primary antibody (MP Biomedicals, Catalogue 64737) Estradiol Standards ranging from 2000 pg.mL^{-1} to 0 pg.mL^{-1} (4 µg.mL^{-1} Estradiol stock, DSL, serially diluted in Full Granulosa Cell Media, Chapter 8 Appendices: 8.2.2 Media) and Immunoassay Biorad Controls 1, 2 and 3 (Table 8-2) all of which were assayed in duplicate. The limit of detection of this assay was 20 pg.mL^{-1} and the interassay coefficient of variation was 12%.

The estradiol radioimmunoassay was carried out in the same way as the progesterone protocol. However the only difference was that after addition of the anti-estradiol antibody the tubes were incubated at 4°C for 20 h in a cold room. 100 µL of 1:30 dilution of donkey anti-sheep serum was used for the estradiol assay in order to separate bound and free fractions. The remainder of the estradiol assay was followed as for the progesterone protocol, section 2.12.1.

Chapter 3. Optimisation of *In Vitro* Culture of Primary-Derived Human Granulosa Cells

3.1 Introduction

In vitro culture of primary-derived granulosa cells provides a good investigative model of a reproductive tissue, with the cells readily isolated from women undergoing ART (Beckman et al. 1991; Chaffkin et al. 1993; Quinn et al. 2006). Although primary-derived granulosa cells will produce large inter-individual variations *in vitro*, under optimised culture conditions they can be controlled to represent the *in vivo* physiological model (Breckwoldt et al. 1996; Chaffkin et al. 1993; Quinn et al. 2006; Sasson & Amsterdam 2002; Ubaldi 2005; Yong et al. 1992). However, the *in vivo* follicle is comprised of a complex network of steroidogenic and non-steroidogenic cells, with follicular tissues normally comprised of 5-15% macrophages and leukocytes (Baranao et al. 1995; Beckman et al. 1991; Lobb & Younglai 2006; Loukides et al. 1990). Nonetheless, *in vitro* toxicity assays are needed to examine and investigate effects on fertilisation capabilities. Thus, the key aspects of GC culture, the conditions conducive to viability and functionality, need to be optimised. Such factors include GC isolation technique, cell culture density, pre-treatment incubation period (adherence), supplementation of medium with serum, and the type of culture vessel.

The follicular fluid needs to be processed in order separate granulosa cells from contaminating factors such as red blood cells in order to minimise the interference to experimental assays, and to obtain a homogenous population of luteinised granulosa cells. The presence of these contaminating factors has the potential to alter the biochemical properties of GC such as progesterone production (Beckman et al. 1991; Lobb & Younglai 2006; Quinn et al. 2006). Various methods have been developed to minimise presence of contaminating factors. These involve one-step methods or a multiple process of density gradients (colloidal silica), incubation in tissue culture plastic dishes with removal of medium and non-steroidogenic cells, hypertonic and hypotonic solutions, and enzymatic treatment with trypsin/EDTA

(Beckman et al. 1991; Figenschau et al. 1997; Lobb & Younglai 2006; McAllister et al. 1990; Quinn et al. 2006; Wang et al. 1995). Although each method has its advantages and disadvantages, no method eliminates all contaminating cell types; however, each effectively reduces the percentage of contaminating cells to less than 5% detectable levels (Baranao et al. 1995; Beckman et al. 1991; Lobb & Younglai 2006; Loukides et al. 1990). In this research, granulosa cells were isolated from the follicular fluid using Ficoll-Hypaque density gradient centrifugation (Lymphoprep, see Chapter 8 Appendices: Reagents, Stock Solutions & Media Preparation). Density gradients are also indirect methods for selection of luteinised granulosa which have increased lipid composition and steroid hormone production raising the density and effectively segregating from un-luteinised cells (Hartshorne 1990). The three types of GC and their densities include; mural (1.004 $g.mL^{-1}$), antral (1.044 $g.mL^{-1}$) and cumulus oophorus (1.032 $g.mL^{-1}$) cells, which when centrifuged over Ficoll-Hypaque (δ = 1.0076 $g.mL^{-1}$) would isolate the mural cells at the interface, so that the majority of cells would be granulosa-lutein cells.

In vivo, the LH surge induces ovulation and luteinisation of granulosa cells. hCG administered *in vivo* during the ART procedure mimics the role of LH. Upon isolation, ≈ 20% of granulosa cells have yet to differentiate; a pre-treatment culture provides an environment for them to differentiate, desensitise from *in vivo* gonadotropin stimulation and transform into granulosa-lutein cells (Breckwoldt et al. 1996; Chaffkin et al. 1993; Edgar et al. 1991; Lobb & Younglai 2006; Schmidt et al. 1984). This pre-treatment period can range from 24 – 96 h, although some studies have used a pre-treatment period of up to 7 days or eliminated it completely (Schipper et al. 1993). Pre-treatment period also allows for the removal of non-adherent cells as medium is changed prior to experimentation being carried out (Best et al. 1994). Although the literature shows that primary-derived granulosa cells are pre-cultured for a minimum of 24 h before treatment, cell lines such as the human uterus carcinoma (HeLa) require only 3 h to attach (Ishiyama et al. 1996). However, the granulosa-like KGN cell line also requires a minimum 24 h pre-treatment (Tsutsumi et al. 2008). Granulosa cells contain cytoplasmic lipid droplets (characteristics of steroidogenic cells), have oval shaped nuclei, with the cells

found spread out in singles, or aggregated together to form clusters, when cultured in the absence of stimulants such as FSH and LH (Aharoni et al. 1995).

Granulosa cells can be cultured in various types of vessels; however, the optimal density to culture granulosa cells has yet to be investigated (Baranao et al. 1995; Beckman et al. 1991; Chaffkin et al. 1993; Figenschau et al. 1997; Schmidt et al. 1984). The closest study was carried out by Chaffkin et al. (1993) who found that at low granulosa cell densities, cells proliferate *in vitro* due to the presence of secretary factors released into the culture medium, reflecting an un-luteinised population of cells. At higher culture densities, granulosa cells differentiated. This chapter aims to investigate the optimum *in vitro* density to culture granulosa cells representing a cell population which is differentiated and capable of steroid hormone production whilst remaining viable throughout the exposure period selected. The optimal density of granulosa cells used in culture also varies between studies. Generally, the greater the surface area of culture vessel, the higher the density of granulosa cells used: 1×10^6 cells in 1 mL in a 6-well plate; $2 – 4 \times 10^5$ cells in 0.5 mL in a 24-well plate; and only $5 – 50 \times 10^4$ cells in 0.1 mL in a 96-well plate (Baranao et al. 1995; Beckman et al. 1991; Chaffkin et al. 1993; Figenschau et al. 1997; Schmidt et al. 1984). At high cell densities progesterone concentrations were directly correlated to cell density, whereas at cell densities of 5×10^4 cells/well this relationship was no longer observed. Hence, there is a need to ensure that granulosa cell density used in culture is optimal to ensure normal steroid hormone production, a key endpoint of toxicity studies (McAllister et al. 1990).

In an attempt to prolong the viability of granulosa cells in culture, the medium is supplemented with serum to provide biological components lacking in artificial nutrient medium, although many of these may not be found in the direct *in vivo* environment of granulosa cells located within the ovary of the maturing follicle (Figenschau et al. 1997). Throughout *in vitro* culture, serum has been found to spontaneously cause luteinisation of granulosa cells (Schipper et al. 1993). However, the presence of serum is essential for maintenance of cell viability and the concentration of serum used ranges between 5 and 30% depending on the endpoint to be assessed post-treatment (Best et al. 1995; Figenschau et al. 1997).

Secretion of steroid hormones is crucial for the nourishment, growth, development and successful fertilisation of the oocyte (Asboth et al. 2001; Breckwoldt et al. 1996). The differentiation process involves transforming into large luteal cells, commonly referred to as hypertrophy, and is also indicated by increased progesterone production and the presence of cholesterol lipids (Chaffkin et al. 1993; Quinn et al. 2006; Stoklosowa et al. 1978; Yu et al. 2005). Steroid hormones released into the culture medium can be detected using antibody-based detection via enzyme immunoassays; however, the predominant method used is radioimmunoassay which is specific and has low cross-reactivity (Beckman et al. 1991; Chaffkin et al. 1993; Kasson et al. 1993; Lobb & Younglai 2006; Lovell et al. 2002; McAllister et al. 1990; Schmidt et al. 1984).

Granulosa cells from mature follicles (15-25 mm in diameter) maintain their differentiated state and continue to secrete progesterone in long-term culture (Chaffkin et al. 1993; Schmidt et al. 1984; Stoklosowa et al. 1978; Yu et al. 2005). Some culture conditions utilise an extracellular matrix, as this has been shown to increase basal progesterone production by granulosa cells in response to hCG stimulation as well as formation of gap junctions which are the intracellular communication pathways between granulosa cells (Figenschau et al. 1997; Kasson et al. 1993; McAllister et al. 1990). However, the use of an extracellular matrix provides granulosa cells with steroid precursors and thus creates difficulty in determining if the endogenous supply of substrate would be a limiting factor to steroid hormone production. For this reason, extracellular matrix was not included in our culture of granulosa cells (Schipper et al. 1993).

In order to assess the suitability of selected culture conditions and cytotoxicity of compounds, granulosa cell viability must be determined. The viability assay selected needs to be rapid, reproducible, high throughput and sensitive (Siddiqui et al. 2006). Viability of cells cultured *in vitro* can be measured by various assays, the most common are those that include staining of membrane structures with dyes or assays which measure metabolic activity of cells with the uptake of salts (Gillies et al. 1986; Ishiyama et al. 1996; Mosmann 1983). Metabolic assays and dye uptake assays (physical method) sometimes result in different determinations of viability,

despite individual correlation between absorbance and cell number. Thus, it is important to assess viability by using two distinct mechanisms simultaneously to increase reliability of results when determining cytotoxic effects (Fotakis & Timbrell 2006; Hansen et al. 1989).

In addition to determining viability of cells as described in section 1.7.4, assessment of cytotoxicity of toxins includes determining whether normal physiological functioning of the cells is affected. In the case of granulosa cells, this is steroid hormone production. This can be directly determined by the presence of cholesterol substrates required for steroidogenesis (ORO Lipid Stain), by quantifying hormone production, and by investigating effects on enzymes involved in steroidogenesis.

Large granulosa-lutein cells have the potential for increased metabolic capability required for cellular function such as production of steroid hormones progesterone and estradiol, in which the mitochondria play a vital role (Zlotkin et al. 1986). The changes to viability and functionality of granulosa cells would be indicated by changes to mitochondrial functioning (Amsterdam et al. 1999). Although a variety of endpoints to determine viability of granulosa cells exist, the MTT assay is dependent on the metabolic activity of the mitochondria as is steroidogenesis, hence viability is best characterised using the MTT or MTS assay, and results are supported by comparing viability determined in the Crystal Violet assay (Gerlier & Thomasset 1986; Mosmann 1983; Patrizio et al. 2007).

Steroidogenic cells capable of producing progesterone and estradiol contain cholesterol lipids and selectively stain with Oil Red O (Hillensjo et al. 1985; Stoklosowa et al. 1978). It is considered that a granulosa cell still capable of proliferation, which is not in a terminal state of differentiation, is incapable of accumulating lipid (Quinn et al. 2006). Granulosa cells used in the experiments described in this chapter were primary-derived, thus the mouse Leydig tumour cell line (MLTC-1) was also used because it has a similar steroidogenic pathway to granulosa cells and is a good *in vitro* model to compare and characterise experimental techniques (Martinat et al. 2005; Yu et al. 2005). The KGN granulosa cell line was not available when the research described in this chapter was

undertaken. Both granulosa cells and MLTC-1 can be used to investigate cholesterol lipid staining via Oil Red O assay (ORO) and hence deduce the cells' capability to produce steroid hormones.

3.2 Chapter Aims and Objectives

In order to determine cytotoxic effects of cylindrospermopsin, the optimal culture conditions for granulosa cells need to be characterised. Viability assays, pre-treatment culture period and identification of cholesterol lipids require optimisation. The following hypotheses were investigated (Figure 3-1: Flow Diagram of Chapter Objectives):

- Granulosa cell mitochondrial dehydrogenase enzymes will reduce the MTT tetrazolium salt to formazan in which absorbance will be proportional to cell number.

- Granulosa cells cultured *in vitro* will stain with the Crystal Violet dye in which absorbance will be proportional to cell number.

- Primary-derived granulosa cells will require a minimum of 24 h pre-treatment culture period to attach and exhibit characteristics of a differentiated population capable of steroidogenesis.

- Granulosa cells which are in a differentiated state will stain with Oil Red O indicating presence of steroid hormone precursors and capability of initiation of steroid hormone production.

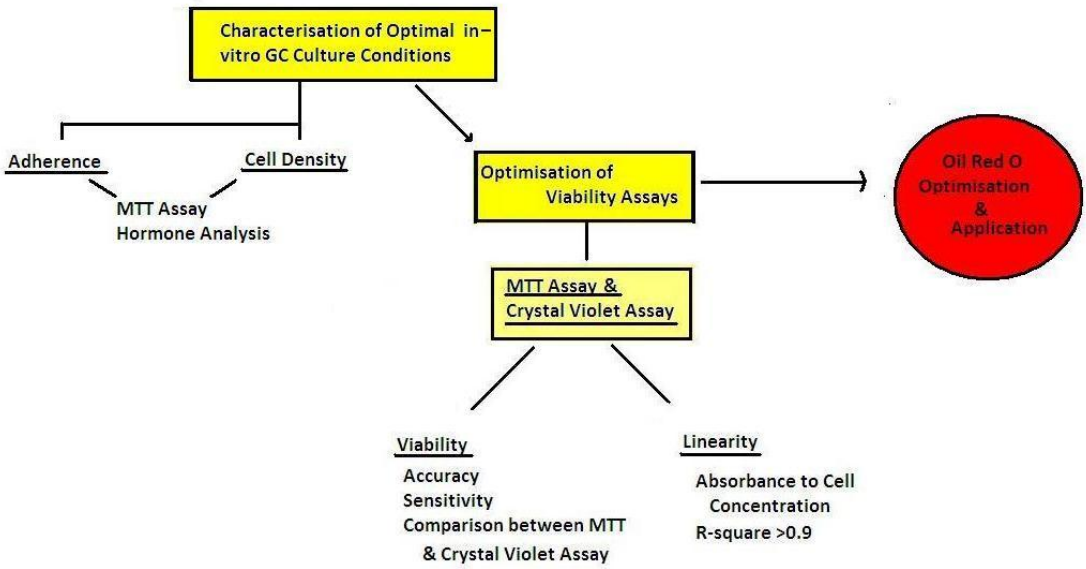


Figure 3-1: Flow Diagram of Chapter Objectives

Flow diagram represents sequence of experimental approaches undertaken in this chapter. GC were classified as healthy or unhealthy. Secondly, culture of GC were characterised in terms of adherence time and cell density based on optimal MTT assay and steroid hormone results. Thirdly, viability assays for GC were optimised and finally the optimisation of Oil Red O lipids stain was carried out.

3.3 Materials and Methods

3.3.1 Characterisation of Granulosa Cells

Women were recruited from June 2006 until November 2008. Data regarding various fertility factors were obtained from individual case records and included demographic, medical and fertility histories (Table 8-1).

3.3.2 Isolation of Primary- derived Human Granulosa Cells

Granulosa cells were isolated from ovarian follicular fluid as outlined in 2.3 Isolation of Human Granulosa Cells. A total of 27 cases were included, 11 cases were IVF and 16 cases were ICSI. The sources of primary cells were classified as cells used for "optimisation" under the "Experimental Endpoint" column in (Table 8-1). Couples that underwent ICSI but had a female infertility factor (women 49 and 53) were excluded; woman 44 was included as her infertility factor was tubal ligation and not a hormonal issue.

3.3.3 KGN Cell Line Maintenance

Refer to section 2.5.1 KGN Cell Line Maintenance.

3.3.4 Trypan Blue Exclusion Assay

Immediately after isolation of GC the number of live (viable cells) was determined using Trypan Blue (2.8.1 Trypan Blue Exclusion Assay Method). This was used to set-up adherence standard curves for MTT assay, Crystal Violet assay and ORO staining.

3.3.5 Optimisation of Pre-treatment Culture Period and Cell Density of Primary-derived Human Granulosa Cells *in vitro*

3.3.5.1 Adherence Standard Curves

In order to determine the optimal adherence time and cell density required for GC culture, a series of adherence standard curves were setup as shown in Figure 3-2. Three adherence times were to be investigated: 2, 4 and 24 h. The adherence plate was setup identical to a standard curve plate. A standard curve plate was comprised of granulosa cell concentrations of $0 - 4 \times 10^4$ cells/well in replicates of six which were dispensed into wells of a flat-bottomed 96-well plate (Nunc, *in*Vitro Australia)

in DMEM/F12 + 10% FCS+ ITS of 100 µL final volume. The internal controls were the replicate six wells which did not contain any granulosa cells. The adherence standard curve plates were cultured for 2, 4 or 24 h. The MTT assay (refer to section 2.8.2.1 MTT Assay) was carried out and a linear regression analysis was undertaken to assess relationship of formazan production with cell number for each attachment time. The number of granulosa cells originally seeded was then compared to the number of granulosa cells remaining after attachment period, based on comparing formazan production in the samples to the prepared standard curve, Figure 3-2.

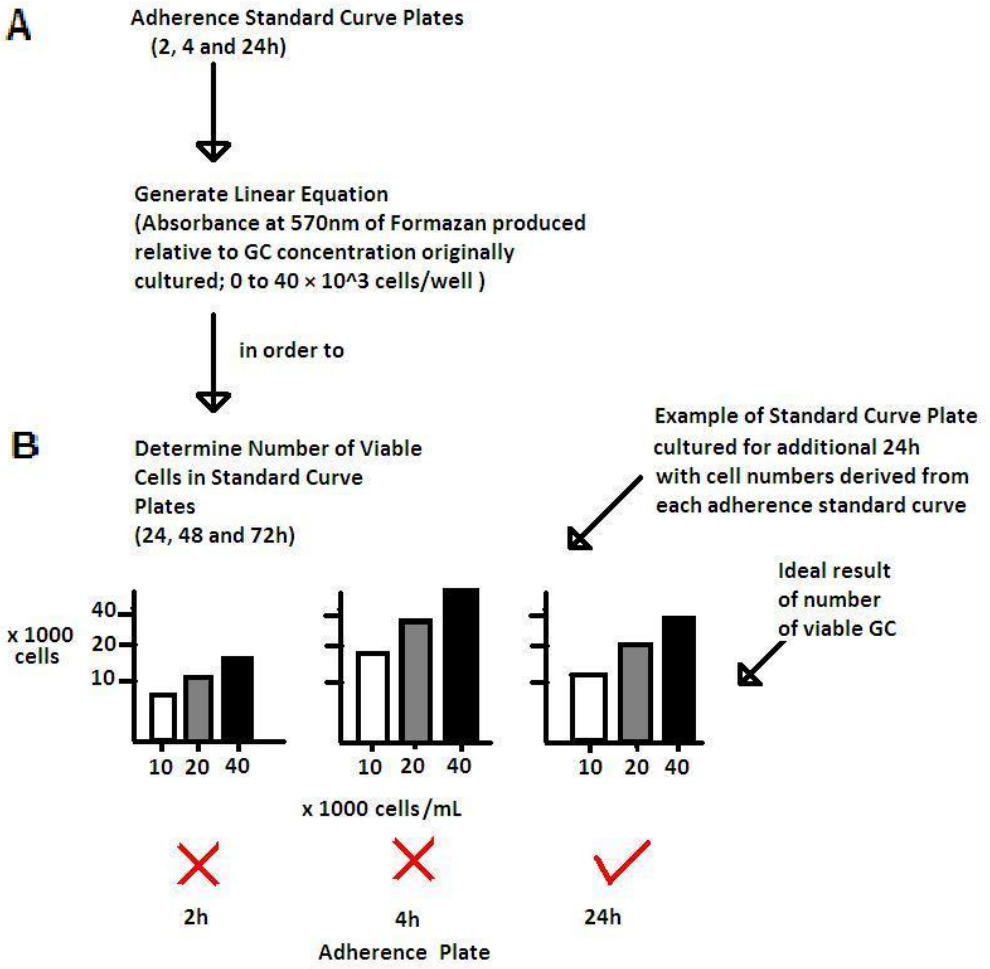


Figure 3-2: Adherence Standard Curves

Flow diagram representing how the adherence standard curves were used to determine optimal time required for granulosa cells to adhere to flat bottom 96-well plates. (A) Each adherence standard curve (2, 4 and 24 h) was used to generate a linear equation derived from absorbance of formazan produced by cultured GC of concentrations up to 40×10^3cells/well.(B) Each equation was then used to calculate the number of viable cells produced after 24, 48 and 72 h culture in standard curve plates. The ideal result is an adherence standard curve plate that represents the original GC concentration added to each well (shown on x-axis of graphs). This is represented by the 24 h standard curve as it estimated the number of viable cells close to that originally seeded, whereas the 2 h and 4 h show not enough and too many respectively.

3.3.5.2 Culture Plates

In addition to measuring cell numbers immediately following the adherence periods, extra plates were established for each adherence protocol and then further cultured for 24, 48 or 72 h (Figure 3-2, Part B). These plates were used to assess the adherence protocols for their ability to preserve the relationship with initial seeded cell numbers over the course of the experiment. Conditioned media post GC culture were collected and stored at -20°C for steroid hormone analysis by radioimmunoassay (RIA, Figure 3-2).

3.3.5.3 KGN Cells

KGN cells were allowed to adhere for 24 h prior to *in vitro* culture. Standard curve plate (post 24 h adherence period) and culture plates were setup as detailed in section 3.3.5.2. Refer to KGN MTT Standard Curve for the standard curve. This represents 3 repeat independent assays.

3.3.6 Optimisation of Crystal Violet Assay for Primary-derived Human Granulosa Cells

For each independent experiment 4 culture plates were setup, each comprised of GC of $0 – 4 \times 10^4$ cells/well in replicates of six wells in a flat-bottomed 96-well plate (Nunc, inVitro Australia) in DMEM/F12 + 10% FCS+ ITS of 100 µL final volume. The 4 culture plates consisted of 3 standard curves and 1 adherence standard curve (24 h). The negative controls were six wells that did not contain any GC. Granulosa cells were allowed to adhere for 24 h before Crystal Violet processing. Media conditioned by GC were collected and stored at -20°C for steroid hormone analysis by RIA. At the end of each culture period (24, 48 and 72 h) the standard curve plates were assayed by Crystal Violet (section 2.8.4). The linear regression equation from absorbance at 570 nm relative to GC concentration of the 24 h standard curve plate was then used to determine the number of viable cells at the end of culture 24, 48 and 72 h. This was repeated for the KGN cell line.

3.3.7 Radioimmunoassay (RIA)

Radioimmunoassays were carried out on conditioned cell culture media containing hormone collected after culture of GCs. Concentrations of progesterone ($ng.mL^{-1}$) and estradiol ($pg\ mL^{-1}$) were determined as outlined in Section 2.12 Radioimmunoassay. Each culture well was independently assayed.

3.3.8 Oil Red O Lipid Stain Assay (ORO Assay)

3.3.8.1 MLTC-1 Cell Line

MLTC-1 cells were used to optimise the Oil Red O lipid stain. Cells were obtained from a research colleague within the Department of Biotechnology and were maintained as detailed in General Material and Methods section 2.6 Mouse Leydig Tumour Cell Line (MLTC-1).

3.3.8.2 Primary-derived Human Granulosa Cells

Granulosa cells were cultured in LabTekII 8-well glass chamberslides (NUNC, In vitro Australia) at a density of 4×10^4 cells/well in final volume of 300 µL. They were allowed to adhere for 24 h in DMEM/F12 + 10% FCS supplemented with ITS at 37°C humidified incubator with 5% CO_2. Post-culture period granulosa cells were rinsed with PBS, fixed with 10% formalin for 10 min, rinsed with 60% isopropanol for 30 s, rinsed with MQ water for 1 min and stained with ORO/isopropanol. Two stain preparations were examined: ORO Stain 1 from Kinkel et al. 2004 and ORO Stain 2 (Casselman 1959). Two incubation times were examined (15 min/30 min) and two temperatures (22°C / 37°C) as shown in Table 3-1. Cells were then counter stained with Mayer's Haematoxylin (SIGMA CA,USA) for 10 min, rinsed with MilliQ water, and haematoxylin stain was intensified with sodium phosphate solution (Kinkel et al. 2004) for 5 min, followed by 2 × 30 s rinses with MQ water. Slides were allowed to air-dry and mounted with aqueous mounting medium Aquatex (Merck, Australia). Slides were visualised on a brightfield microscope at × 200 magnification for presence of red droplets. MLTC-1 cells (2.6 Mouse Leydig Tumour Cell Line (MLTC-1) were used to optimise Oil

Red O staining (2.9 Identification of Lipids Using the Oil Red O Stain) (Manna et al. 2004; Manna et al. 2001; Panesar et al. 2003).

3.3.9 Image Analysis of Oil Red O Lipids

Refer to 2.11 Image Analysis of Oil Red O & 3β-Hydroxysteroid Dehydrogenase.

3.3.10 Statistical Analysis

Absorbance was related to number of granulosa cells by fitting either a linear or exponential regression curve or both. Independent-T test was used to compare means of data from two groups. Significance was accepted when *P*-value< 0.05 unless otherwise indicated.

ORDER OF OPTIMISATION →

Cells	Denisty (cells/well)	ORO Stain	Time (min)	Temperature (°C)
MLTC-1	4.00E+04	1	15	22
MLTC-1	4.00E+04	2	15	22
MLTC-1	4.00E+04	1	30	22
MLTC-1	4.00E+04	2	30	22
MLTC-1	4.00E+04	1	15	37
MLTC-1	4.00E+04	2	15	37
MLTC-1	4.00E+04	1	30	37
MLTC-1	4.00E+04	2	30	37

Table 3-1: Oil Red O Optimisation

Optimisation of ORO staining (n=3). MLTC-1= mouse Leydig tumour cell line, at a density of 4×10^4 cell/well in LabTekII 8-well chamberslide. ORO stain 1& 2 refer to stains adopted from Kinkel et al. (2004) and Casselman (1959). Cells were allowed to adhere for 6 h prior to staining. Arrow indicates order in which parameters were then optimised. Slides were then processed for image analysis using ImageJ analysis (WCIF) software.

3.4 Results

3.4.1 Establishment of Optimal Culture Conditions of Primary-derived Human Granulosa Cells *in Vitro*.

3.4.1.1 Determination of Adherence Period for Granulosa Cells

The culture periods of 2, 4 or 24 h were investigated to determine the optimal conditions, and whether a shorter pre-treatment adherence period for granulosa cells could be used as a better alternative. The MTT assay was carried out and both an exponential and linear regression was fitted to the data to determine the type of relationship between absorbance and granulosa cell number, whereby the R-square value represented the relative strength of the association. The adherence period that produced the highest formazan production and the strongest correlation to cell number were the first criteria initially used to determine the optimal adherence period for GC. The results showed that the 24 h adherence exponential standard curve had the highest correlation of R^2=0.99, followed closely by 4 h (R^2=0.96) and 2 h (R^2=0.94) (Figure 3-3). Although there was a strong linear relationship at all adherence time points investigated (24 h, 0.96; 48 h, 0.98 and 2 h, 0.99), the data was also well modelled by an exponential regression, due to sub-linear curvature at the lower cell densities. The curvature suggests that the amount of formazan produced is not entirely dependent on the number of granulosa cells and that other factors contribute. The amount of tetrazolium salt metabolised by granulosa cells was higher after the 24 h adherence than the 2 or 4 h standard curves (Figure 3-3). The linear regression was the preferred model since the cell range of 0.5 – 4 × 10^4 cells were to be investigated when determining granulosa cell viability after CYN treatment and this range was well covered by the linear portion of the curve.

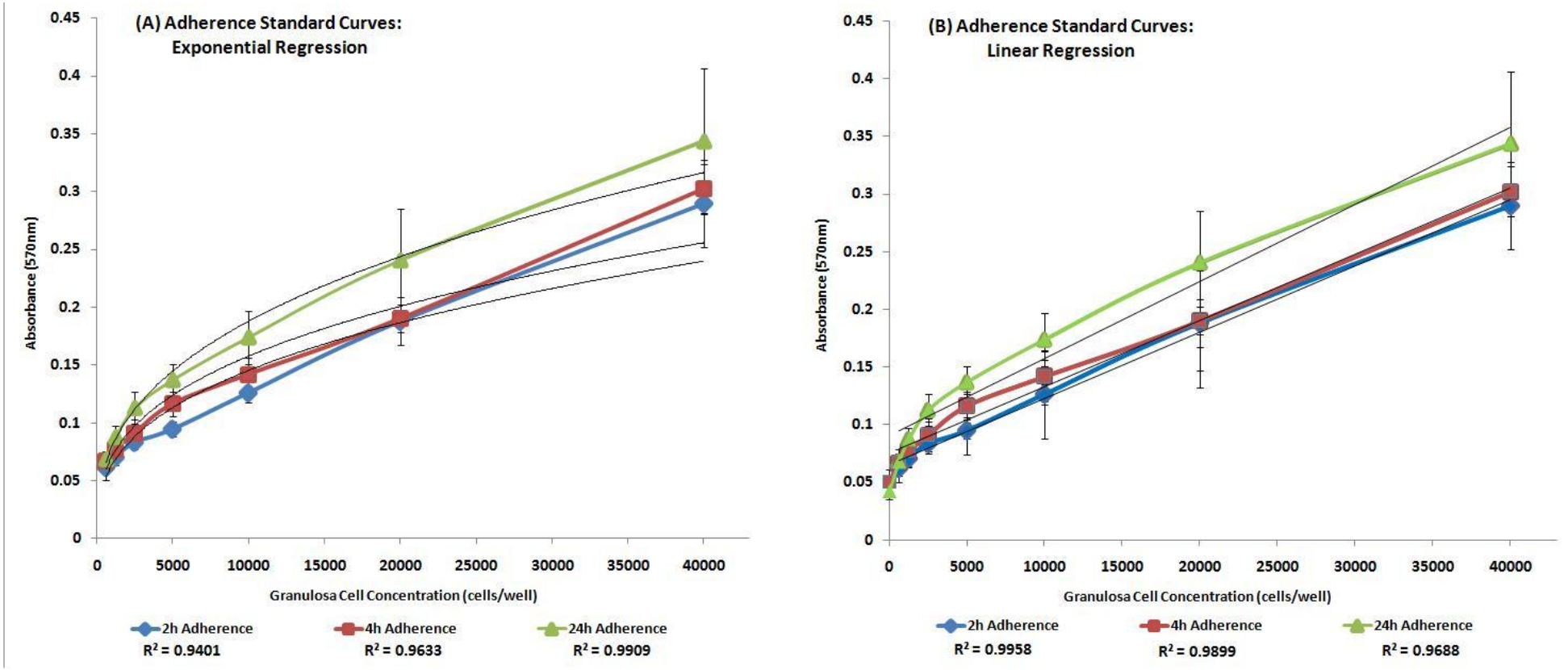


Figure 3-3: Formazan production by GC after 2, 4 or 24 h adherence

Granulosa cells seeded at concentrations from 0 to 4× 10^4 cells/well were allowed to adhere for 2 h (n=3), 4 h (n=4) or 24 h (n=4) in DMEM/F12 + 10% FCS. GC were incubated with MTT (0.5mg/mL) for 24 h, and formazan production (absorbance of 570nm) correlated to granulosa cells concentration. Each line represents adherence time with respective R-square value determined by (A) Exponential regression or (B) Linear regression analysis. Error bars represent mean absorbance ± 1 SEM.

The intra-assay coefficient of variance (intra CoV) of $OD_{570\ nm}$ between replicate wells at each cell density was calculated, refer Figure 3-4. Lower granulosa cell concentrations of 2.5×10^3 and 5×10^3 cells/well resulted in higher intra CoV in comparison to 1×10^4 and 4×10^4 cells/well densities which had intra CoV less than 10%. The 24 h adherence standard curve had lower intra CoV in comparison to the 2 and 4 h adherence periods at all concentrations except 625 and 2,500 cells/well. This indicates that granulosa cells cultured for a 24 h culture post-isolation may indicate a granulosa cell population which has similar metabolic capabilities.

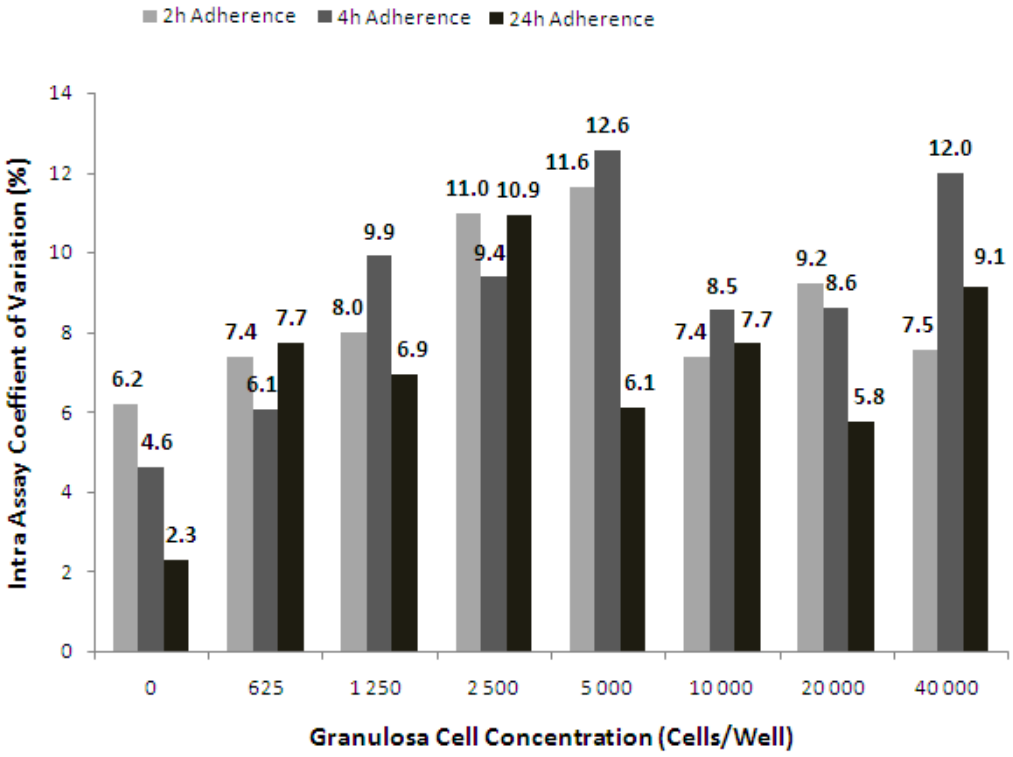


Figure 3-4: Intra Assay Coefficient of Variation for Adherence Standard Curves

Granulosa cells (GC) seeded at concentrations from 0 to 4× 10^4 cells/well in DMEM/F12 + 10% FCS, at adherence periods of 2, 4 or 24 h. GC were incubated with MTT (0.5mg/mL) for 24 h, formazan production was quantified by measuring absorbance at 570nm. The OD_{570nm} is means of triplicate wells within each assay. The intra CoV was calculated by expressing the standard deviation by the mean of triplicate wells at each GC density and multiplying by 100 to present as percentage (%). Bars represent the mean of the intra CoV of n=3 repeat assays and data values for each bar are located on the outside.

The second criterion investigated was which linear equation generated from each adherence period would accurately represent the cell number of granulosa cells cultured for 24 h, as determined by MTT assay (Figure 3-5). All adherence standard curves showed a strong correlation between the following variables: the number of viable GC after 24 h culture and initial GC concentration seeded. Figure 3-6, shows that the number of viable granulosa cells generated by the 24 h adherence standard curve equation was more accurate in reflecting number of viable granulosa cells initially seeded after 24, 48 and 72 h of culture because of the lower intra CoV, higher metabolic capability and absorbance reading and produced a strong linear region of the range of cell densities which are of interest.

Thus 24 h was determined to be optimal adherence period to culture GC prior to treatment exposure.

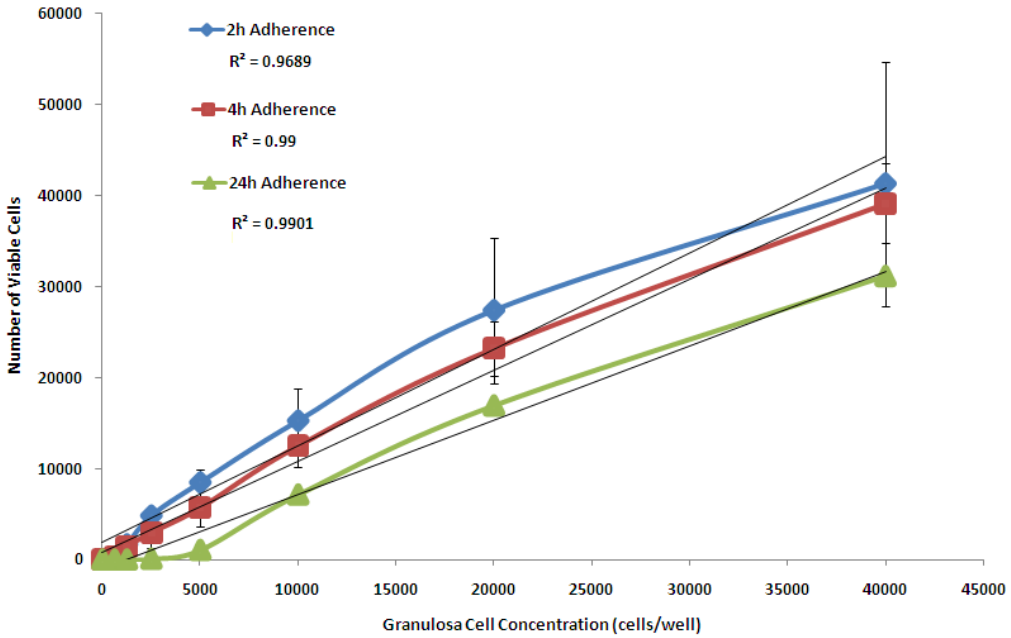


Figure 3-5: Standard Curves for Determining Number of Viable Cells by MTT Assay

Granulosa cells (GC) seeded at concentrations from 0 to 4× 10^4 cells/well in DMEM/F12 + 10% FCS, and allowed to adhere for 24 h and further cultured for 24 h. GC were then incubated with MTT (0.5mg/mL) for 24 h, and formazan production was quantified by measuring absorbance at 570nm. The OD_{570nm} was used to generate the number of viable cells from the equation for each adherence time 2 h (n=3), 4 h (n=4) or 24 h (n=4). Number of viable cells generated was correlated to the granulosa cell concentration originally seeded. Each line represents linear regression relationship between number of viable cells and GC concentration. Error bars represent mean Number of viable cells ± 1 SEM.

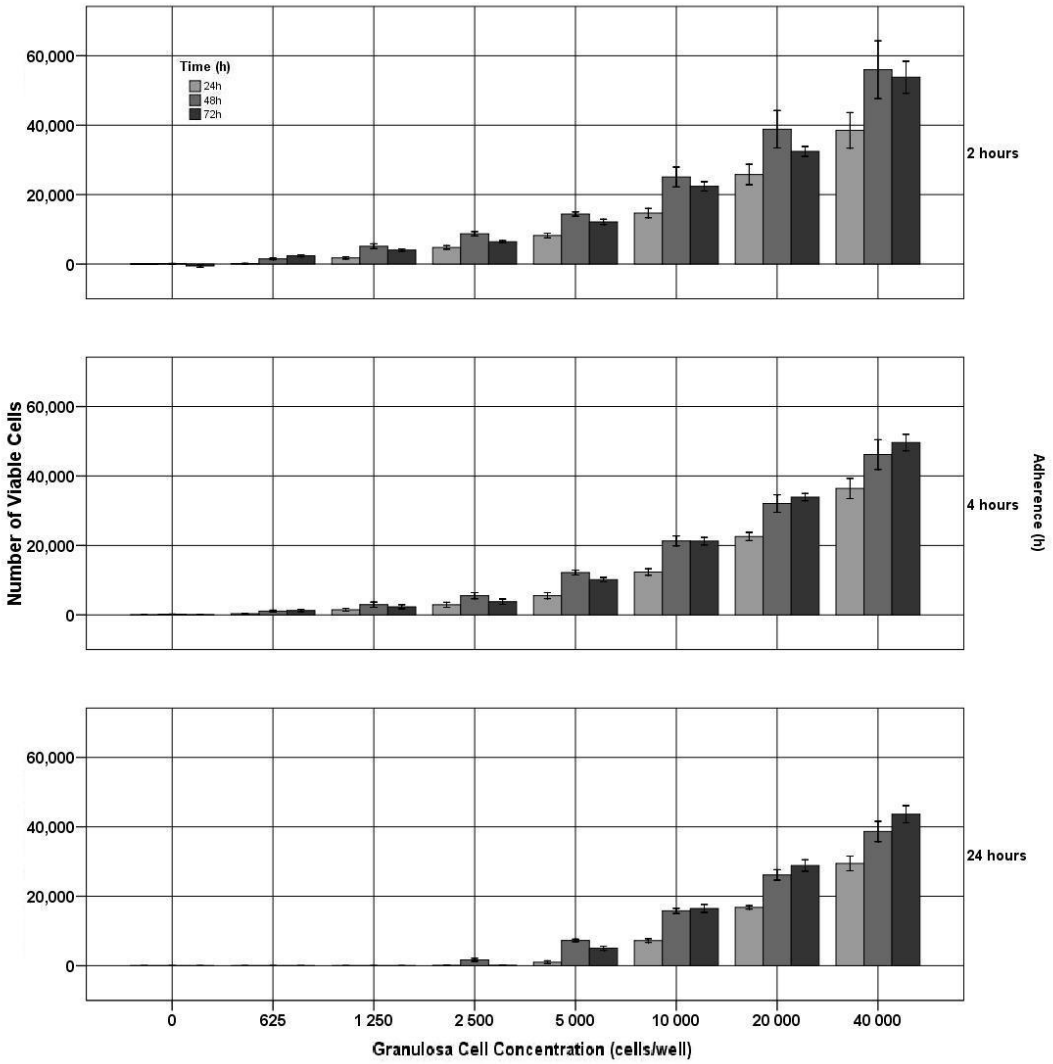


Figure 3-6: Number of Viable Cells after 24, 48 and 72 h as determined by each Adherence Standard Curve

Granulosa cells seeded at concentrations from 0 to 40× 10^3 cells/well were cultured for 24 h, 48 h or 72 h in DMEM/F12 + 10% FCS. GC incubated with MTT (0.5mg/mL) for 24 h, and formazan production was quantified by measuring absorbance at 570nm. The OD_{570nm} was used to calculate the number of viable cells present at 24 h, 48 h and 72 h using the linear regression equation for each adherence time 2 h (n=3), 4 h (n=4) or 24 h (n=4). One-way ANOVA was used to determine significant differences in number of viable cells over 72 h culture period. Each bar represents number of viable cells ± SEM.

3.4.1.2 Determination of Optimal Granulosa Cell Concentration for *in vitro* culture

A 24 h pre-treatment culture was applied to the subsequent experiments including the standard curve (a linear equation was used to determine cell numbers) and the three culture plates (24, 48 and 72 h). Specifically, granulosa cell concentrations of 1×10^4, 2×10^4 and 4×10^4 cells/well were investigated to determine the optimal GC density (cells/well) to use for *in vitro* toxicity studies over a 72 h culture period, Figure 3-7 and Figure 3-8. Viability, progesterone ($ng.mL^{-1}$) and estradiol ($pg.mL^{-1}$) were quantified at 24 h intervals as these were deemed important factors in the characterisation of optimal *in vitro* culture conditions for GC.

The standard curve was not sensitive in determining the number of viable cells ranging between the GC concentration range of 625 to 2,500 cells/well as $OD_{570\ nm}$ was below the 0 cell/well control wells, Figure 3-7. The number of viable cells after 72 h remained the same or only slightly increased compared with that observed at the 48 h culture period, Figure 3-7. The MTT assay indicates that an initial density of 2×10^4 cells/well was maintained over the 72 h period. At 2×10^4 cells/well progesterone concentrations were optimal, being significantly higher than at 1×10^4 cells/well and did not differ from wells containing 4×10^4 cells/well (Figure 3-8). Estradiol concentrations were less variable and lower at 2×10^4 cells/well in comparison to the 4×10^4 density, (Figure 3-8). A time-dependent decrease in estradiol concentrations occurred at 2×10^4 and 1×10^4 cells/well, however at the latter cell concentration, progesterone concentrations did not increase with increased culture time. The optimal GC concentration for use in culture after an initial 24 h adherence period was the 2×10^4 cells/well due to stability and reproducibility of the number of viable cells, lower estradiol and higher progesterone production- indicating the presence of granulosa-lutein cells.

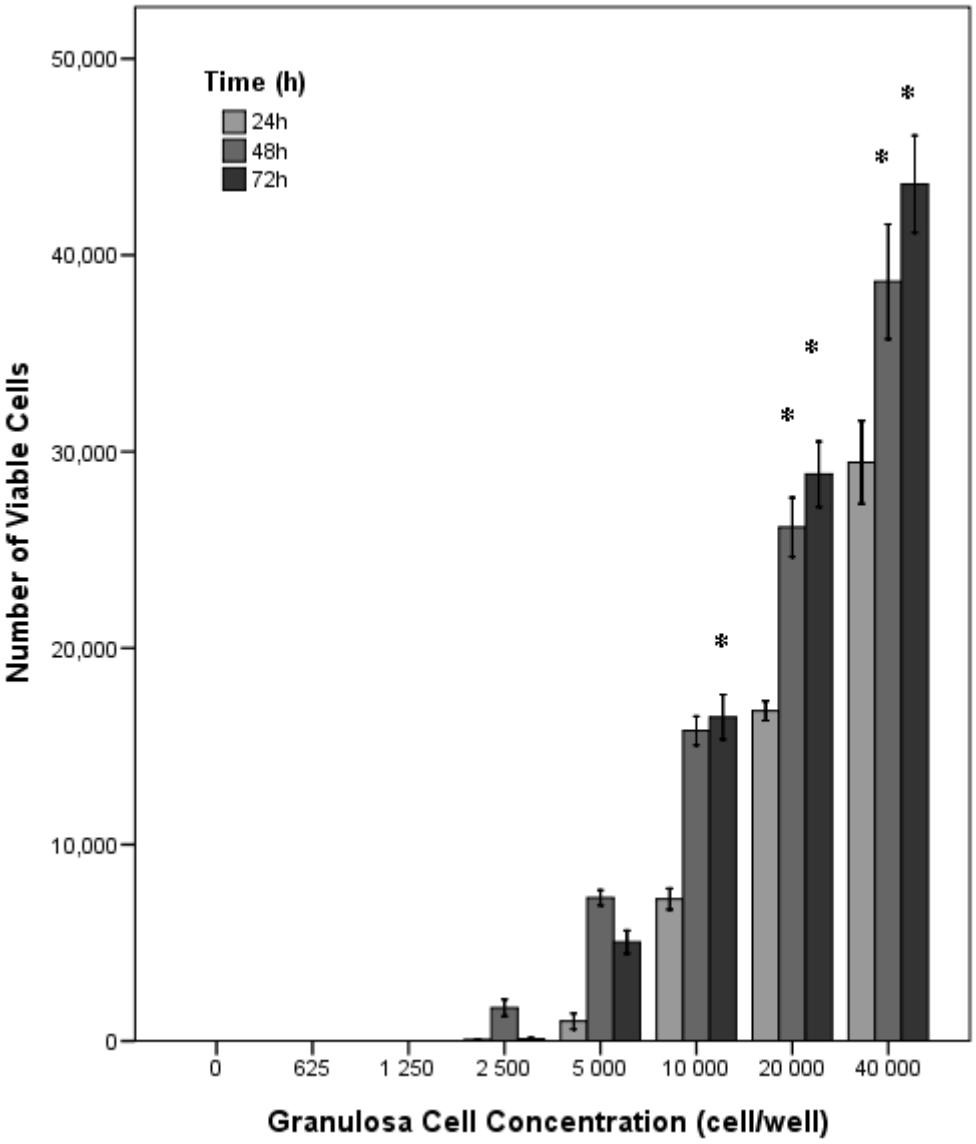


Figure 3-7: Number of Viable Granulosa Cells after 24, 48 and 72 h

Granulosa cells (n=4) seeded at densities in the range of 0 to 4× 10^4 cells/well were cultured for 24 h, 48 or 72 h in DMEM/F12 + 10% FCS. GC were incubated with MTT (0.5mg/mL) for 24 h, and formazan production (absorbance at 570nm) was used to calculate the number of viable cells from the 24 h adherence linear regression equation. One-way ANOVA was used to determine significant differences in number of viable cells over 72 h culture period. Each bar represents number of viable cells ± SEM, $p<0.05$.

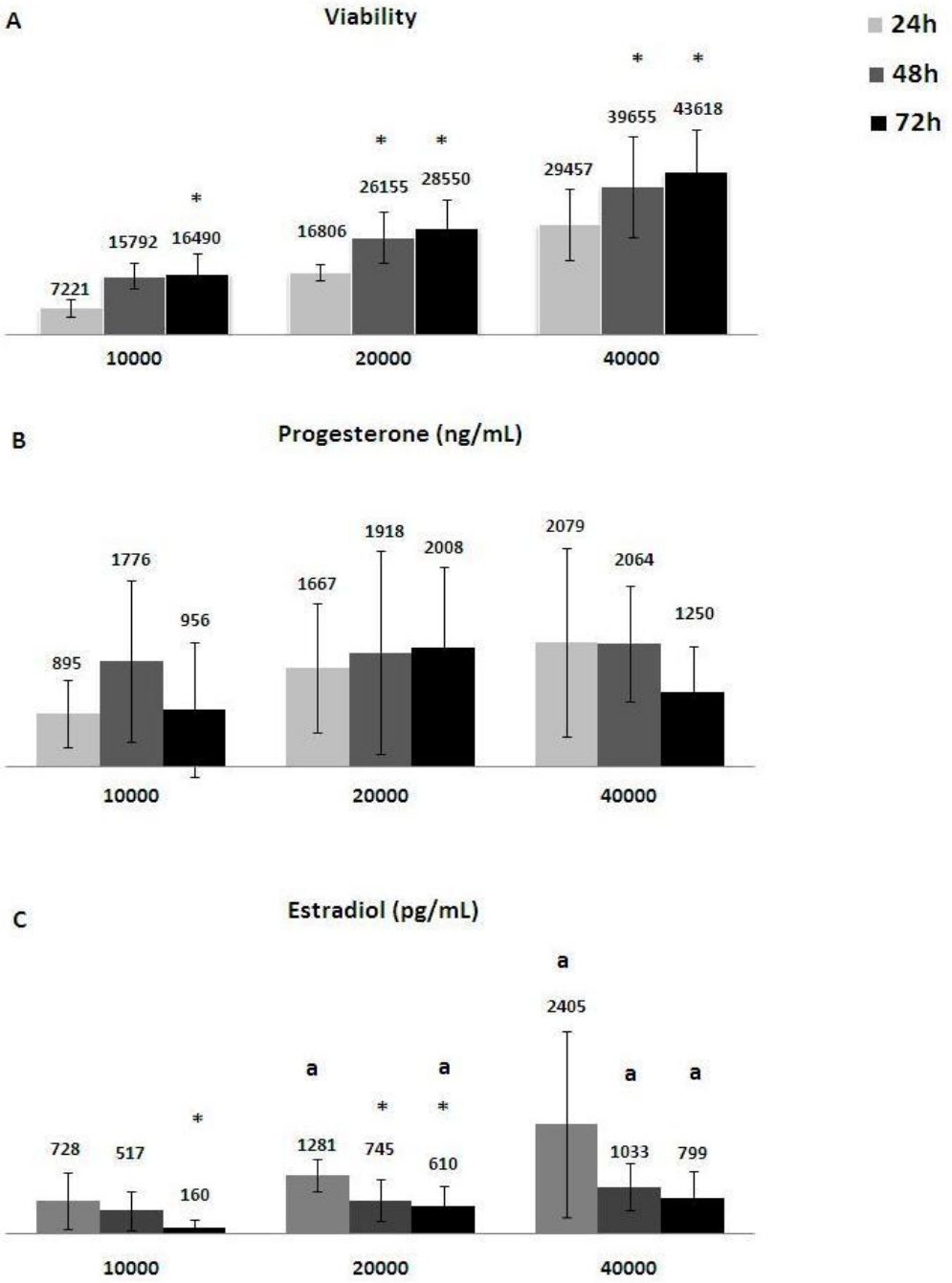


Figure 3-8: Comparison of Variables from Granulosa Cells Cultured at Various Denisties

Number of viable granulosa cells (A), progesterone concentration (B, $ng.mL^{-1}$), and estradiol concentration (C, pg/mL) (n=4). GC were seeded at a densities of 10×10^3 cells/well, 20×10^3 cells/well or 40×10^3 cells/well and then cultured for 24 h, 48 or 72 h in DMEM/F12 + 10% FCS. GC were incubated with MTT (0.5mg/mL) for 24 h, and formazan production (absorbance of 570nm) was used to calculate the number of viable cells from the 24 h adherence linear regression equation. Steroid hormone concentrations were determined by RIA. Statistical significance was determined by one-way ANOVA, p-values <0.05 for each variable. * Represents significant differences from 24 h within each GC density; [a] represent significant differences from 10×10^3 cells/well within time point.

3.4.1.3 Standard Curves: Comparison of the MTT and Crystal Violet Assays in Primary-derived Human Granulosa Cells

The MTT assay, which is dependent on metabolic production of formazan, and the Crystal Violet assay, which stains live adhered cells were compared in terms of their accuracy, sensitivity and reproducibility in determining the number of viable cells. The absorbance after 24 h in culture and the number of granulosa cells initially seeded was strongly linearly correlated in both the MTT assay, (R^2= 0.98, n=15) and the Crystal Violet assay (R^2=0.99, n=9), Figure 3-9. Further investigation found that when data points were fitted with an exponential regression the R-square value of the MTT assay and Crystal Violet were similar R^2=0.98 and R^2=0.99, respectively, Figure 3-9. The sub-linear formazan production in the MTT assay at the lower cell densities in comparison to the higher cell densities which resulted in the linear range of absorbance may be attributed to the limited amount of tetrazolium substrate available and changes in metabolic capability of cells at higher densities. Nonetheless, both assays produced absorbance's which were strongly linearly correlated to GC concentration.

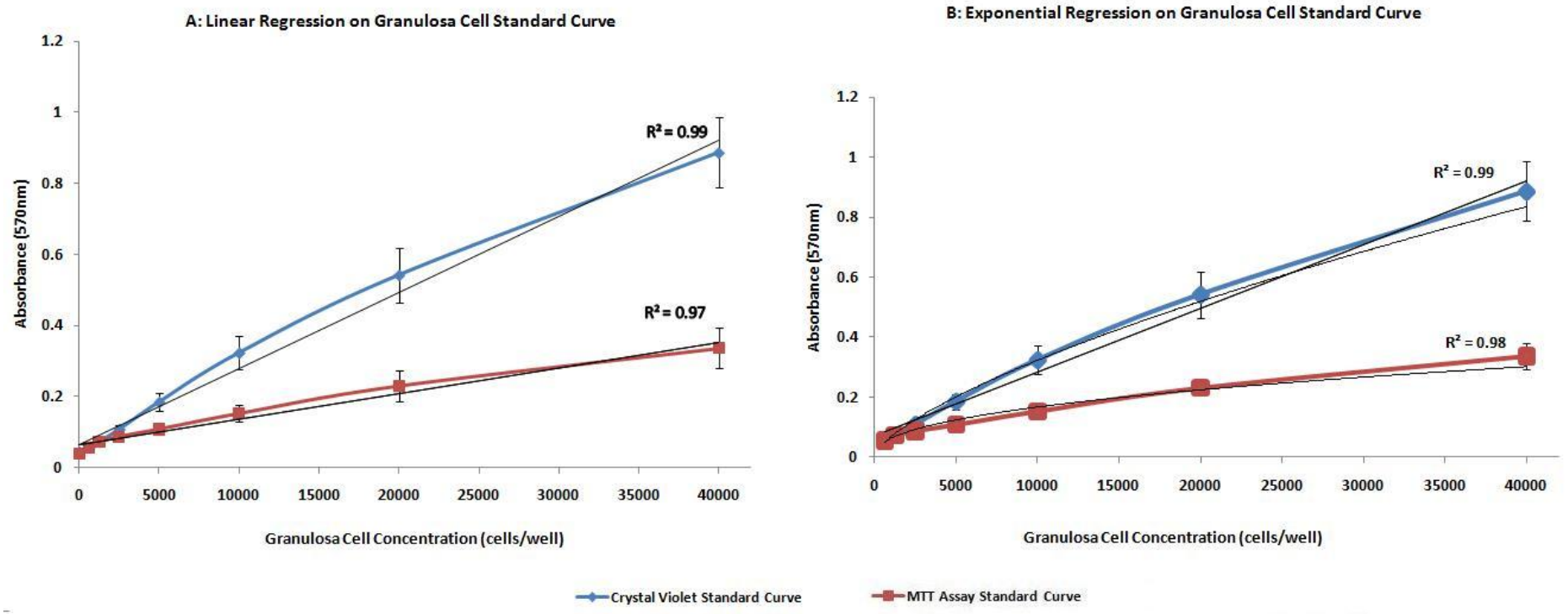


Figure 3-9: Comparison of MTT and Crystal Violet Standard Curves

Granulosa cells seeded at densities in the range of 0 to 4× 10^4 cells/well in DMEM/F12 + 10% FCS. GC were incubated with MTT (0.5mg/mL, n=16) for 24 h or stained with Crystal Violet (0.5% w/v in 50% methanol, n=8). Inset graph (A) represents linear regression of data points, main graph (B) represents data points fitted with exponential regression. Each data point represents mean absorbance (570nm) ± SEM at each GC concentration (cells/well).

3.4.1.4 Standard Curves: Comparison of the MTT and Crystal Violet Assay in the KGN Granulosa Cell Line

Inter-individual variability associated with using primary-derived human granulosa cells may interfere with the strength of association between absorbance and cell number, and thus both viability assays were optimised in KGN cells. As a cell line they are not subject to significant biological variability between repeats and would be less variable than primary-derived cells. The MTT assay (n=3) had previously shown a strong linear relationship between formazan production and KGN cell concentrations in our laboratory (Figure 8-6, KGN MTT Standard Curve).

The Crystal Violet assay also produced an R-square value of 0.99 which shows a good correlation between absorbance and KGN cell concentration from three independent repeats (Figure 3-10).

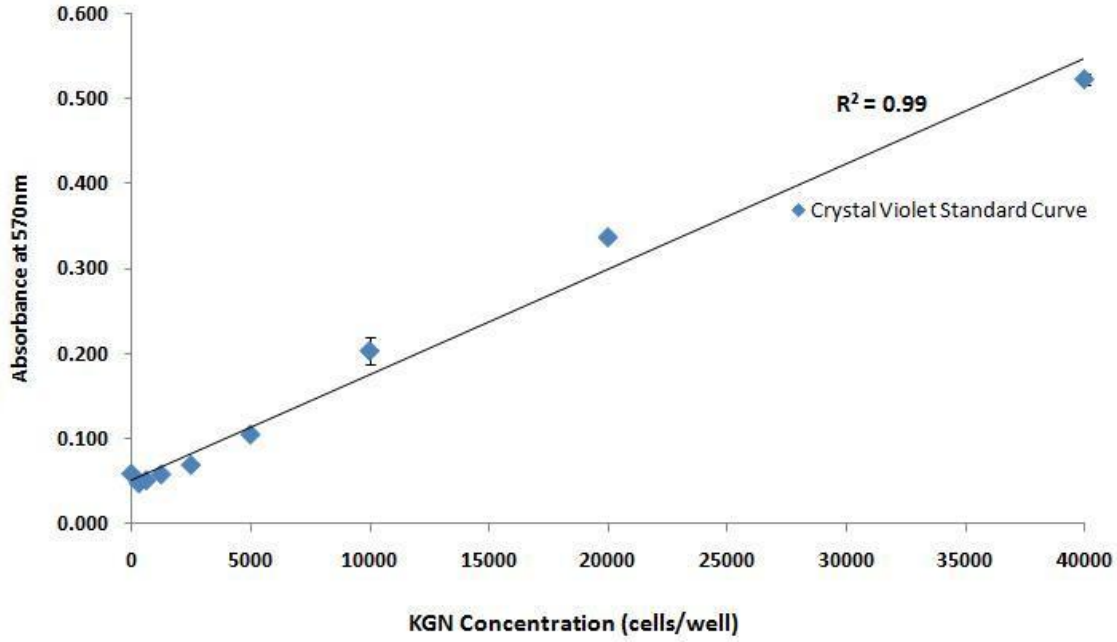


Figure 3-10: Crystal Violet Standard Curve of KGN

KGN (n=3) seeded at densities in the range of 0 to 4× 10^4 cells/well were allowed to adhere for 24 h in DMEM/F12 + 10% FCS and then processed for the Crystal Violet assay. Linear regression of mean absorbance (570nm) ± SEM in relation to KGN concentration (cells/well).

3.4.1.5 Viability: Comparison of the MTT and Crystal Violet Assays in Primary-derived Human Granulosa Cells

A comparison between the numbers of viable cells calculated from the MTT and Crystal Violet assays was carried out after 24, 48 or 72 h at an initial density of 2×10^4 cells/well (Figure 3-11). There were no statistically significant differences between viable cell numbers calculated from either assay. Both assays showed higher numbers of viable cells than the 2×10^4 cells/well that was originally seeded (Figure 3-11).

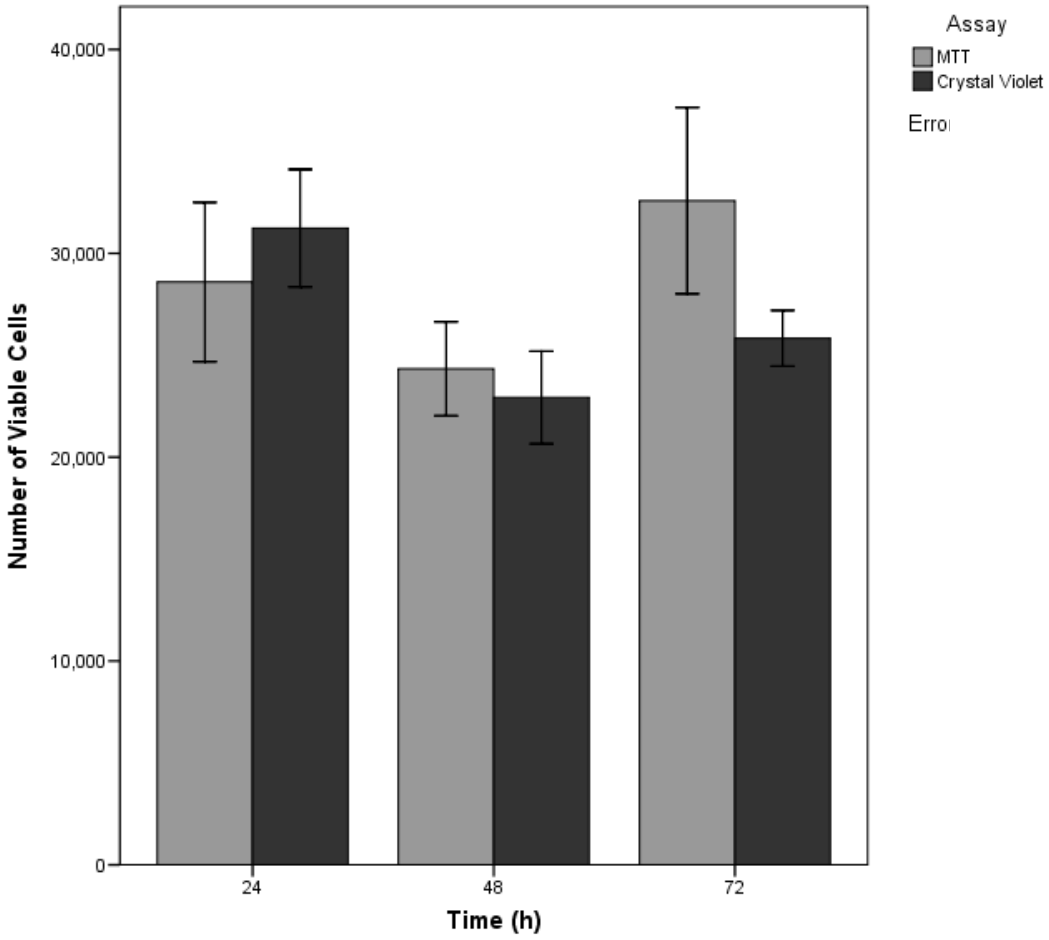


Figure 3-11: Comparison of Number of Viable Cells determined by MTT and Crystal Violet Assay over 72 h Culture Period

Granulosa cells were cultured at 2× 10^4 cells/well in DMEM/F12 + 10% FCS. GC were incubated with MTT (0.5mg/mL, n=18) for 24 h or stained with Crystal Violet (0.5% w/v in 50% methanol, n=8). Absorbance at 570nm was used to calculate the number of viable cells from the 24 h adherence linear regression equation. Calculated number of viable cells at each incubation time was correlated with the granulosa cell concentration originally seeded. Each bar represents the number of viable cells ± SEM.

3.4.2 Optimisation of ORO staining for use with Primary-derived Human Granulosa Cells

3.4.2.1 Optimal Oil Red O Staining Conditions

Three variables were required to be characterised in order to identify positive ORO lipid staining in MLTC-1 cells. These were the ORO stain concentration, incubation time and temperature. The key determining factor of ORO positive staining was the ORO stain preparation (significantly higher staining with Stain 2 preparation, $P < 0.0005$, Figure 3-12). There was no difference in positive ORO cells exposed to 15 min or 30 min incubation with Stain 2 at either 22°C or 37°C ($P > 0.05$, Figure 3-13 & Figure 3-14). The parameters of the ORO staining were selected in the following order: ORO stain 2, followed by an optimal incubation time of 15 min, as temperature had no effect on staining, 22°C was selected.

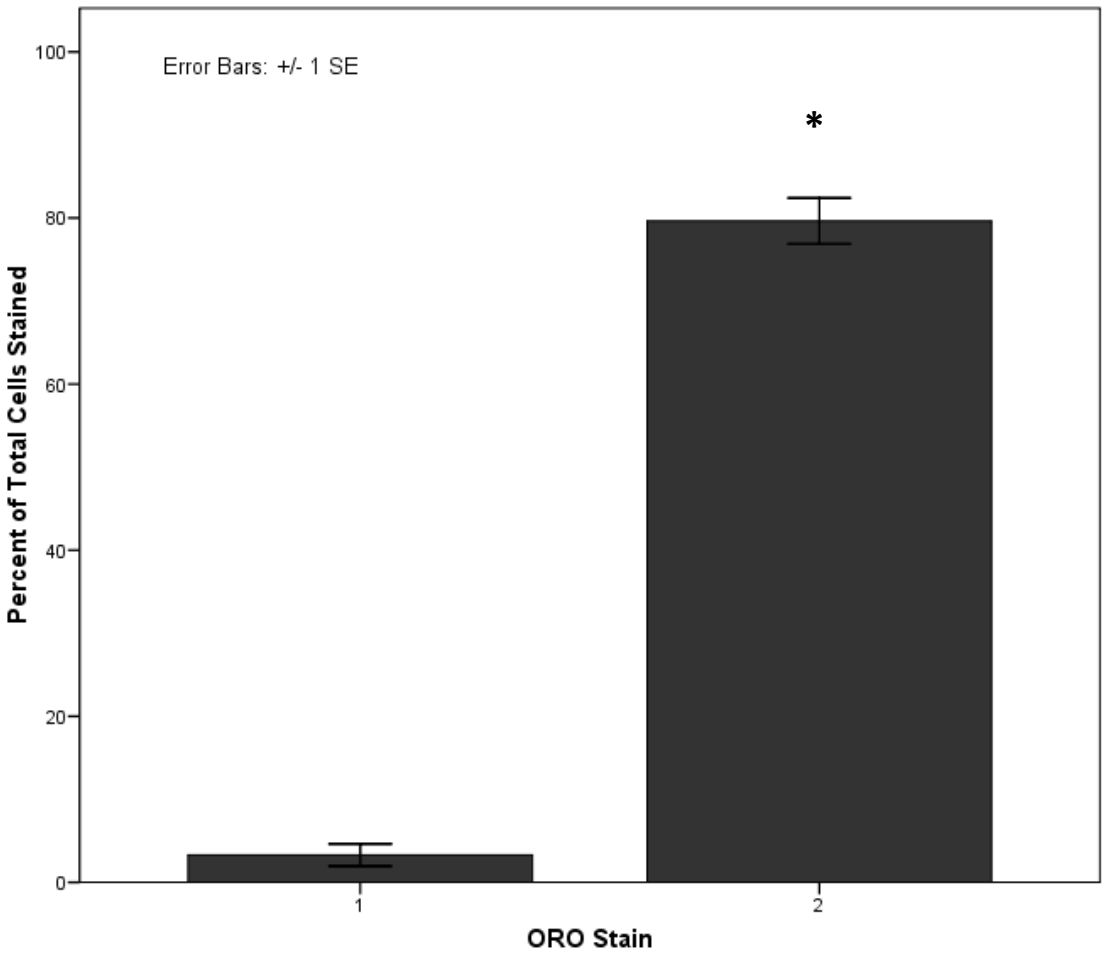


Figure 3-12: Comparison of ORO positive hCG-stimulated MLTC-1 cells in ORO Stain 1 and 2

MLTC-1 (n=4) were cultured at 4× 10^4 cells/well for 4 h in 300 µL RPMI + 10% FCS +1000mIU/mL hCG. MLTC-1 cells were stained with ORO for presence of lipids with two preparations of ORO Stain (ORO Stain 1 or 2). Four images were acquired per well and cells were scored for presence or absence of ORO-stained lipids. Each bar represents stained cells as a percentage of total cells counted ± SEM, **P* <0.0005 by Independent t-test.

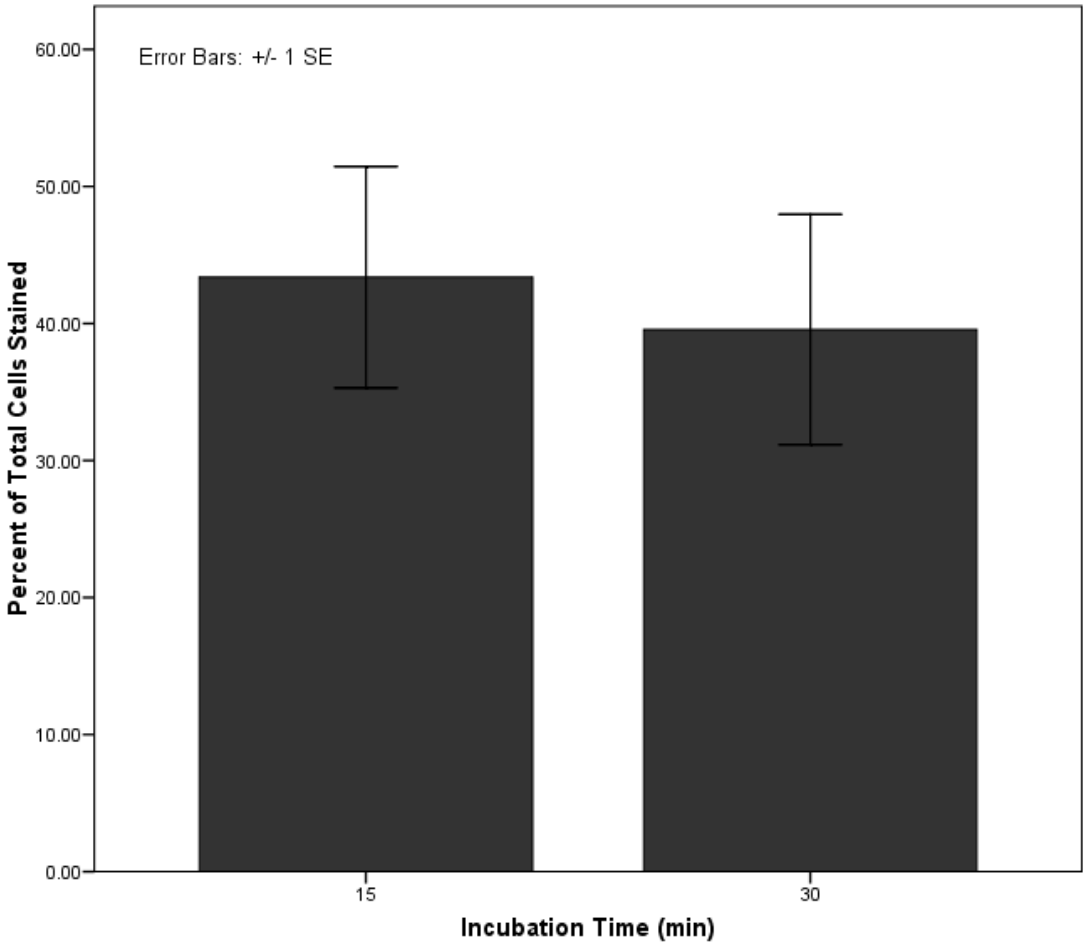


Figure 3-13: Comparison of ORO positive hCG-stimulated MLTC-1 cells after 15min and 30 min

MLTC-1 (n=4) were cultured at 4× 10^4 cells/well for 4 h in 300 µL RPMI + 10% FCS +1000mIU/mL hCG. MLTC-1 cells were stained with ORO for presence of lipids using ORO stain 2 for 15 or 30 min. Four images were acquired per well and cells were scored for presence or absence of ORO lipids. Each bar represents stained cells as a percentage of total cells counted ± SEM.

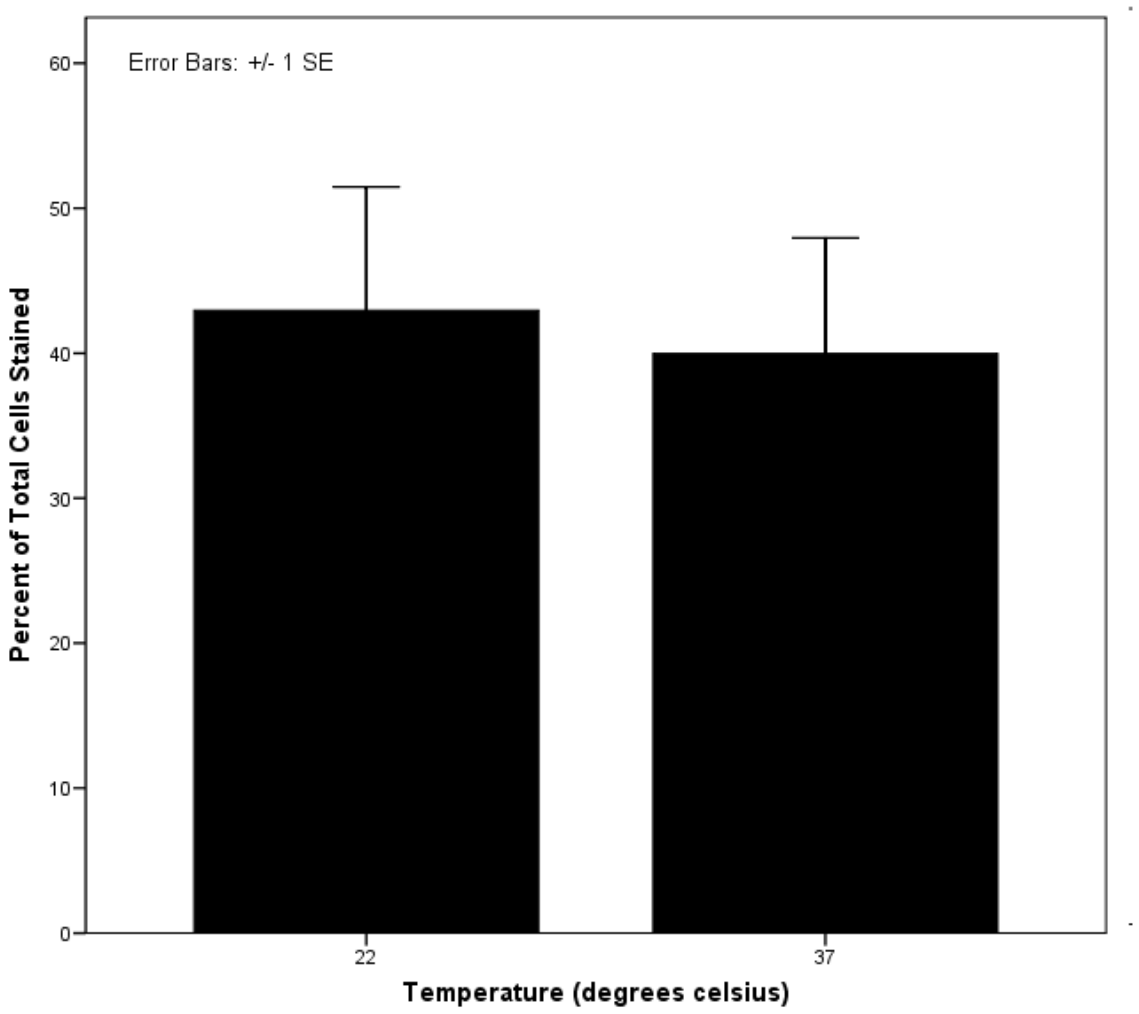


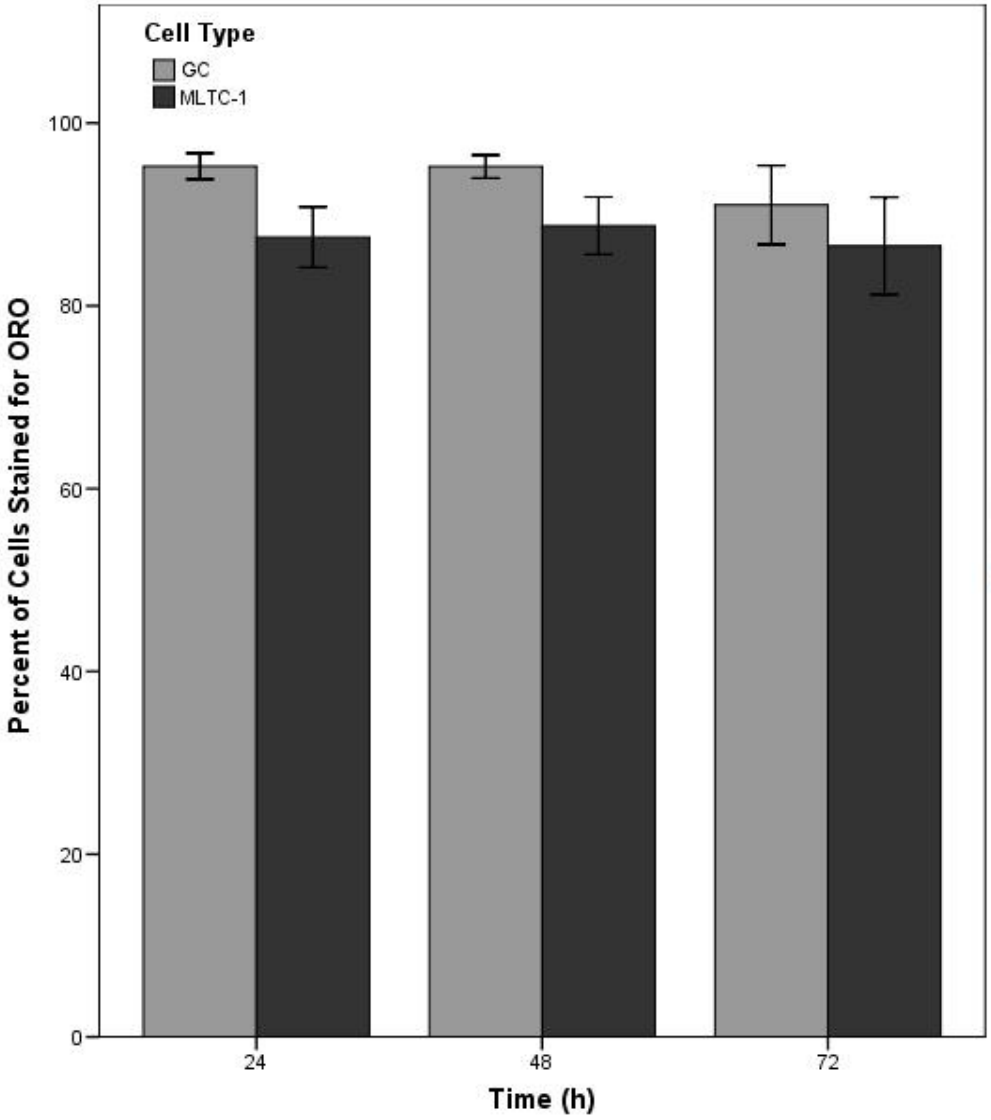
Figure 3-14: Comparison of ORO positive hCG-stimulated MLTC-1 cells at 22 and 37 degrees Celsius

MLTC-1 (n=4) were cultured at 4× 10^4 cells/well for 4 h in 300 µL RPMI + 10% FCS +1000mIU/mL hCG. MLTC-1 cells were stained with ORO for presence of lipids using ORO stain 2 for 15min at either 22^{o}C or 37^{o}C. Four images were acquired per well and cells were scored for presence or absence of ORO lipids. Each bar represents stained cells as a percentage of total cells counted ± 1 SEM.

3.4.2.2 Comparison of Oil Red O in MLTC-1 Cells and Primary-derived Human Granulosa Cells

MLTC-1 cells were used to optimise ORO staining conditions as they are a cell line with low variability between passage numbers and were readily available for experimental use. The optimised conditions were trialled in human primary derived granulosa cells and compared to MLTC-1 ORO staining over a 72 h culture period. Independent t-test showed that there was no significant difference in percent positive ORO staining between MLTC-1 and human primary-derived granulosa cells after 72 h using optimised conditions outlined in 3.4.2.1 (Figure 3-15).

.


Cell Type
GC
MLTC-1
Percent of Cells Stained for ORO
100
80
60
40
20
0
24
48
72
Time (h)

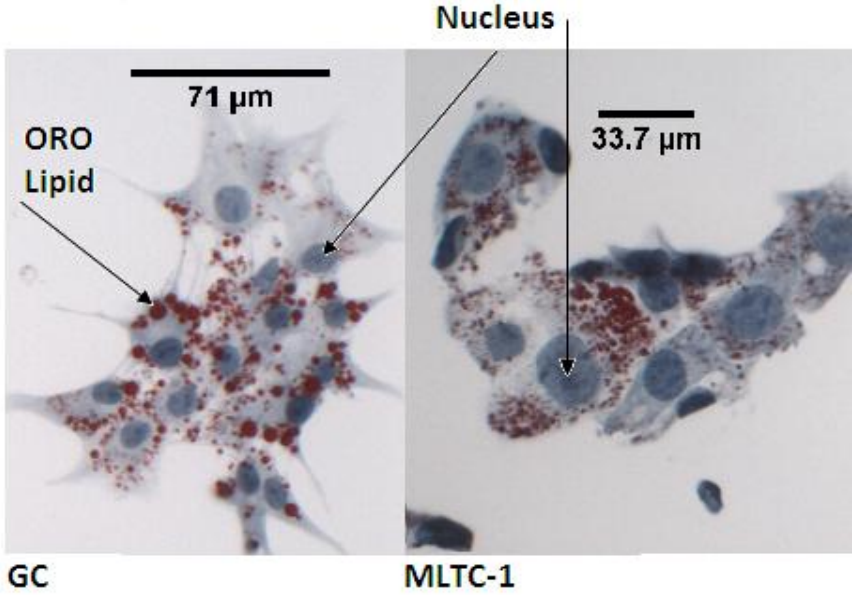

Nucleus
71 µm
ORO
Lipid
33.7 µm
GC
MLTC-1

Figure 3-15: Comparison of ORO positive hCG-stimulated MLTC-1 cells and human primary-derived Granulosa Cells (GC)

MLTC-1 cells were cultured at 40× 10^3 cells/well in 300 µL RPMI + 10% FCS +1000mIU/mL hCG and human primary-derived Granulosa cells (GC) were cultured at 40× 10^3 cells/well in 300 µL DMEM/F12 + 10% FCS (n=4) for 24, 48 or 72 h. Cells were stained for presence of cholesterol lipids with ORO stain 2 for 15 min at 22^{o}C. Four images were acquired per 8-well LabTek II chamber slide and cells were scored for presence or absence of ORO lipids. Each bar represents percentage of total cells counted ± 1 SEM. An example of ORO staining is also shown; arrows indicate the nucleus of each cell. The GC image shows 14 cells and the MLTC-1 image shows 13 cells with identifiable nuclei ×200 magnification on brightfield microscope. Magnification x 400.

3.5 Discussion

Viability assays are the usually the first step of toxicity assessment; correct interpretation relies on their accuracy, sensitivity, reproducibility and swiftness, attributes found in both the MTT and Crystal Violet assays (Fotakis & Timbrell 2006; Gillies et al. 1986; Ishiyama et al. 1996; Siddiqui et al. 2006). A 24 h adherence period was found to be the optimal culture period for human-primary derived granulosa cells *in vitro*. An exponential and linear model could be fitted to the data obtained by the MTT assay, representing a strong association between OD_{570nm} and cell number after 24 h adherence.

A plateau of absorbance in wells at the higher granulosa cell densities cultured may be attributed to a combination of increased metabolic activity and limited substrate availability. This was indicated when comparing 2, 4 and 24 h adherence standard curves; whereby the 2 h and 4 h produced similar OD_{570nm} at the highest GC density. At 24 h the OD_{570nm} readings suggest that there was an increase in metabolic capability. Further experiments are required in which extended adherence periods, examination of higher granulosa cell densities and increased tetrazolium concentrations are needed to determine if substrate availability was a limiting factor or granulosa cells gained increased metabolic capabilities with prolonged time in culture. Granulosa cell density plays a key role in metabolic activity of cells whilst in culture, whereby at low densities (5×10^3 cells/well) GC maintain mitotic capabilities and can proliferate, whereas at higher cell densities (5×10^4 cells/well) are committed to differentiation (Chaffkin et al. 1993). It is unlikely that the GC at higher densities were deprived of substrate as the concentration of MTT used was 0.5 $mg.mL^{-1}$, which was previously optimised by Young et al. (2005c) and was found to be an optimal concentration that balances cytotoxicity and maximum formazan production by granulosa cells (Young et al. 2008; Young et al. 2005).

The presence of other cell types and factors in the culture medium may interfere with the MTT assay. Granulosa cells isolated from follicular aspirates will always be a part of a heterogeneous cell population which contains leukocytes, lymphocytes and macrophages (Antczak & Van Blerkom 2000; Baranao et al. 1995; Loukides et al. 1990). A comparative assay between an ovarian cell line (HeLa

cells) and a murine macrophage cell line (Raw 264.7) found that macrophage activation in the presence of NADPH interferes with the ability of the mitochondria to reduce MTT thus the MTT assay is at risk of underestimating the number of viable cells in what were similar culture conditions used in our experimental model (Ahmad et al. 2006). At the same time studies suggest that formazan is spontaneously produced as an artefact of the MTT assay due to the possibility of other sites than the mitochondria for reduction of MTT, whereby inducible nitric oxide synthase (iNOS) which has a high affinity for the MTT salt, also spontaneously generates formazan (Pozzolini et al. 2003). Studies have shown that presence of albumin used in culture medium at a 10% concentration reduced absorbance readings of the MTT assay by 50% affecting cell number determinations (Huang et al. 2004). However in our experimental model it is essential that 10% FCS is used to maintain granulosa cell viability.

Thus the term 'optimal adherence period' may not necessarily indicate GC are at optimal metabolic functioning in which they have regained sensitivity from *in vivo* gonadotropin stimulation, have luteinised or are at their optimal steroidogenic output. As demonstrated the most we can extrapolate from the adherence period is the time required for cells to attach in the culture vessel under the described conditions to allow for experimental research.

The linear regression model of the 24 h adherence standard curve was used to determine the number of viable granulosa cells cultured at 24, 48 and 72 h. A 24 h adherence is a minimum period for which granulosa cells can attach, continue to luteinise and potentially reach optimum mitochondrial metabolic activity indicated by their ability to produce formazan (Zlotkin et al. 1986). The selection of 24 h allows granulosa cells to desensitize from *in vivo* gonadotropin stimulation and is supported by published literature of routinely used *in vitro* culture methods for primary-derived granulosa cells (Beckman et al. 1991). The 2 h adherence period compromised the accuracy of the cell number determination despite having the highest sensitivity (625 cells/well). The required cell concentration range of 0.25 – 4×10^4 cells/well was in the linear region of the 24 h adherence standard curve,

further supporting the 24 h as the optimal GC pre-treatment culture period, with high accuracy in representation of number of viable cells.

It is accepted that studies involving granulosa cell culture use cell densities that range from 0.5 – 5 × 10^4 cells/well in 96-well culture plates (Beckman et al. 1991; Chaffkin et al. 1993). Granulosa cell densities of 1, 2 and 4 × 10^4 cells/well were investigated to determine optimal viability and steroid hormone production for culture in 96-well plates. A cell density of 4 × 10^4 cells/well cultured for 72 h generated an overestimation of cell numbers by the MTT assay. This is most likely attributable to hypertrophy of cells rather than an indication of proliferation. Hypertrophy is a result of GC differentiation into large luteal cells with the potential for increased hormone production involving the mitochondria and increased MTT metabolic activity (Chaffkin et al. 1993; Zlotkin et al. 1986). Granulosa cells undergo marked hypertrophy over the 24 to 72 h period after hCG exposure *in vivo*, in which there is an abundant increase in cytoplasm, increased nuclei size and cessation of proliferation (Landefeld et al. 1997). Interestingly granulosa cells obtained from women who were over 38 years of age had substantially less mitochondria compared to women less than 34 years of age (Fatum et al. 2009). The mean age of women used in the entire research was 35 ± 5 years of age and unlikely to be the main reason for the varied formazan produced. The link with MTT metabolism is supported by Gerlier and Thomasset (1986), who found that the MTT assay can determine a cell's ability to produce formazan independent of proliferation. Thus it is difficult to determine if granulosa cell numbers have increased, have differentiated or if cells have hypertrophied. Progesterone concentrations were consistently higher at 2 × 10^4 cells/well while estradiol concentrations significantly decreased over the culture period in comparison to other cell densities, indicating that luteinisation occurred under these culture conditions. The 2 × 10^4 cells/well was the optimal density for *in vitro* culture of GC.

The MTT assay determines viability of granulosa cells on the basis of their metabolic activity, whereas the Crystal Violet assay determines viability by measuring the uptake of Crystal Violet stain by attached cells (Gillies et al. 1986;

Hansen et al. 1989; Ishiyama et al. 1996; Prochaska & Santamaria 1988; Russell & Robker 2007; Saotome et al. 1989; Siddiqui et al. 2006; Steuerwald 2007; Zlotkin et al. 1986). Fitting the absorbance data of both assays with a linear and exponential regression produced a strongly linear association (R^2-values were above 0.97) between OD_{570nm} and number of cells. For this research, the viability of cells cultured for cytotoxicity assays required the linear region to be within the range of $0.25 – 4 \times 10^4$ cells/well, for which the equation generated by the linear regression was most suitable.

It can be concluded first, that the MTT and Crystal Violet assay both accurately determine viability, are reproducible and these results support that of published literature (Best et al. 1995; Fotakis & Timbrell 2006; Hansen et al. 1989; Lambert et al. 2000; Mosmann 1983). Secondly, the repeat assays involve granulosa cells from different women, the high inter-individual variability which exists in using primary-derived cells reflected by high standard error bars in the higher cell densities by both viability assays despite their different mechanisms for determining viability (Fotakis & Timbrell 2006; Ishiyama et al. 1996). The variance within the MTT assay may be attributable to mitochondrial aging, whereby GC isolated from different women of varying ages will have mitochondria that have differing metabolic activities (Fatum et al. 2009; Steuerwald 2007). Mitochondrial deletions in the nucleotide sequence of mtDNA may reach up to 73% in oocytes of women older than 37, and up to 53% in women younger than 32 (Hsieh et al. 2004). The women in our study were 35±5 years old. Thus production of mitochondrial dehydrogenase enzymes and their metabolic functioning may be altered (Hsieh et al. 2004).

Determining granulosa viability, defined by a cell's capability to carry out metabolic processes as opposed to cell staining by assessing mitochondrial activity using the MTT assay, was a particularly important end point. The sub-optimal functionality of the mitochondria and the mitochondrial dehydrogenase enzymes will also reflect capability of steroid hormone production, as the mitochondria play a key role in steroidogenesis. This creates further difficulty in expressing hormone production relative to viable cell number determined by MTT assay, hence the need

to assess viability via two distinct mechanisms simultaneously to increase reliability of results when determining cytotoxic effects (Amsterdam et al. 1999; Arthur & Boyd 1974; Fotakis & Timbrell 2006; Hansen et al. 1989).

It is expected that GC obtained from pre-ovulatory follicles exposed to *in vivo* hCG are luteinised (McAllister et al. 1990) and it is unlikely that proliferation was a cause of the curvature as luteinised GC do not proliferate (Amsterdam et al. 1999; Chaffkin et al. 1993). Thus, there are differences in the metabolic capacity and cellular commitment by cells between the densities investigated. The rate at which luteinisation occurs is asynchronous, only one-third of follicular cells isolated after *in vivo* hCG administration are classified as granulosa cells with the remainder being luteinised (Chaffkin et al. 1993; Steuerwald 2007) hence a small proportion of cells will metabolise MTT at different reates depending on their state of differentiation.

The use of both the MTT and Crystal Violet assays is required when determining the number of viable granulosa cells. Both viability assays generated R-square values greater than 0.97, and were equally accurate, sensitive and reproducible in determining viability of primary-derived granulosa cells and KGN cells.

The presence of steroidogenic cells was identified by the measurement of the steroid hormones progesterone and estradiol. Their synthesis is dependent on the availability of cholesterol lipid substrates. A positive stain by Oil Red O (ORO) is an indication of the cholesterol substrate material within cells and that they are no longer in their proliferative state but have differentiated (Kinkel et al. 2004; Stoklosowa et al. 1978). There are many ORO staining techniques available so the key parameters needed to be optimised for use with GC (Casselman 1959; Disbrey & Rack 1970; Fischer & Kahn 1972; Kinkel et al. 2004; Stoklosowa et al. 1978). Taking this into account and the limited yield of granulosa cells after isolation, the optimisation of staining parameters of the assay were carried out in Mouse Leydig Tumour cell line (MLTC-1) which stain positive to ORO. The optimal staining parameters were that of ORO stain 2 by Kinkel et al. (2004) and Stoklosowa et al. (1978), for 15 min at room temperature. These conditions were then trialled in GC

and were adopted as there were no significant differences in ORO staining between the two cell types.

3.6 Conclusion

The key characteristics for optimal GC culture that will be used in subsequent chapters in order to investigate the toxic effects of the cyanobacterial toxin, cylindrospermopsin, *in vitro* include a cell density of 2×10^4 cells/well with a 24 h "adherence" period prior to treatment in 96-well plates for cytotoxicity assays. In addition to the aforementioned reasons, the 2×10^4 cell/well density also allowed more treatment combinations to be examined compared to using 4×10^4 cells/well. Determination of viability of GC after treatment with CYN will include both the MTT and Crystal Violet assays. Furthermore, this model for *in vitro* culture of GC in our experiments is similar to other GC toxicity models established in the literature (Breckwoldt et al. 1996; Sasson & Amsterdam 2002; Young et al. 2008). Steroidogenic capabilities are maintained over the 72 h culture time investigated, suggesting that hormone production in GC can continue until cell death occurs. In addition to endpoints that determine steroid hormone production, steroidogenic functionality ORO Lipid Stain and 3β- hydroxysteroid dehydrogenase assay will also be carried out (Amsterdam et al. 1999; Breckwoldt et al. 1996; Ho et al. 2004).

Chapter 4. The Effects of the Blue-Green Algal Toxin Cylindrospermopsin on Human Granulosa Cells

4.1 Introduction

Acute human exposure to CYN can occur by accidental ingestion of contaminated drinking water and recreational water (Codd et al. 1999). However what is more of a concern is the low dose- chronic exposure of CYN through the drinking water, which is more likely to occur. To date the only human death involving direct oral consumption of cyanobacterial toxins was a teenage boy from Madison Wisconsin, USA. The coroner found the likely cause of death to be linked to the exposure of the neurotoxin- anatoxin-a (Stewart et al. 2006). However it is more likely that non-direct routes such as that encountered by the second human poisoning 1997 in Caruaru, Brazil, where 76 of the 131 patients undertaking treatment at the dialysis centre developed acute liver failure and died due its contamination with the cyclic peptide microcystin and CYN (refer to section 1.7). In Australia, pollution of water systems with cyanobacterial toxins is becoming a major concern (McKay & Moeller 2001), with the real risk of CYN reaching the reproductive organs and associated cells if the toxin were to enter the circulatory system. CYN is a highly soluble stable molecule and is resistant to degradation due to changes in pH or temperature (See section 1.6 for references). If ingested orally, CYN may survive the acidic environment (pH 1-2) of the stomach (Thompson et al. 2011) which will depending on the rate of gastric emptying and thus has the possibility of entering the bloodstream (Bryant & Knights 2010, pg 130-135). The levels of CYN in blood plasma have yet to be determined and were not a focus of this study. The maturing follicle is comprised of a family of cells including granulosa cells (Figenschau et al. 1997; Peluso 2003). Within the ovary, the follicular fluid of the developing follicle is comprised of secretions from granulosa cells and plasma exudates (Edwards 1974). The follicular fluid and plasma are also connected due to permeability of the membrane; thus, granulosa cells and the oocyte within the follicle are exposed to

components of the circulatory system (Edwards 1974; Hess et al. 1998), including toxins and pharmaceuticals such as nicotine, antibiotics, alcohol and chlorinated hydrocarbons (Macklon & Fauser 1998; Wagner et al. 1990). Interestingly the chlorinated hydrocarbons were detected in 152 samples of follicular, seminal and cervical fluids from sterility patients undergoing IVF (Wagner et al. 1990).

Cylindrospermopsin mainly affects the liver of where foreign chemicals undergo detoxification. Hence the metabolites produced during the detoxification of CYN cause toxicity, specifically toxic CYN metabolites produced by CYP enzymes (Froscio et al. 2003). CYN toxicity occurs in four phases: protein synthesis inhibition, membrane proliferation, fat droplet accumulation and cell death (Terao et al. 1994). A more detailed review of the *in vitro* CYN toxicity studies can be found in section 1.7.3. If CYN was to be ingested orally it will need to be filtered through the liver before entering the systemic blood and although CYN has non-specific cytotoxicity in the body the primary site of toxicity occurs in the liver. All CYP enzymes belong to the same family but differ in functionality as discussed in section 1.1.3. The CYP enzymes found in the liver are different to those required for steroid hormone biosynthesis, so although CYN has the potential to enter the bloodstream and reach the ovaries which is highly vascularised, CYN requires to be metabolised into its toxic metabolites to confer its toxicity. Whether the CYP enzymes involved in steroid hormone synthesis can generate the toxic CYN metabolites have yet to been determined. The identification of CYN metabolites was not a focus of this study.

Currently, the recommended guideline value of a safe concentration of CYN in drinking water is 1 $\mu g.L^{-1}$ as determined by *in vivo* studies which found the NOAEL 30 $\mu g.kg^{-1}.day^{-1}$ of CYN (Humpage & Falconer 2003). The two proposed mechanisms of action of CYN are the production of metabolites via CYP oxidation and the second is via direct effect on protein synthesis (Froscio et al. 2001; Terao et al. 1994).

Cytotoxicity is a result of one or more effects on a cell, including DNA damage, protein synthesis inhibition, destruction of the cell membrane, and inhibition of enzymatic reactions (Ishiyama et al. 1996). Cytotoxicity can be evaluated by

various assays such as the lactate dehydrogenase (LDH), MTT, MTS and Crystal Violet assays. These assays have been used to investigate the effects of CYN toxicity and are commonly used in determining the viability of granulosa cells (Bain et al. 2007; Fessard & Bernard 2003; Froscio et al. 2003; Knaggs et al. 1998; Lambert et al. 2000; Runnegar et al. 1994; Soboloff et al. 2001; Young et al. 2005). Cytotoxicity caused by CYN may be dependent on the mechanism of CYN toxicity, which can vary in different cell types and according to CYN concentration. Recent studies suggest that sensitivity to the cytotoxic effects of CYN differs between cell lines and primary-derived cells (Bain et al. 2007; Chong et al. 2002; Froscio et al. 2009).

4.2 Chapter Aims and Objectives

This chapter aims to investigate the effects of CYN on viability and steroid hormone production by human primary-derived granulosa cells using concentrations ranging from 1-3 µM (416- 1248 $\mu g.L^{-1}$), similar to the range tested on primary hepatocytes (Froscio et al. 2003; Runnegar et al. 1994). These concentrations are significantly higher than what would be expected to naturally occur; however there have been instances when CYN concentrations have reached 100,000 $\mu g.L^{-1}$-, which is 80% greater than the highest concentration tested on human granulosa cells in this study. These set of results also aim to reproduce the data obtained by Young et al. (2008) and further extend the work by comparing sensitivity of granulosa cells to toxin exposure segregated on clinical ART infertility factors. The effects of CYN on viability of a granulosa-like tumour cell line, KGN cells will also be investigated. Finally, histochemical analysis of granulosa cells exposed to CYN will be used as a tool to monitor whether cholesterol lipids or 3βHSD activity are affected by CYN, both of which are required for steroidogenesis. The working hypotheses underpinning these investigations are:

- Cylindrospermopsin will be cytotoxic to granulosa cells, in a time and dose-dependent manner.

- Cylindrospermopsin will decrease basal granulosa cell steroid hormone production.

- Changes to steroid hormone production will be reflected in percentage of the cells staining positive to the presence of cholesterol.

- Decreased progesterone production will be reflected by decreased 3βHSD staining due to reduced activity in granulosa cells cultured *in vitro*.

- Granulosa cells classified as unhealthy will be more sensitive to the adverse effects of CYN in comparison to their healthy counterparts, with cytotoxicity and decreases in basal granulosa cell steroid hormone production being observed.

- Cylindrospermopsin will adversely affect the granulosa tumour-cell line KGN at similar concentrations and exposure periods to that of primary derived human granulosa cells.

-

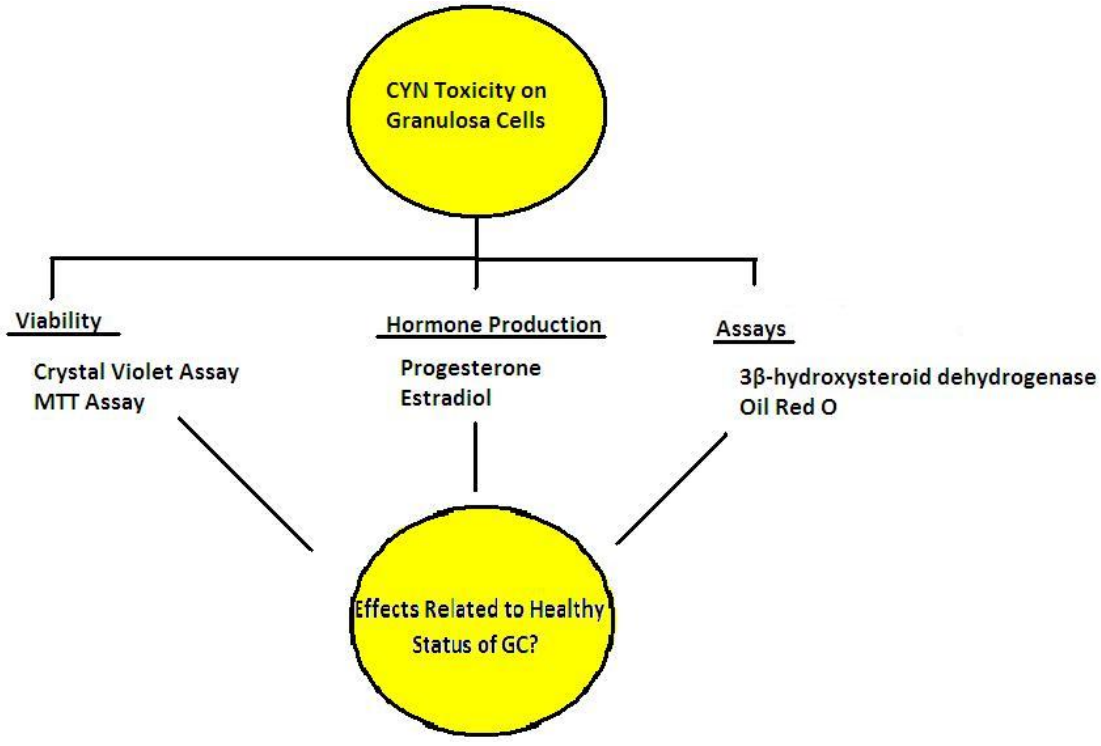


Figure 4-1: Flow Diagram of Chapter Objectives

4.3 Materials and Methods

Granulosa cells were isolated from ovarian follicular fluid as outlined in 2.3 Isolation of Human Granulosa Cells. A total of 13 cases were included, two cases were FIF and 11 cases were MIF/unknown (but underwent ICSI). The sources of primary cells were classified as cells used for "CYN and GC" under the "Experimental Endpoint" column in (Table 8-1). Preparation of Cylindrospermopsin Treatments

CYN stock solution of 1 $mg.mL^{-1}$ (2400 µM) was prepared by dissolving freeze-dried toxin in sterile water, which was stored at -20°C. Concentrations tested were 0.1, 0.3, 1 and 3 µM CYN, Table 2-5. Serial dilutions were made in Ham's F12/Dulbecco's Minimum Essential Medium (F12/DMEM, 1:1; ThermoTrace, Melbourne, Australia) supplemented with 10% fetal calf serum (FCS, Trace Biosciences) and insulin, transferrin and selenium (Sigma) and stored at -20°C in the dark. Control was DMEM/F12 + 10% FCS/ITS (0 µM CYN).

4.3.1 Statistical Analysis

Statistical Package for the Social Sciences (SPSS, v15.0) was used to undertake statistical analyses. One-way ANOVA was used to determine statistical significance in the GC viability (MTT and Crystal Violet assay), steroid hormone production (progesterone and estradiol) and functionality assays (ORO lipid stain and 3βHSD activity) after CYN exposure. If analysis showed a significant interaction effect ($P \leq 0.05$) between CYN concentrations, a follow-up analysis was conducted in which data were grouped by exposure period or CYN concentration, and analysed by Tukey's post-hoc analysis to compare the means of different treatments within each time point against the control. A t-test was carried out to determine statistical significance in GC viability (MTT assay), steroid hormone production (progesterone and estradiol) between unhealthy and healthy cohorts. *P* values $\leq$ 0.05 were accepted as significant. Data in graphs represent mean ± SEM.

4.4 Effects of CYN on Primary-derived Human Granulosa Cells

As discussed in section 1.7.2, CYN is known to have a wide range of *in vivo* effects, particularly as a developmental toxicant (Rogers et al. 2007). More recently, Young et al. (2008) investigated the effects of CYN on the viability and steroid hormone production of human primary-derived granulosa cells *in vitro*. Viability was assessed by the MTT assay, while basal and human chorionic gonadotropin (hCG)-stimulated estradiol and progesterone production was quantified by radioimmunoassay. *In vitro*, hCG (a placental homologue of LH) has the ability of activating LH receptors and increasing progesterone output by granulosa-lutein cells (Devoto et al. 2002; Figenschau et al. 1997; Huhtaniemi 2004).

The study by Young et al. (2008) found CYN to be cytotoxic to primary-derived granulosa cells after 24 h exposure to 1 $\mu g.mL^{-1}$ (2.4 µM) CYN. Basal progesterone production decreased after 24 h exposure to 0.0625 $\mu g.mL^{-1}$ (0.3 µM) CYN, and 1 $\mu g.mL^{-1}$ (2.4 µM) caused a significant decrease in hCG stimulated progesterone production after 6 h exposure, without inducing cell death. CYN may have inhibited progesterone production by reducing substrate availability for steroidogenesis or by down-regulating or inhibiting the activity of the steroidogenic enzyme 3βHSD (Young et al. 2008). Effects on progesterone production per well did not occur in granulosa cells which did not respond to hCG stimulation (control wells) *in vitro*. This suggests that a heterogeneous population of cells existed in which some cells were not only hCG non-responsive, but whose steroid hormone production was unaffected by exposure to CYN.

The granulosa cells used in the study by Young et al. (2008) were obtained from women seeking ART for their infertility, however information on their fertility and reproductive were not provided. Thus, it is unclear if the granulosa cells were isolated from women with normal reproductive physiology, who were undergoing ART due to MIF, that might be considered healthy (refer to section 2.2.1). Healthy and unhealthy granulosa cells may differ in their sensitivity to further gonadotropin stimulation or exposure to toxins *in vitro*, as was observed by Young et al. (2008).

It is important to determine the toxic effects of CYN in cells that are representative of the normal, healthy women.

Primary-derived granulosa cells were exposed to CYN concentrations of 0.1 – 3.0 µM similar to in the study of Froscio et al. (2003) to determine if there would be a dose- and time-dependent decrease in viability and basal steroid hormone production. Granulosa cells used in cytotoxicity assays came from 12 different donors and were cultured at a density of 2×10^4 cells/well. CYN exposure periods were 24 h, 48 h and 72 h, following a pre-treatment adherence period of 24 h, and were cultured under the conditions described in section 2.3.1. Conditioned media post-treatment was collected and stored at -20°C for progesterone and estradiol analysis by RIA (section 2.12 Radioimmunoassay). Viability was determined by MTT (n=5, section 2.8.2.1 MTT Assay) or Crystal Violet assay (n=7, section 2.8.4 Crystal Violet Assay). Results are shown for the combined cohort and then further segregated into healthy and unhealthy cohorts to determine if there are differences in sensitivity to CYN toxicity.

4.4.1 Non-differentiable differences in Clinical Data Segregated Based on Infertility Factors

In this group of women there were no differences in the clinical data obtained for the MIF and FIF cohorts, with both groups suggesting that the granulosa cells were healthy (Table 4-1). There was 1 successful pregnancy from the cohort of women used in this study. This woman was classified as having FIF and underwent IVF treatment. The FIF cohort produced higher GC yield than the MIF cohort, however the inter-woman variation was also high ($9.0 \times 10^5 \pm 4.3 \times 10^5$ SD cells/mL). A comparison between ovarian response parameters from MIF and FIF derived granulosa cells are detailed in Table 4-1. Data collected from both groups were comparable; the minimal differences between the data collected suggest that none of the factors which segregate cells into healthy or unhealthy could be directly indicative of the sensitivities to be observed in granulosa cell responsiveness after CYN exposure. Hence, viability and hormone data (where applicable) are presented combined and then further subdivided into healthy and unhealthy to all allow for interpretation of differences due to healthy status of cells.

Parameter	MIF	FIF
Age of Patient At Isolation	35 ± 2	34 ± 2
Treatment Cycle Number	3 ± 2	3 ± 2
Total FSH Administered (Units)	2648 ± 1035	1890 ± 1118
Maximum Plasma E_2	9059 ± 7545	7840 ± 4998
Number of Mature Follicles (>15mm)	6 ± 1	6 ± 3
Number of Oocytes Aspirated	10 ± 6	9 ± 3
Number of Embryos Transferred	2 ± 1	1 ± 1
Pregnancy Outcome	0	1
Number of Full Follicular Fluid Tubes Collected (per woman)	7 ± 2	6 ± 2
Granulosa Cells Isolated (cells/mL)	$6.0 \times 10^5 \pm 2.1 \times 10^5$	$9.0 \times 10^5 \pm 4.3 \times 10^5$

Table 4-1: Ovarian Response Parameters for Male and Female Infertility Factor Cohorts

Granulosa cells isolated from women used in this chapter (n=12) were divided into two cohorts based on whether they had male infertility factor (MIF) or female infertility factor (FIF). Ovarian response factors collected include; age of patient at time of isolation; previous treatment cycles, and total units of FSH administered; maximum plasma estradiol (IU/mL) detected before oocyte aspiration, number of mature follicles produced, number of oocytes aspirated, number of embryos transferred and pregnancy outcome; number of follicular fluid tubes collected per woman, number of isolated $GC.mL^{-1}$. Data represent mean ± standard deviation.

4.4.2 CYN was Cytotoxic to Primary-Derived Granulosa Cells

In the combined data, results showed that exposure to 0.1, 0.3, 1 or 3 µM CYN for 72 h caused a time-dependent decrease in the number of viable cells observed in both the MTT and the Crystal Violet assays. After exposure to 0.1 µM CYN for 72 h a 40% loss of cells was observed determined by the MTT assay, $P < 0.01$ (Figure 4-2). A 48 h exposure to 1 µM CYN was the effective concentration where a 50% loss in viability (EC_{50}) in GC was observed (MTT assay, $P < 0.001$). CYN continued to be significantly cytotoxic throughout the 72 h exposure period (Figure 4-2). The unhealthy granulosa cells were more sensitive to the cytotoxic effects of CYN after 72 h exposure, but not at earlier time points. 72 h exposure to 0.1 µM CYN resulted in a significant decrease in viability as determined by the MTT assay, whereas a 1 µM CYN concentration was required to observe a loss of cell viability in the healthy granulosa cells group (MTT assay, Figure 4-2). At 24 h, CYN appears to have stimulated increased formazan production causing an overestimation of viable cell number which was not apparent in the Crystal Violet assay (Figure 4-2).

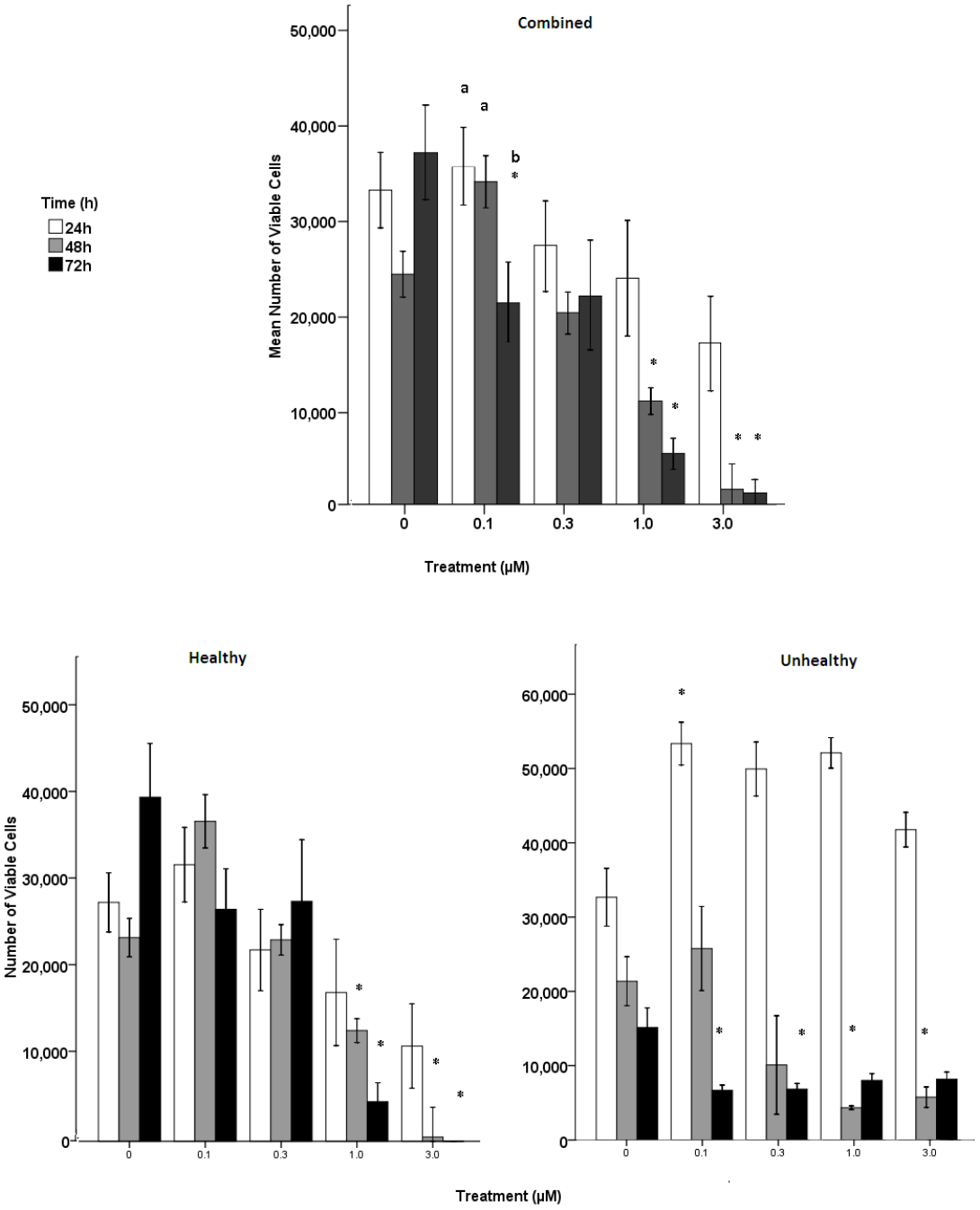


Figure 4-2: GC Viability Assay As Determined by MTT Assay

Granulosa cells seeded at a density of 20,000 cells/well, in triplicate wells which were treated with 0, 0.1, 0.3 1.0 or 3.0µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. The number of viable granulosa cells was determined by the MTT assay. Top graph is for combined data (n=5), bottom left graph depicts data for women whose GC classified as healthy (n=3), bottom right graph represents data for women whose GC classified as unhealthy due to female infertility factors (n=2). Error bars represent mean number of viable cells ± 1 SEM, significance indicated by *p<0.05 as determined by one-way ANOVA. a & b represent significant differences within a specific concentration over 72 h-exposure period investigated where a and b are significantly different to each other p<0.01.

In comparison, the Crystal Violet assay detected a 72% decrease in number of viable GC after 72 h exposure to 1 µM CYN, (P <0.001 Figure 4-3). After 48 h exposure to 3 µM CYN there was a 80% loss of viability of granulosa cells as determined by the Crystal Violet assay, P <0.001 (Figure 4-3). The Crystal Violet data also suggest that granulosa cells classified as unhealthy were more sensitive to CYN than their healthy counterparts. Exposure to 0.3 µM CYN for 72 h caused a significant decrease in cell viability in the unhealthy cohort. In comparison, the healthy cohort required 72 h exposure to 1 µM CYN for a significant loss in viability to be observed (Figure 4-3).

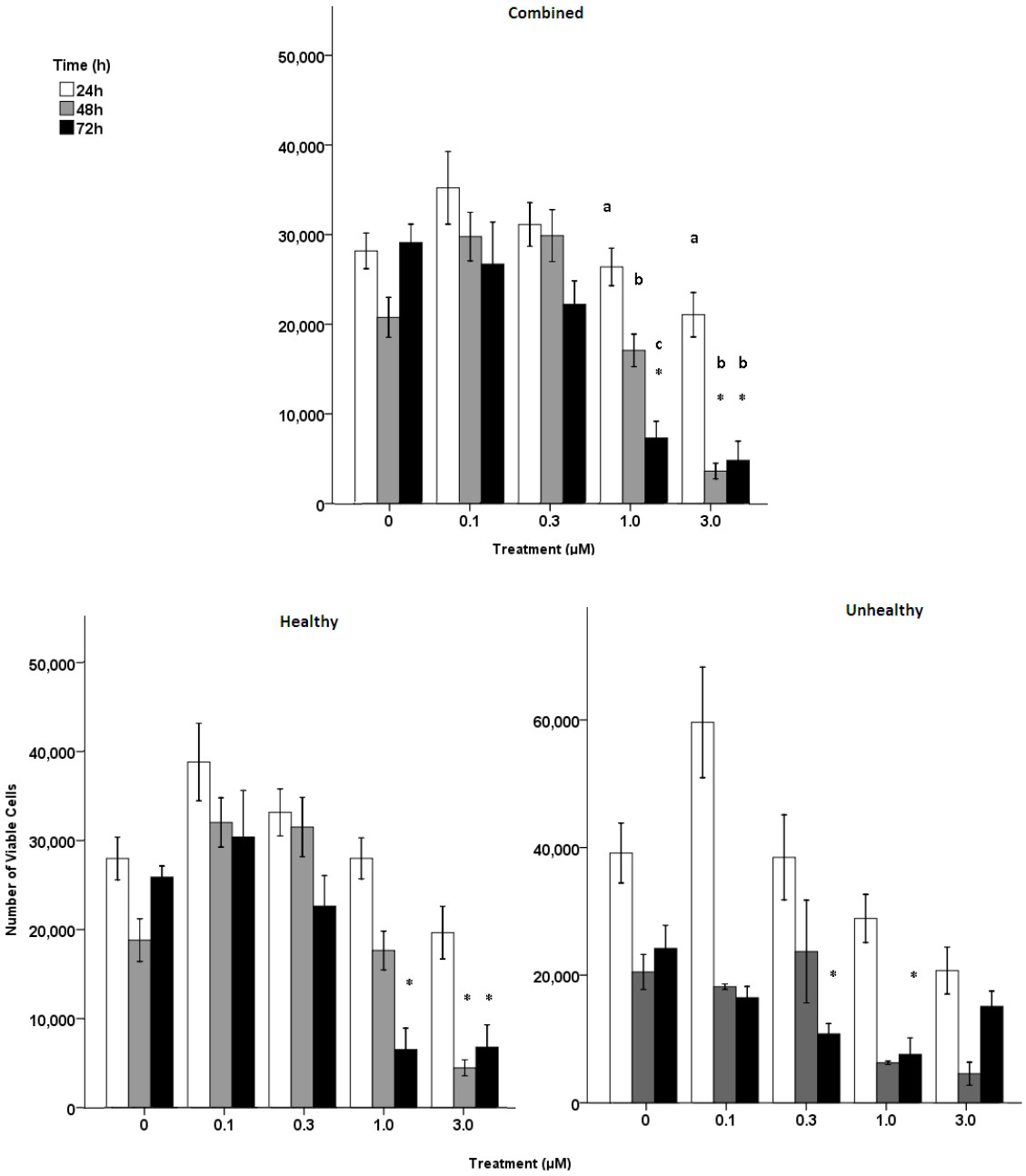


Figure 4-3: GC Viability Assay As Determined by Crystal Violet Assay

Granulosa cells seeded at a density of 20,000 cells/well (n=5), in triplicate wells which were treated with 0, 0.1, 0.3 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. The numbers of viable granulosa cells were determined by the Crystal Violet assay. Top graph is for combined data (n=5), bottom left graph depicts data for women whose GC classified as healthy (n=3), bottom right graph represents data for women whose GC classified as unhealthy due to female infertility factors (n=2). Error bars represent mean number of cells ± 1 SEM, significance indicated by *P <0.05 as determined by one-way ANOVA. a, b & c represent significant differences within CYN concentration over the 72 h period time point where a, b and c are significantly different to each other P <0.01

Overall, the data suggests that the MTT assay was more sensitive in the detection of the metabolic changes within granulosa cells caused by exposure to CYN, and that the unhealthy cohort were more sensitive to CYN toxicity.

4.4.3 CYN Decreased Steroid Hormone Production by Primary-Derived Granulosa Cells

Granulosa cells were cultured at 2×10^4 cells/well, with culture medium analysed after 24, 48 and 72 h exposure to measure hormone concentrations. Data was analysed for combined women as well as being subdivided into healthy and unhealthy cohorts. The magnitude of decreased progesterone and estradiol concentrations were not solely related to loss of cell viability. In the combined data, there was a time- and dose-dependent decrease in progesterone concentration after 72 h exposure to 0.1 (IC_{50}), 1 and 3 µM CYN ($P < 0.05$, Figure 4-4).

The steroid hormone data was then examined in the healthy and unhealthy cohorts. Figure 4-4 and Figure 4-5 (bottom panels) show that steroid hormone production in the unhealthy group was more sensitive to low CYN concentrations than the healthy counterparts. Progesterone concentration fell below the limit of detection after 72 h exposure to 0.1 µM CYN (Figure 4-4). This was not correlated to a loss of viability as determined by either the MTT assay or Crystal Violet assay and was not observed in the healthy cohort.

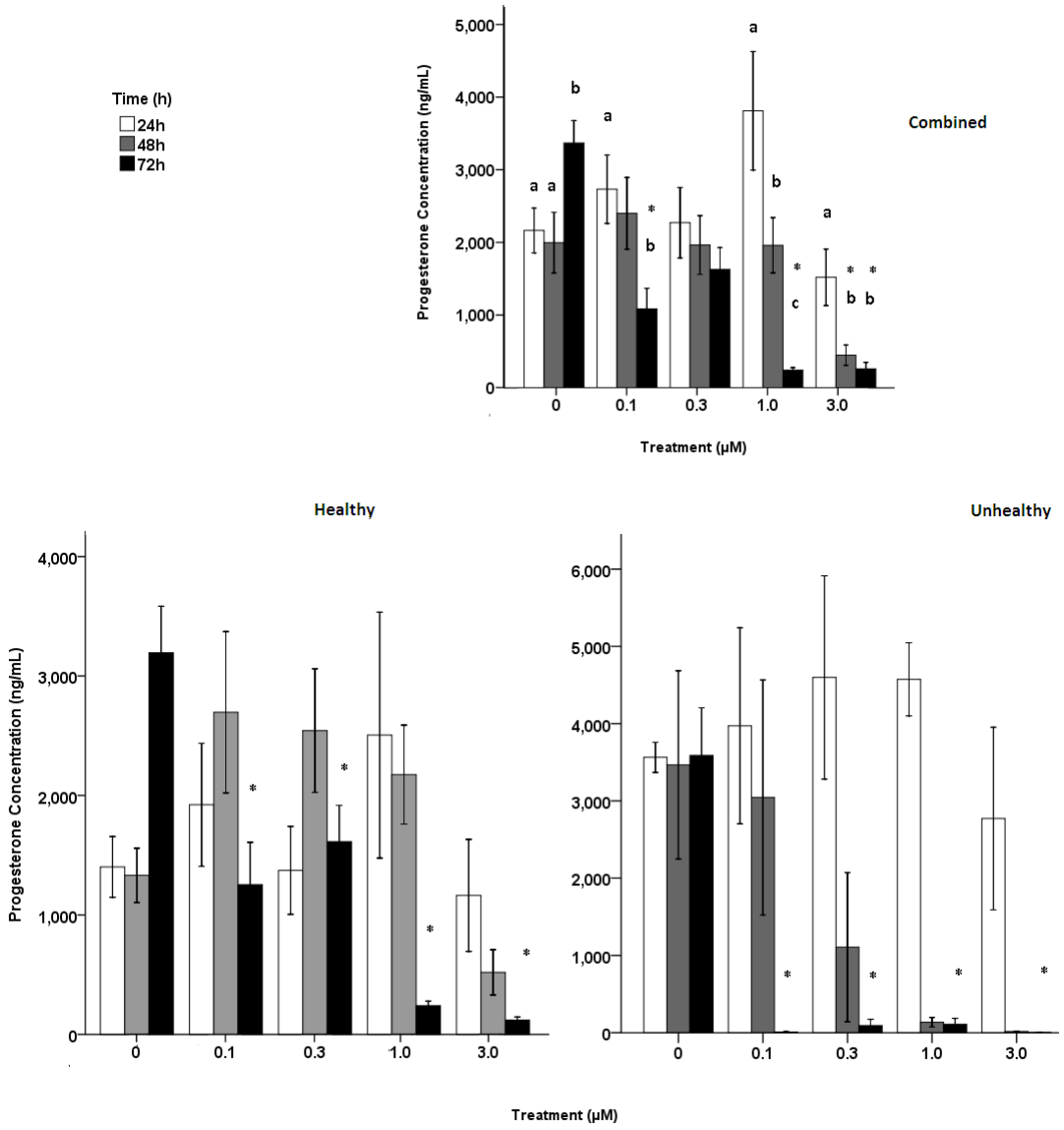


Figure 4-4: Progesterone Production (ng/mL) by GC

Granulosa cells seeded at a density of 20,000 cells/well, in triplicate wells which were treated with 0, 0.1, 0.3 1.0 or 3.0µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Progesterone concentration (ng/mL) was determined by RIA. Top graph is for combined data (n=5), bottom left graph depicts data for women whose GC classified as healthy (n=5), bottom right graph represents data for women whose GC classified as unhealthy due to female infertility factors (n=2). Error bars represent progesterone concentration ng/mL ± 1 SEM, significance indicated by *P <0.05 as determined by one-way ANOVA. a, b & c represent significant differences within CYN concentration over the 72 h period where a, b and c are significantly different to each other P <0.05.

Similarly, estradiol concentrations decreased after 72 h exposure to 0.1, 0.3, 1 and 3 µM CYN, $P < 0.001$ except at 3 µM where $P < 0.05$ (Figure 4-5). Similar to the data for progesterone, the magnitude of decrease in estradiol concentrations could not be accounted for by the loss of cell viability.

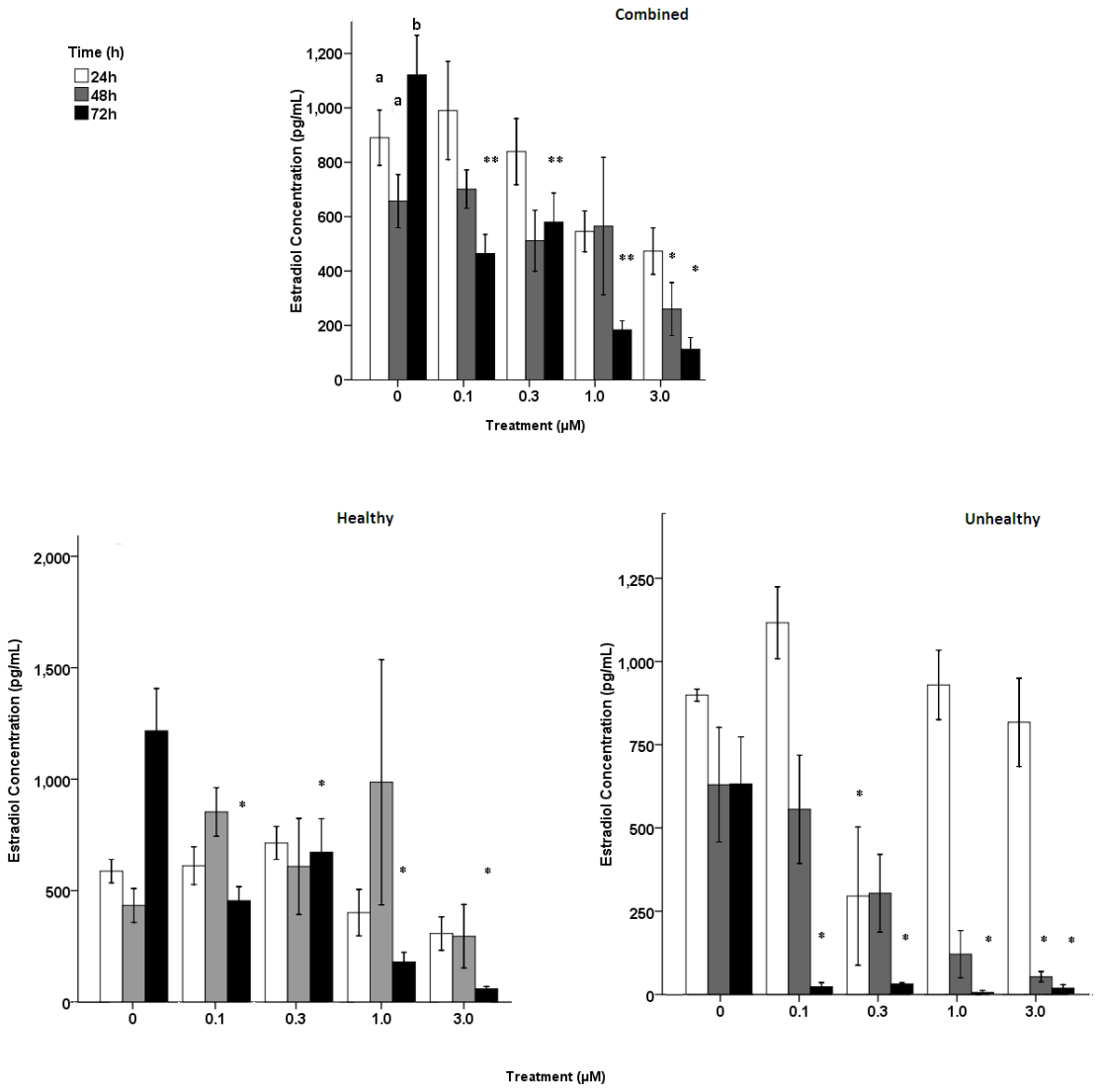


Figure 4-5: Estradiol Production (pg/mL)

Granulosa cells seeded at a density of 20,000 cells/well, in triplicate wells which were treated with 0, 0.1, 0.3 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Estradiol concentration (pg/mL) was determined by RIA. Top graph is for combined data (n=7), bottom left graph depicts data for women whose GC classified as healthy (n=5), bottom right graph represents data for women whose GC classified as unhealthy due to female infertility factors (n=2). Error bars represent estradiol concentration pg/mL ± SEM, significance indicated by **P* <0.05, ***P* <0.001 as determined by one-way ANOVA.

4.4.4 Increased Sensitivity to CYN Toxicity in the Unhealthy Granulosa Cell Cohort

The loss of granulosa cell viability and steroid hormone production after 72 h exposure to CYN in the healthy and unhealthy cohorts (section 4.4.2) were further investigated. The data after 72 h from the two cohorts in Figure 4-2, Figure 4-4 and Figure 4-5 were represented as percentage of 0 µM CYN control. Steroid hormone data were not expressed per viable cell number because the final cell numbers calculated at the end of treatment exposure do not factor in the steroid hormone production by cells which were metabolically active prior to cell death at the end of treatment exposure.

The MTT assay cell viability data were used, as this assay was more sensitive in the detection of decreased cell number than the Crystal Violet assay (section 4.4 and Figure 4-2). The percent decrease in the number of viable cells and steroid hormone production relative to the 0 µM CYN control in the unhealthy cohort was more sensitive to the toxic effects at low CYN concentrations (0.1 and 0.3 µM CYN) than the healthy cohort, Figure 4-6.

Cylindrospermopsin concentrations of 1 and 3 µM were still cytotoxic to primary-derived granulosa cells, but the percent decrease in viability was not significantly different between unhealthy and healthy cohorts, Figure 4-6. Thus, the unhealthy cohort was more sensitive to low CYN concentrations than the healthy cohort.

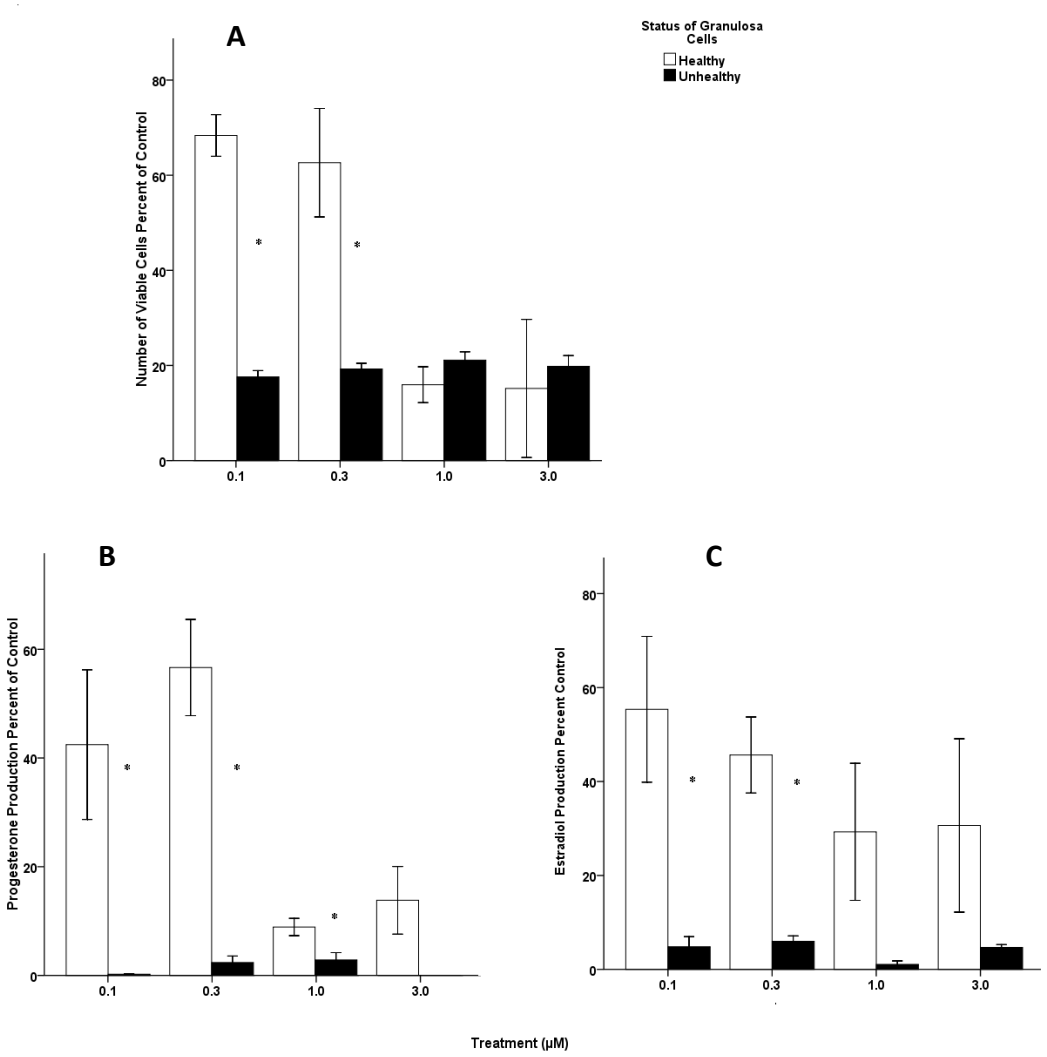


Figure 4-6: Percentage of Steroid Hormone Production and Viable Granulosa Cells in Healthy and Unhealthy Cohorts after 72 h Exposure to CYN

Granulosa cells seeded at a density of 20,000 cells/well were treated with 0, 0.1, 0.3 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Steroid hormone production after 72 h exposure to CYN in both healthy (n=5) and unhealthy (n=2) cohorts from Figure 4-2, Figure 4-4 and Figure 4-5 were represented as percent of media control (0µM CYN). The number of viable cells (A) was represented as percent of media control (0µM CYN) derived from the MTT assay after 72 h exposure to CYN in both healthy and unhealthy cohorts in Figure 4-2, progesterone production (B) as percent of control and the estradiol production (C) as percent of control. Error bars represent mean percent of control ± SEM, significance indicated by *P <0.05, as determined by t-test.

4.5 Effects of CYN on KGN Granulosa-like Cell line

Although primary-derived human granulosa cells are a good *in vitro* reproductive model to investigate the effects of toxins as they reflect the *in vivo* physiological state, granulosa-like cell lines are increasingly being used as a comparative tool. KGN are a human granulosa tumour cell line first developed and characterised by Nishi et al. (2001). Cells were obtained from a 63 year old woman with stage III ovarian cancer (Nishi et al. 2001). KGN cells require cAMP to stimulate progesterone production *in vitro* (Nishi et al. 2001; Tsutsumi et al. 2008). They maintain FSH receptors and the same pattern of steroidogenesis and are used as a comparative model to identify mechanisms that control the response in primary derived cells, with minimal biological variability and are particularly useful when yields of primary-derived granulosa cells are low (Chu et al. 2004; Macklon & Fauser 1998; Minehata et al. 2007; Nishi et al. 2001).

KGN (n=3 independent experiments) were cultured at a density of 2×10^4 cells/well in triplicate wells were cultured as for primary-derived granulosa cells. Conditioned media was removed and replaced with fresh medium containing treatments of 0.1, 0.3, 1 or 3 µM + 1 mM cAMP, as detailed in Table 2-5 (Tsutsumi et al. 2008). Control wells for CYN treatment and cAMP inclusion were also carried out- referred to as 1 mM cAMP. Media post-treatment was collected and stored at -20°C for progesterone analysis by RIA (2.12 Radioimmunoassay). The number of viable cells after treatment was then determined by Crystal Violet assay (section 2.8.4 Crystal Violet Assay).

4.5.1 CYN was Cytotoxic to KGN Cells

The KGN cells were exposed to CYN concentrations detailed in Table 2-5, cAMP (1 mM) was added to detect changes in stimulated progesterone production. First, it was important to determine if 1 mM cAMP would be cytotoxic to cells up to 72 h exposure. By this time, the number of viable KGN cells had decreased by 40% when compared to the media control DMEMF12, Figure 4-7. However this appears to be due to an inhibition of an increase in cell numbers in the control rather than a loss of cells *per se*. Thus, in order to determine if CYN was cytotoxic to KGN cells,

data was directly compared to 1 mM cAMP control. The KGN cells were exposed to all CYN concentrations and were unaffected after 24 h of treatment exposure, though 3 µM CYN caused a 55% and 90% loss of cell viability after 48 h and 72 h exposure respectively, $P < 0.05$ (Figure 4-7). Thus, the KGN cells and healthy primary derived granulosa cells had similar sensitivity to CYN, as determined by the Crystal Violet assay.

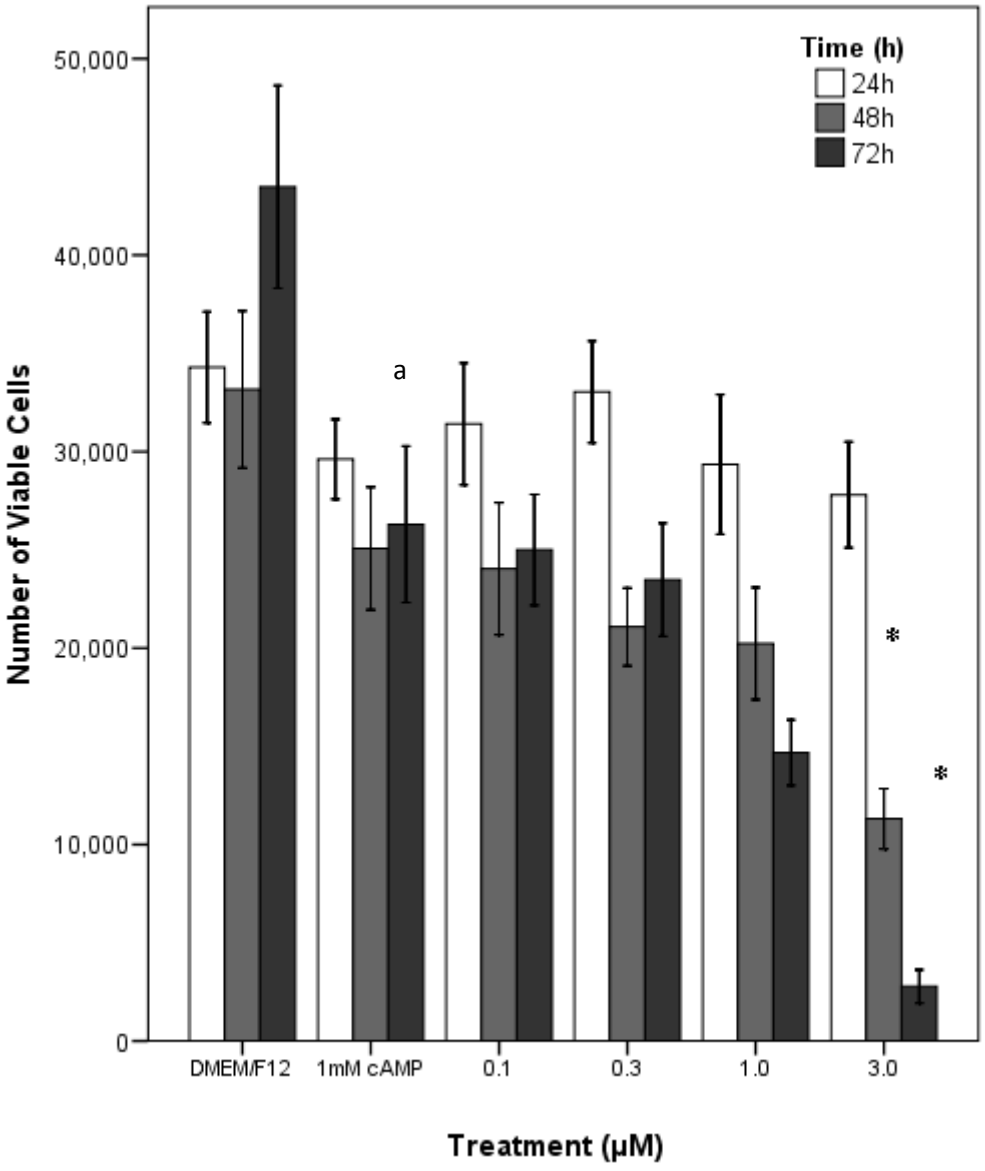


Figure 4-7: Viability of KGN after Exposure to CYN

KGN cells were seeded at a density of 20,000 cells/well (n=3) and exposed to 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 1mM cAMP + 10% FCS for 24 h, 48 h or 72 h. Experimental controls were DMEM/F12 + 10% FCS (DMEM/F12), and DMEM/F12 + 1mM cAMP + 10% FCS (1mM cAMP). The number of viable granulosa cells were determined by the Crystal Violet assay. Error bars represent ± 1 SEM, significance indicated by "a" and * P <0.05 as determined by one-way ANOVA, in comparison to DMEM/F12and to 1 mM cAMP, respectively.

4.5.2 CYN Decreased Progesterone Production by KGN Cells

In the absence of cAMP stimulation, KGN cells did not produce progesterone throughout the 72 h period (RIA limit of detection 1ng.mL^{-1}). However, when KGN cells were stimulated with 1 mM cAMP, progesterone production significantly increased at each 24 h culture interval over a total of 72 h of culture (48 h, 18.99 ± 16.33 ng.mL^{-1} $P < 0.05$ and 72 h 56.65 ± 24.48 ng.mL^{-1}, $P < 0.001$), Figure 4-8. KGN cells maintained the capability to produce progesterone when stimulated with cAMP in the presence of CYN. However, the magnitude of progesterone production decreased after 48 h and 72 h exposure to 0.1 – 3 µM CYN ($P < 0.001$, Figure 4-8). Decreases of 66% and 93% were observed after 48 h exposure to 0.1 µM and 0.3 µM CYN respectively, $P < 0.001$. Furthermore, after 24 h exposure to 1 µM CYN, progesterone concentrations remained at the same level as the unstimulated controls at the same time-point (Figure 4-8). Thus, the decrease in cAMP-stimulated progesterone production by KGN after CYN exposure was not correlated to loss of cell viability, Figure 4-7.

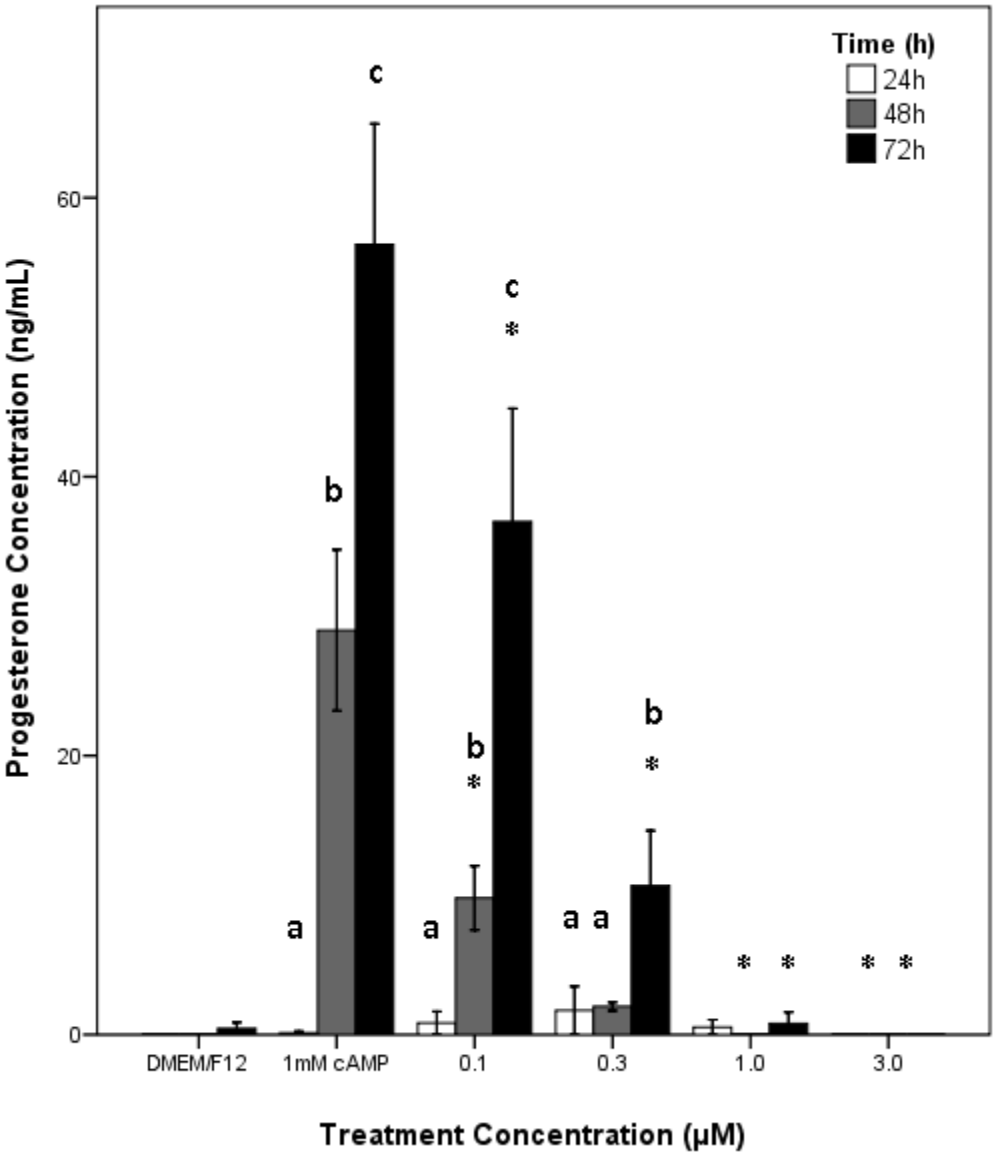


Figure 4-8: Progesterone Production (ng/mL) by KGN after CYN Exposure

KGN cells were seeded at a density of 20,000 cells/well (n=3) and exposed to 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 1mM cAMP + 10% FCS for 24 h, 48 h or 72 h. Experimental controls were DMEM/F12 + 10% FCS (DMEM/F12), and DMEM/F12 + 1mM cAMP + 10% FCS (1mM cAMP). Progesterone concentration (ng/mL) was determined by RIA. Error bars represent ng/mL ± 1 SEM *P <0.001 as determined by one-way ANOVA. a, b & c represent significant differences within time point where a, b and c are significantly different to each other P <0.001 as determined by one-way ANOVA.

4.6 Effects of CYN on Oil Red O Lipid Staining & 3β- Hydroxysteroid Dehydrogenase Enzyme in Primary-derived Human Granulosa Cells

The granulosa cells used in these functionality experiments were isolated from different women to the viability assays (refer to section 4.4.2) and represent only healthy women. Primary-derived granulosa cells were exposed to the same CYN concentrations as viability assays. For functionality assays, granulosa cells (n= 3 donors for ORO and n= 3 donors for 3βHSD) were cultured in LabTekII 8-well glass chamberslides at a density of 4×10^4 cells/well. CYN exposure periods were 24 h, 48 h and 72 h, following the pre-treatment adherence period of 24 h. Media post-treatment was collected and stored at -20°C for progesterone and estradiol analysis by RIA (section 2.12 Radioimmunoassay). Analysis of images taken of GC cultured for ORO stain and 3βHSD activity was carried out, refer to section 2.11 (Image Analysis of Oil Red O & 3β-Hydroxysteroid Dehydrogenase). In addition to determining the presence of cholesterol substrates (O Red O staining) and 3βHSD functionality, the number of attached cells was used to measure viability.

4.6.1 Cytotoxicity of CYN on Primary-Derived Granulosa Cells in Functionality Assays

There was a significant loss of cell viability after 48 h exposure to 3 µM CYN in pooled ORO and 3βHSD cell counts, P <0.001 Figure 4-9. This was similar to that detected by the MTT and Crystal Violet assays (Section 4.4.3)

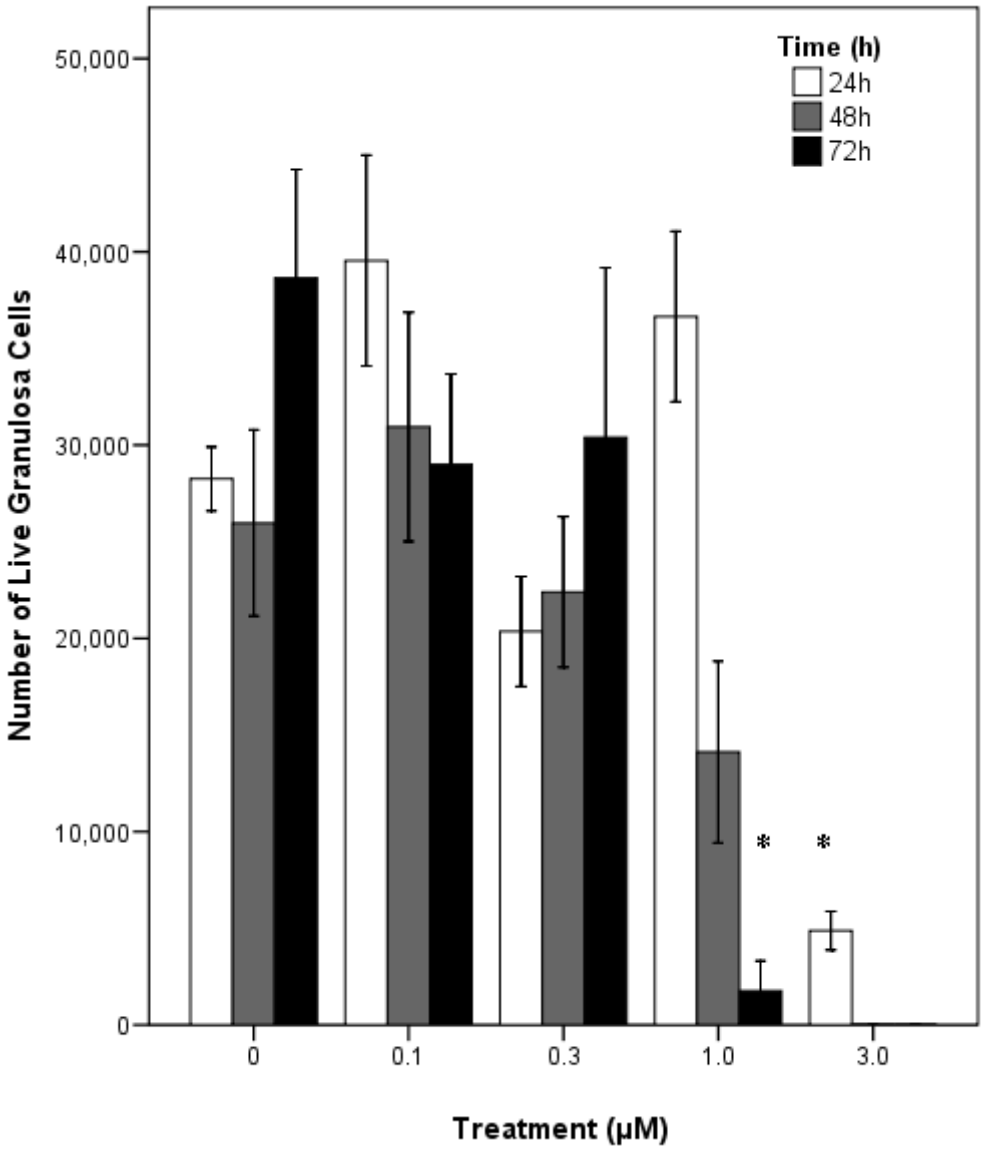


Figure 4-9: Number of Attached GC from pooled ORO & 3βHSD Data

Average number of attached granulosa cells from combined ORO (n=3) and 3βHSD (n=3) assays. This was derived from the total number of viable cells in each assay. Mean total counts for both assays from 4 random photos representing 1.37% of chamber slide well were transformed into total number of viable cells by multiplying by 100/1.37. Error bars represent ± 1 SEM, significance indicated by $P < 0.001$ as determined by one-way ANOVA.

4.6.2 Effects of CYN on Cholesterol Substrate Availability in Primary-derived Human Granulosa Cells

The type and rate of steroidogenesis in primary-derived granulosa cells and in other steroidogenic cells, is dependent on the intracellular availability of cholesterol and the translocation of cholesterol substrate into the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) (Bar-Ami 1994). Cholesterol lipid substrates can be detected by a positive Oil Red O (ORO) stain (Kinkel et al. 2004). Thus, these experiments were designed to replicate the findings of Young et al. (2008) which suggest the decrease in progesterone production might have been the result of decreased cholesterol substrate availability.

ORO lipid staining significantly decreased after 24 h exposure to 3 µM CYN, in which 76 ± 22% cells remained positively stained, in comparison to 96 ± 4% in the control, P <0.05. No cells were present to assess ORO staining after 48 h and 72 h exposure to 1 µM and 3 µM CYN. At lower CYN concentrations, no decrease in ORO staining was observed. Thus, exposure to CYN results in loss of cell viability rather than actual decreases in cholesterol substrates required for steroidogenesis.

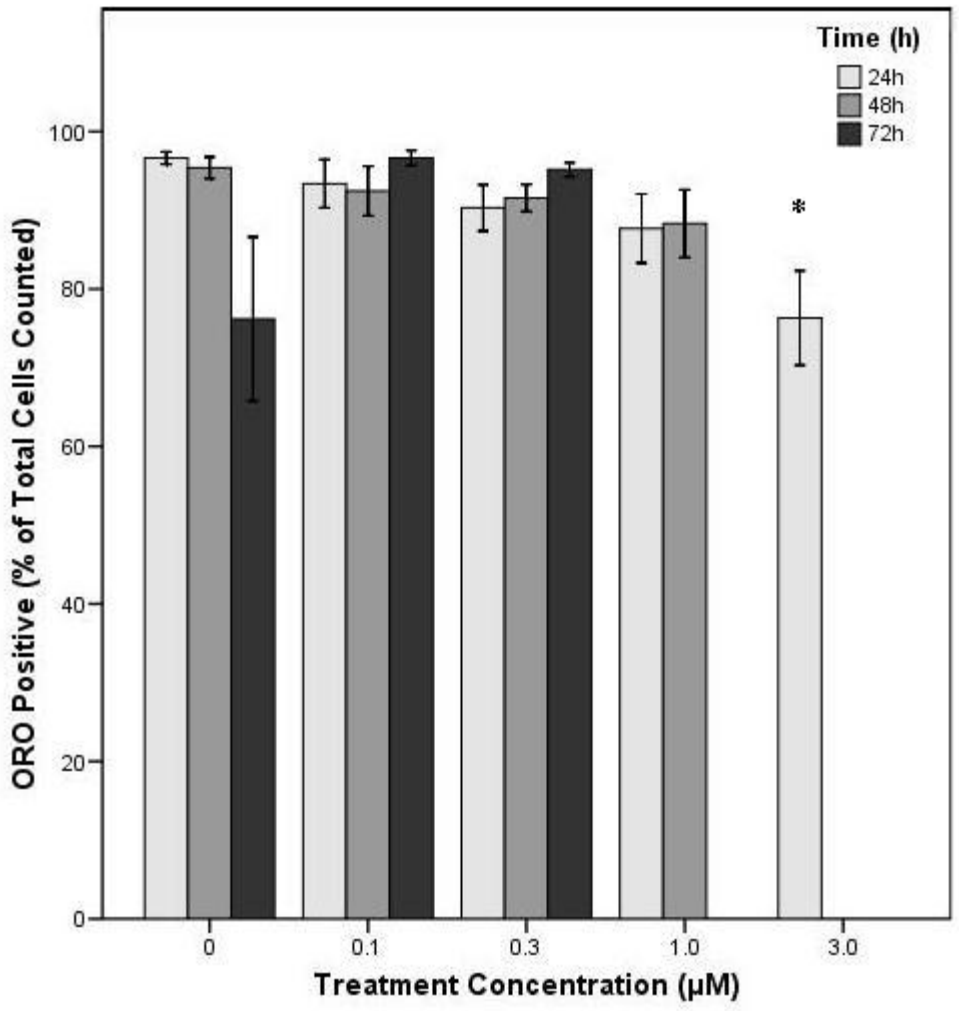


Figure 4-10: Percentage of ORO Positive Cells

Granulosa cells seeded at a density of 40,000 cells/well (n=3), allowed to adhere for 24 h followed by exposure to 0, 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Post treatment media was collected and stored for steroid hormone production and cells were then stained for ORO. Staining observed under bright field microscope at × 200 magnification for positive ORO cells and negative ORO cells. Error bars represent ± 1 SEM of percentage of cells stained positive to ORO, significance indicated by **P* <0.05 as determined by one-way ANOVA on cell counts positive to ORO

4.6.3 Effects of CYN on 3βHSD Activity in Primary-derived Human Granulosa Cells

These experiments were designed to further develop the findings by Young et al. (2008) which suggest the decrease in progesterone production might have been the result of decreased 3βHSD enzyme activity. Progesterone production by luteinised granulosa cells is dependent on the presence and activity of 3βHSD enzyme. The activity of 3βHSD in granulosa cells was identified by the reduction of DHA substrate and subsequent production of blue formazan deposits. This was investigated after exposure to CYN treatments over a 72 h culture period. Overall, 3βHSD activity was unaffected after exposure to CYN throughout the period examined.

In the absence of formazan deposits granulosa cells were classified as 'negative staining' indicating either the absence of 3βHSD, or activity of the enzyme. The presence of blue formazan deposits was further classified as being either light or intensely stained as detailed in Table 2-6 and small dense cells were also present as shown in Figure 4-11.

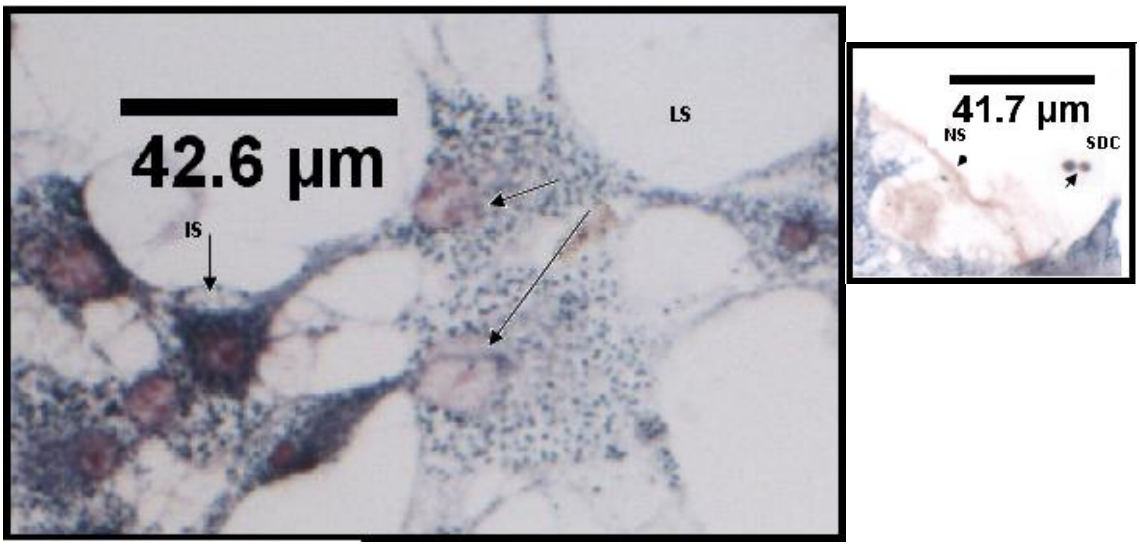


Figure 4-11: Example of various 3βHSD staining classifications

Classification of 3βHSD staining of granulosa cells seeded at a density of 40,000 cells/well (n=3), after exposure to 0, 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. 3βHSD reaction mixture (0.5mg/mL NBT, 2mg/mL NAD & 0.25mg/mL DHA) was added for 1hr. Staining was then observed under bright field microscope at × 200 magnification, classification as detailed in Table 2-6. Images analysed in ImageJ WCIF software.

The proportion of cells which did not stain in the cell population did not change as a result of CYN exposure over time. The total percent of positive 3βHSD staining significantly increased after the first 24 h of culture in DMEM/F12 (Figure 4-13). This suggests that a majority of unstained cells acquired 3βHSD activity and potentially steroidogenic capabilities to produce progesterone during this period, an indication of luteinisation. This was further supported by progesterone concentrations increasing after a 72 h culture period in culture medium only, Figure 4-14.

Although the net number of staining cells did not change, there were shifts between the percentages of intense 3βHSD stained large cells (IS) and light 3βHSD stained large cells (LS). In the control treatment medium, intensely stained cells significantly increased to 87 ± 18% after 72 h of culture when initially only 44 ± 22% were intensely stained, P <0.01,Figure 4-13. This suggests that with increasing time in culture, granulosa cells had increased 3βHSD enzyme activity and capability to produce progesterone.

There was no significant difference in intense and light staining observed in the first 24 h of culture in DMEM/F12 (Figure 4-12). The intense staining was defined as greater than 90% of the cell covered with blue-formazan deposits in comparison to light staining, which was approximately 20-30% of the cell. These were differentiated by visual inspection and ImageJ was used as a cell counter. The change in intensity of staining was observed at 0.3 µM CYN after 72 h exposure, where intense staining increased to 92 ± 18% and light staining decreased to 7± 19% (Figure 4-12) suggesting that there was increased 3βHSD activity. The presence of dense cells was consistent and did not change significantly in percentage after exposure to increasing CYN concentrations. There was no time-dependent or dose-response change in number of dense cells after CYN exposure (Figure 4-12 & Figure 4-13).

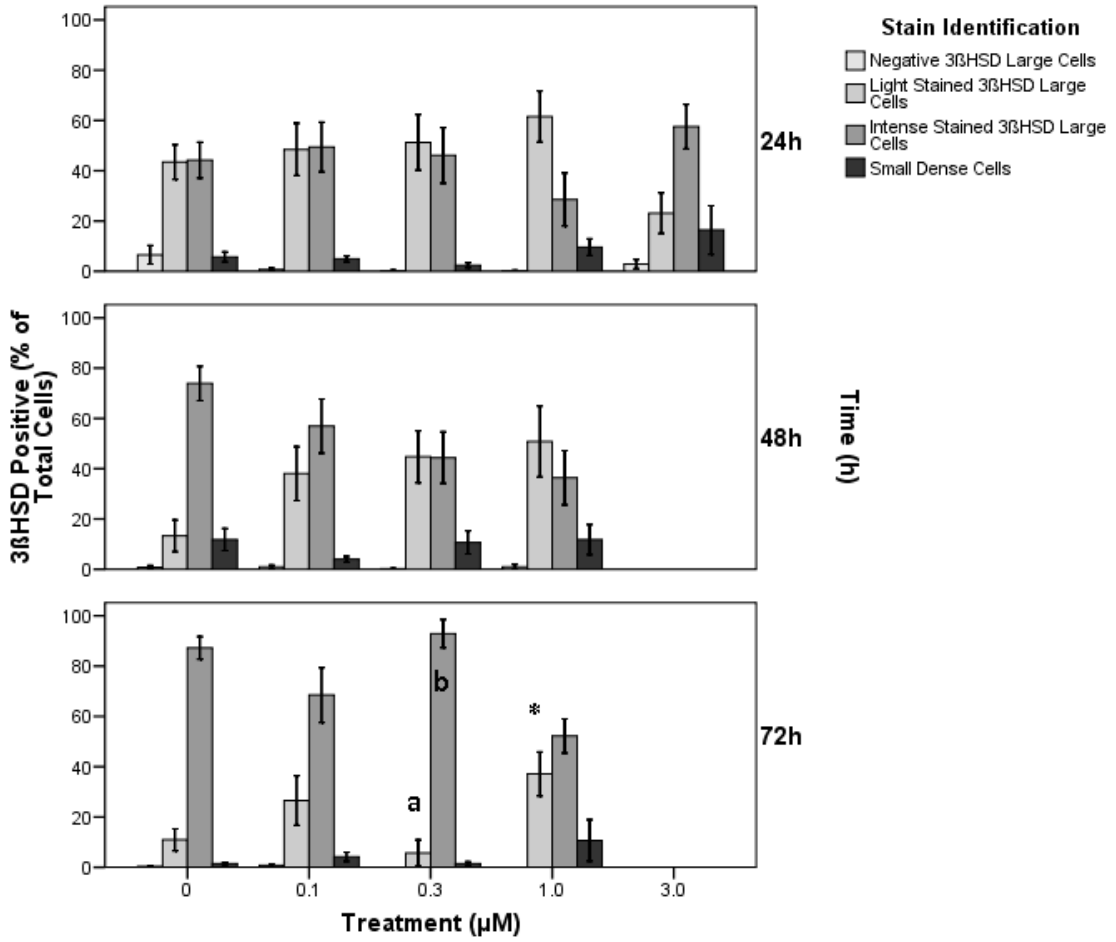


Figure 4-12: Percent (%) of Positive 3βHSD Stained Cells after 24, 48 and 72 h

Granulosa cells seeded at a density of 40,000 cells/well (n=3), allowed to adhere for 24 h followed by exposure to 0, 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Post treatment media collected and stored for steroid hormone production, 3βHSD reaction mixture (0.5mg/mL NBT, 2mg/mL NAD & 0.25mg/mL DHA) was added for 1hr. Staining observed under bright field microscope at × 200 magnification for Negative 3βHSD large cells: Light stained 3βHSD large cell: Intense stained 3βHSD large cells and small dense cells. Error bars represent Percent 3βHSD positive staining ± 1 SEM, significance indicated by a & b representing significant differences within time point where a and b are significantly different to each other $p<0.05$ by one-way ANOVA on cell counts positive to 3βHSD for each category.

.

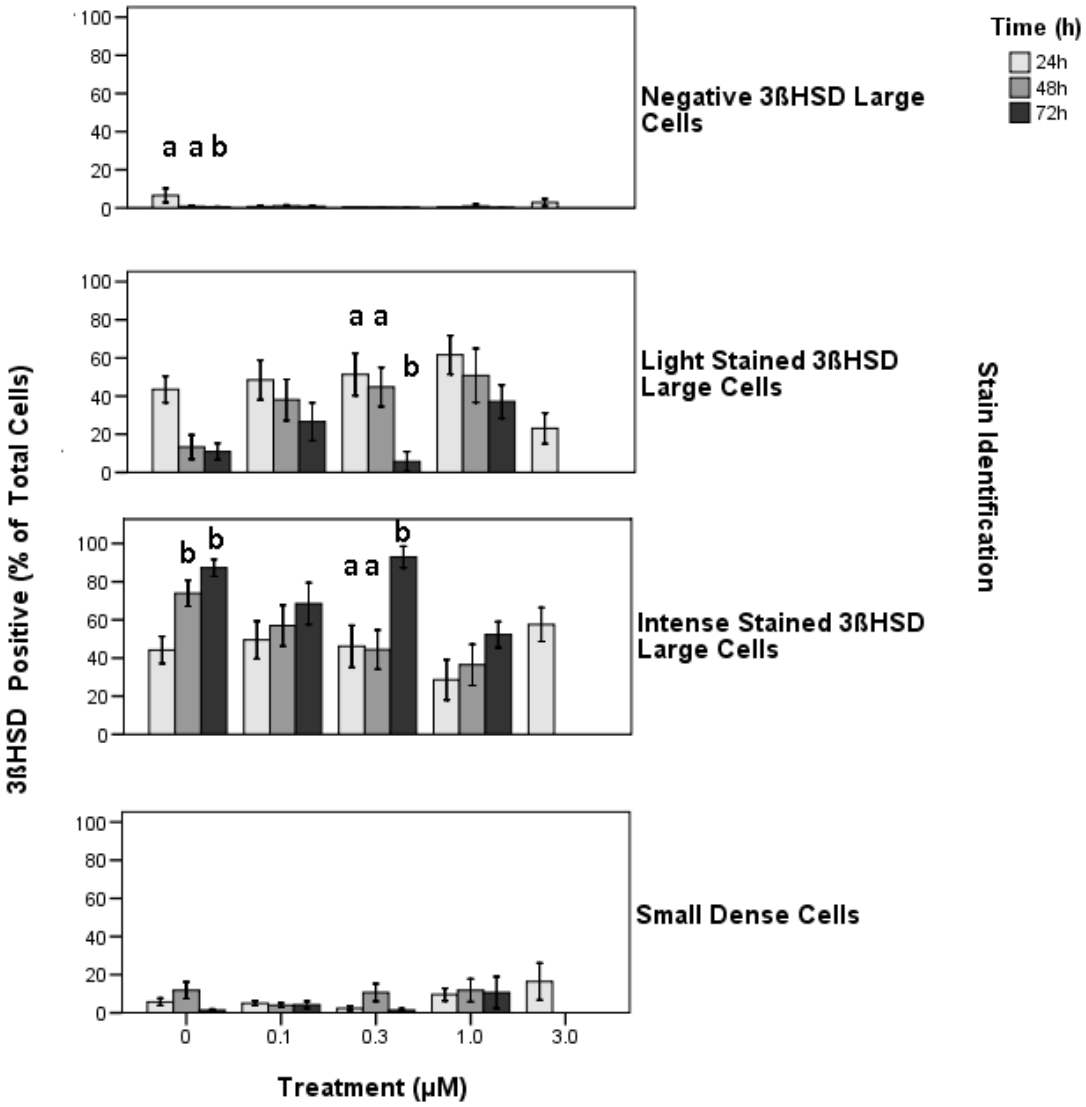


Figure 4-13: Change in Positive Percent 3βHSD Stained Cells

Granulosa cells seeded at a density of 40,000 cells/well (n=3), allowed to adhere for 24 h followed by exposure to 0, 0.1, 0.3, 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Post treatment media collected and stored for steroid hormone production, 3βHSD reaction mixture (0.5mg/mL NBT, 2mg/mL NAD & 0.25mg/mL DHA) was added for 1hr. Staining observed under bright field microscope at × 200 magnification for Negative 3βHSD large cells: Light stained 3βHSD large cell: Intense stained 3βHSD large cells and small dense cells. Error bars represent percent 3βHSD positive staining ± 1 SEM, significance indicated by a & b representing significant differences within time point where a and b are significantly different to each other P <0.05 by one-way ANOVA on cell counts positive to 3βHSD for each category.

4.6.4 CYN Decreased Steroid Hormone Production by primary derived- Granulosa Cells Cultured for Functionality Assays

There was a time-dependent decrease in progesterone concentrations after 72 h exposure to 0.1 (58%), 1.0 (82%) and 3.0 µM CYN (90%), $p<0.05$ Figure 4-14.

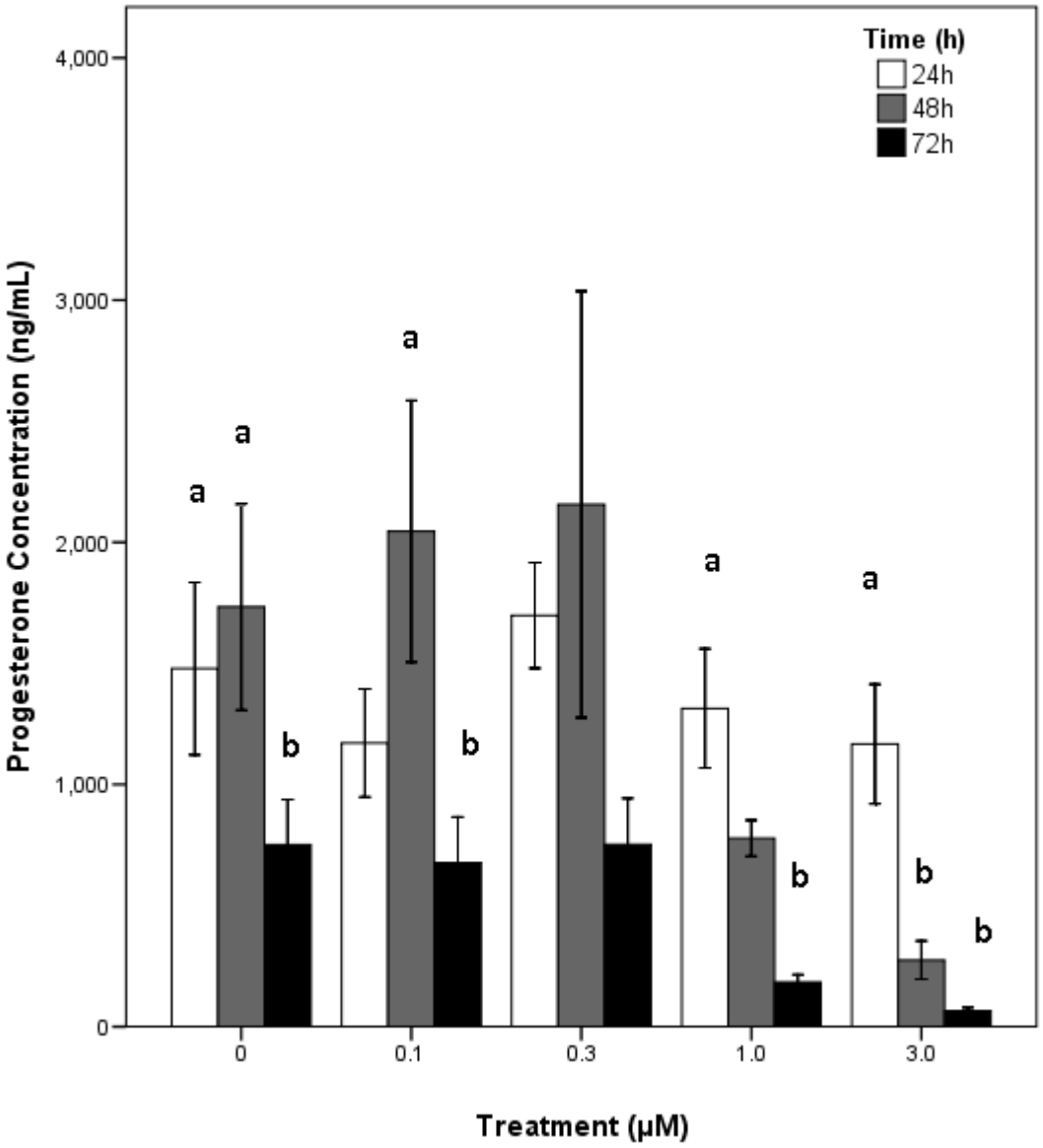


Figure 4-14: Progesterone Concentration (ng/mL) produced after CYN Exposure by GC cultured in Chamberslide Wells

Granulosa cells seeded at a density of 40,000 cells/well (n=10) were treated with 0, 0.1, 0.3 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Progesterone concentration (ng/mL) was determined by RIA. Error bars represent ± 1 SEM, significance indicated by a & b representing significant differences within time point where a and b are significantly different to each other $P<0.05$ as determined by one-way ANOVA.

Similarly, time-dependent decreases were also observed for estradiol concentrations after 72 h exposure to 1 µM (75%) and 3 µM CYN (83%), P <0.05 Figure 4-15.

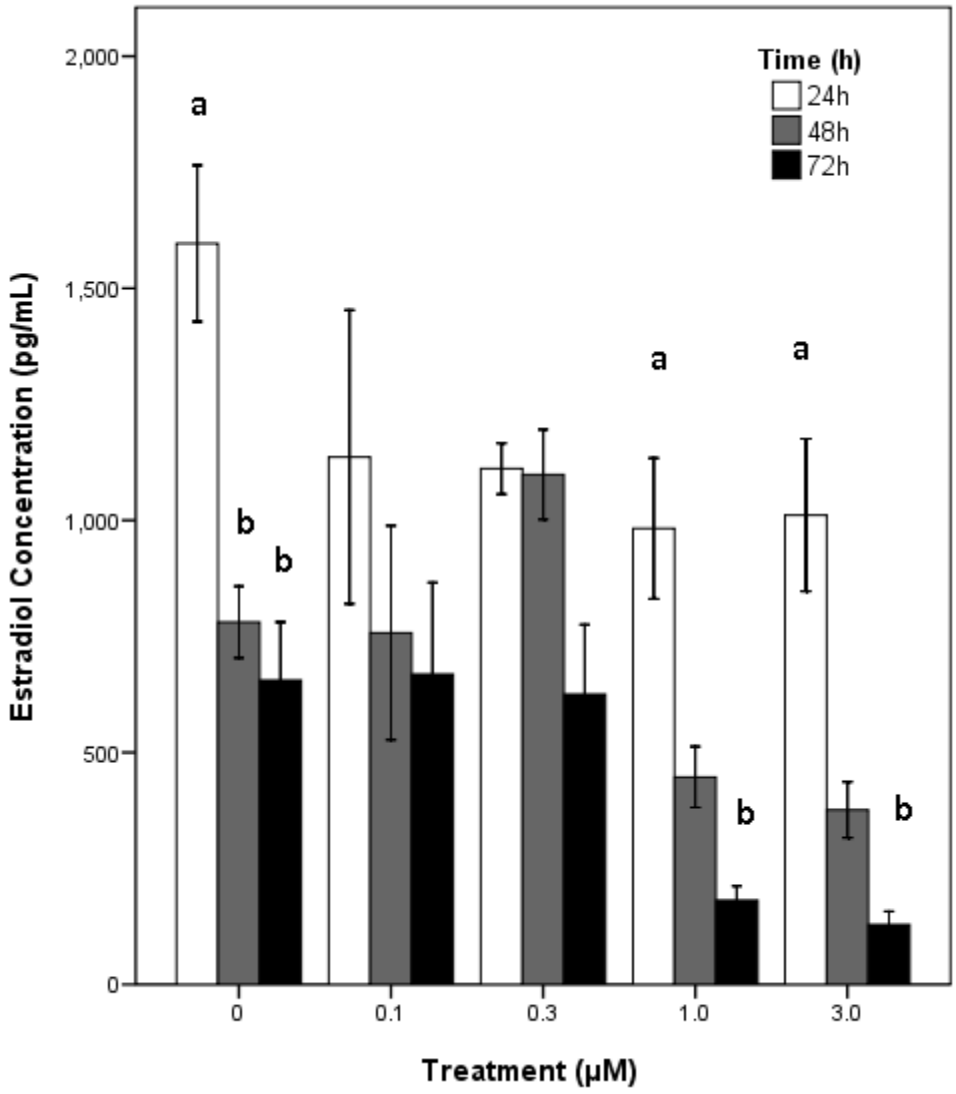


Figure 4-15: Estradiol Concentration (pg/mL) by GC cultured in Chamberslide Wells after CYN Exposure

Granulosa cells seeded at a density of 40,000 cells/well (n=10) were treated with 0, 0.1, 0.3 1.0 or 3.0 µM CYN in DMEM/F12 + 10% FCS for 24 h, 48 h or 72 h. Estradiol concentration (pg/mL) was determined by RIA. Error bars represent ± 1 SEM, significance indicated by a & b which represent significant differences within time point where a and b are significantly different to each other p<0.05 one-way ANOVA.

The magnitude of the decrease in steroid hormone concentration was greater than can solely be attributed to loss of cell viability. The decrease in steroid hormone production was similar to that of viability assays (Section 4.4.1).

4.7 Discussion

The infertility factors MIF or FIF give an indication to which type of ART treatment will be undertaken (IVF or ICSI). Couples undergoing IVF are likely to have female infertility factors (Ng et al. 2000). In contrast, couples undergoing ICSI have an increased chance of having male infertility factor (MIF) (refer to Section 2.2). Women with FIF are likely to have unhealthy granulosa cells and are at risk of having poor ovarian development *in vivo* in response to hyper-stimulation (Ng et al. 2000; Russell & Robker 2007). Thus, experimental results were represented as combined data or separated into two cohorts, FIF and MIF, which represented healthy or unhealthy granulosa cells respectively. The two women whom comprised the unhealthy cohort, women 5 and 7 (Table 8-1) were on their second ART treatment cycle, and had previously been diagnosed with ectopic and tubal ligation fertility problems respectively. Both women were classified as having FIF (unhealthy GC), had a total of 6 follicular fluid tubes collected, an average of 4 mature follicles and a total of 11 oocytes from which an average of 9.75×10^5 granulosa cells mL^{-1} were isolated. Although this may indicate a pattern to which describes unhealthy granulosa cells, it only represents data from 2 women, and when compared to the MIF group (n=10) a similar pattern was also observed. The data regarding the clinical factors collected for each woman (Table 4-1) involved in this research did not differ between the FIF and MIF groups. The lack of difference may be attributed to the sample size (Yie et al. 1995), or the parameters selected were not sensitive enough to detect differences in ovarian responsiveness and development to *in vivo* hyper-stimulation – between healthy and unhealthy cohorts. Furthermore a sample size of two may not be representative of the wider population, thus the women may not actually be more sensitive to CYN due to their female infertility factor classification. A larger number of women would be needed for reproducibility of the results obtained. Additional parameters to those listed in Table 8-1 to investigate that may indicate differences in ovarian response include increasing the number of women in the FIF group from which data is collected from, and matching the granulosa cells to its respective oocytes, at time of collection. The segregation of granulosa cells as unhealthy and healthy based on ART clinical data which segregated women into FIF and MIF groups indicate that

they could possibly pre-determine differences in sensitivity GC to CYN exposure and possibly reflect the *in vivo* response to toxin exposure by the general population.

In the three major experiments undertaken, the effects of CYN on primary-derived granulosa cells and KGN cells have generated three different viability data sets which can be compared.

The MTT assay allows the detection of early cytotoxic changes, reflecting decreases in metabolic capability to produce formazan prior to complete cell loss, whereas the Crystal Violet assay cannot (Amsterdam et al. 1999; Zlotkin et al. 1986). A key aspect of using the MTT assay to determine number of viable cells is the involvement of mitochondrial enzymes. The ability of the succinate dehydrogenase enzyme to reduce the tetrazolium salt to formazan represents live and metabolically active cells and may indicate a cell's capability for steroidogenesis, which also occurs at the mitochondria (Arnould et al. 1990; Carmichael et al. 1987; Mosmann 1983; Mueller et al. 2004; Petty et al. 1995; Young et al. 2005). The viability of primary-derived granulosa cells as determined by the MTT and Crystal Violet assays showed that CYN was cytotoxic to granulosa cells after 72 h exposure to 0.1 – 3 µM CYN, which was comparable with the results obtained by Young et al. (2008). When comparing between the two viability assays, the MTT assay was more sensitive than the Crystal Violet assay in its ability to detect loss of viability of granulosa cells after CYN exposure (section 3.4.1.5). The study by Young et al. (2008) found that 2.4 µM CYN was cytotoxic to granulosa cells after 24 h exposure (MTT assay); whereas the combined data from this study suggest that granulosa cells were more sensitive to CYN at lower concentrations (1 µM) after 48 h exposure. In comparison, Froscio et al. (2003) found that 1 µM CYN decreased the viability of mouse hepatocytes by 52% after 18 h exposure, as measured by the LDH assay. What we can conclude from this is that in comparison to the study by Young et al. (2008), the toxicity to granulosa cells was delayed, occurring after 48 h, but at only half the concentration. When comparing to Froscio et al. (2003), the CYN toxicity also appears to be delayed. Possible explanations include:

- different toxic CYN metabolites produced by the CYP enzymes found in granulosa cells (which are dedicated to steroidogenesis);
- differences in uptake mechanisms between primary-derived mouse hepatocytes and human granulosa cells (Chong et al. 2002; Froscio et al. 2009);
- toxicity might have been due to the inhibition of protein synthesis, which is a slower process;
- the LDH assay may have been more sensitive than the MTT assay in detecting loss of cell viability; or
- the differences in cytotoxicity may be attributable to cell type and or species (Bain et al. 2007; Froscio et al. 2009).

The functionality assays further support the delayed cytotoxicity of CYN as 48 h exposure to 1 µM CYN resulted in a complete loss of granulosa cell viability from chamber slide wells in a time frame similar to that observed by the MTT and Crystal Violet assays. This was attributed to both CYN and decreased ability of GC to attach to chamberslide wells (Alexopoulos et al. 2000). Although the attachment of granulosa cells to the chamber slide wells was not as strong as in 96-well plates (Amsterdam et al. 1999), changes in cell conformation and secretary properties induced by the presence of CYN may have contributed to the complete loss of cells due to detachment. Biological variability based on state of granulosa cell luteinisation at time of isolation may affect adherence efficiency of primary-derived granulosa cells to chamberslide wells. Alexopoulos et al. (2000) found that GC cultured in chamberslides need to have medium changed in gradual step as they remain loosely attached to the surface of chamberslide wells.

Although the clinical and demographic data collected from the ART cases were similar, segregation of cases based on FIF or MIF and classifying the GC as unhealthy and healthy, respectively showed that granulosa cells from the unhealthy cohort had increased sensitivity to CYN cytotoxicity and reduced steroid hormone production after exposure to CYN. This also suggests that other parameters exist

which may determine and influence the health status of GC and need to be investigated. The segregation of GC into FIF and MIF cohorts may reflect the likelihood of unhealthy cells responding similarly *in vivo*, not just to CYN, but other toxins, further affecting fertility success.

An interesting find in our data is that exposure to CYN (0.1 – 3 µM) for 24 h significantly increased the number of granulosa cells detected by the MTT assay (Figure 4-2, unhealthy cohort). This may be attributed to biological variability, different metabolic capabilities by unhealthy cells, or an indication of an un-luteinised cell population only detected by the MTT assay (Breckwoldt et al. 1996; Chaffkin et al. 1993; Edgar et al. 1991; Schmidt et al. 1984). The latter is unlikely because granulosa cells reflected a luteinised population by staining positive to 3βHSD activity and unlikely to proliferate as the increase in cAMP levels associated with increase steroidogenesis activate signalling pathways that inhibit mitotic proliferation (Richards 1994; Seifer et al. 1993) and overestimation of cell numbers. Thus, the increase in cell number observed further supports differences in metabolic processes between healthy and unhealthy granulosa cells (Bar-Ami 1994; Macklon & Fauser 1998; Richards 1994).

The main mechanisms of CYN toxicity are CYP metabolism-dependent cytotoxicity and protein synthesis inhibition (Froscio et al. 2003). Thus, granulosa cells which are healthy would be expected to have optimal mitochondrial capabilities and steroidogenesis, dependent on CYP enzymes (for reviews of the key steroidogenic CYP enzymes refer to Section 1.1.3). If cytotoxicity in granulosa cells occurred by CYP metabolites then the healthy granulosa cells with optimal mitochondrial functionality and CYP enzymes would more likely to be affected. However, this was not the case in our data, but rather the unhealthy granulosa cells with poor ovarian development to *in vivo* hyper-stimulation were more sensitive to CYN toxicity most likely attributed to their decreased capabilities for cellular defence mechanisms (Fatum et al. 2009; Russell & Robker 2007).

Although ART regimes stimulate follicular growth and subsequently the growth and development of granulosa cells; the GC nonetheless are smaller in size and produce less progesterone than their naturally developed counterparts (granulosa

cells from women who did not undertake IVF) (Lobb & Younglai 2001). The other alternative granulosa cell-like cells are the KGN cells, which are a good comparative model for reasons discussed in section 2.5, particularly due to the higher stimulated-progesterone production by these cells. Thus any disruptions to steroid hormone are more likely to be observed and not related to biological variability. The primary-derived granulosa cells were given a pre-treatment culture period of 24 h to desensitise from *in vivo* gonadotropins and regain sensitivity to further stimulation if required and increase progesterone production (Landefeld et al. 1997; Tureck & Strauss 1982) and thus KGN cells were used as an alternative to non-hyperstimulated ART derived cells.

The KGN cells were not used to directly compare the affects of CYN between primary and cell-lines but rather to determine if CYN is a toxin to this cell type. Thus the effects of CYN on primary-derived GC or KGN cells were compared directly to treatment control. The effect on viability and steroid hormone production will indicate the role of CYN as a reproductive toxicant. As a comparative tool the KGN cells were used; exposure to 3 µM CYN for 48 h decreased KGN cell viability as determined by the Crystal Violet assay. As viability was detected by the Crystal Violet assay, the onset of cytotoxic effects may have been compromised by not using the MTT assay. If the true onset of toxicity (unaffected by choice of viability assay) was after 48 h exposure to 3 µM CYN then mechanisms exist within KGN cells that make them more resistant to CYN toxicity than primary-derived granulosa cells (1 µM CYN after 48 h) within the same species (Froscio et al. 2009). The absence of toxicity in KGN cells after 1.0 µM CYN exposure may indicate that granulosa cell lines respond differently to CYN and may be more robust or have differing intracellular transport mechanisms than primary derived GC (Froscio et al. 2009). Toxicity or loss of cell viability may have been delayed in KGN cells due to the presence of cAMP. It is known that when levels of cAMP decrease apoptosis occurs (Amsterdam et al. 1999), whereas primary-derived granulosa cells were not cultured in the presence of cAMP.

In addition to the three sets of viability data from the three major experiments, each corresponds to its own set of steroid hormone data.

Results for steroid hormone production by granulosa cells after CYN exposure were also delayed compared to Young et al. (2008) whom found that 0.1 µM CYN significantly decreased progesterone concentrations 6 h, whereas in this study it was only observed after 72 h exposure. The combined data show that loss of cell viability was the major factor to the decreased progesterone production. Granulosa cells maintain steroid hormone production capability whilst undergoing apoptosis-mediated cell death (Amsterdam et al. 1999).

Steroid hormone production in the healthy and unhealthy cohorts was not related to significant decreases in cell number at low CYN concentrations (0.1 and 0.3 µM), whereas at higher CYN concentrations (1 & 3 µM), loss of cell viability was the likely cause of decreased steroid hormone production in both groups. The decreases in cell number alone cannot account for the decreased steroid hormone production at the lower CYN concentrations in the healthy cohort. Inhibition of protein synthesis of key steroidogenic enzymes may be a contributing factor to decreased steroid hormone production as studies have shown the inhibition of aromatase and StAR *de novo* synthesis to occur by 6 h (Chan & Tan 1987; Landefeld et al. 1997; Lin et al. 2009).

Both the viability and steroid hormone production data indicated that unhealthy granulosa cells were more sensitive to the toxicity of CYN at lower concentrations after 72 h exposure in comparison to the healthy cohort. However, a higher number of repeats would be required to confirm this, especially as biological variability contributed to the high SEM, which has also been an issue in other studies (Beckman et al. 1991).

The research carried out in this chapter was to further explore if the decrease in progesterone concentrations found in the research by Young et al (2008) were attributable to decreases in cholesterol substrate availability or 3βHSD functionality. First, it was important that primary-derived granulosa cells in culture were luteinised. The degree of luteinisation and maturation of GC is dependent on responsiveness to gonadotropins administered *in vivo* and indicates their ability to produce steroid hormones *in vitro* (Conti 2002). Luteinisation is marked by an increase in progesterone production (dependent on 3βHSD activity), a decrease in

estradiol production and cessation of proliferation (Bar-Ami 1994; Conti 2002; Macklon & Fauser 1998; Richards 1994). The *in vitro* culture model employed in this research was found to be conducive to granulosa cell luteinisation over the 72 h period.

The presence of positive ORO staining indicates the endogenous supply of substrate for steroid hormone production, and the presence of 3βHSD activity suggests that live cells remaining after CYN exposure maintain properties for steroidogenic functionality (Bar-Ami 1994; Fischer & Kahn 1972). The decrease in both progesterone and estradiol production after exposure to 3 µM CYN in the functionality assays were attributed to the decreased cholesterol availability detected by the ORO lipid stain after 24 h. However, progesterone production decreased after 72 h exposure to only 0.1 µM CYN, and was unlikely to be attributed directly to the decrease in substrate availability. Similarly, estradiol concentrations decreased after 72 h exposure to 1 µM CYN. The loss of cells after 72 h exposure to > 0.1 µM CYN (Figure 4-10) may have caused the significant decrease in estradiol. Furthermore, decreases in substrate availability, would be immediately observed in decreased capability of steroid hormone production where studies have shown that decreases in estradiol concentrations are most likely a result of reduced substrate availability for aromatase, rather than due to a direct effect on enzyme activity or protein level (Huang et al. 2004).

The presence of small dense cells, lacking cytoplasm remained constant throughout culture and may represent apoptotic bodies which did not change after exposure to CYN (Fatum et al. 2009). The presence of blue formazan granule deposits was not easily distinguishable within these cells due to the decreased cytoplasmic space. Nonetheless, it has been shown that 3βHSD staining remains present in theca cells of follicles undergoing atresia, thus maintaining ability to synthesise progesterone (Teerds & Dorrington 1993). Alternately, the small dense cells may represent an un-luteinised granulosa cell population that are known to be characterised by their high nuclear to cytoplasmic ratio (Delforge et al. 1972).

The amount of spontaneous formazan production in the absence of exogenous DHA (substrate for 3βHSD enzyme) was found to be minimal and not significantly

different to negatively stained cells (Fischer & Kahn 1972). The presence of unstained cells in the 3βHSD assay may be attributed to presence of lymphohaemopoetic cells (leukocytes, monocytes, B-lymphocytes and T-lymphocytes) which are also found in the follicular fluid (Beckman et al. 1991; Loukides et al. 1990; Wang et al. 1995) and do not stain positive for 3βHSD activity (Amsterdam et al. 1999; Fatum et al. 2009).

Decreases in progesterone concentrations in primary-derived granulosa cells were related to decreased viability rather than 3βHSD activity or cholesterol substrate availability. Our results did not support the decrease in progesterone production found by Young et al. (2008) to be attributed to a loss of 3βHSD functionality; it appears that enzyme activity was still functional. The significant decrease in light-stained 3βHSD cells after 72 h and the simultaneous increase in intense-stained 3βHSD cells suggest that GC increased or regained 3βHSD activity after 72 h in culture with no apparent effect by the presence of 0.3 µM CYN. The same pattern was observed at 1.0 µM CYN, however, not in the same magnitude nor was it statistically significant. Increased 3βHSD activity would indicate that there would be increased progesterone production due to the apparent increased 3βHSD activity and especially as substrate was supplied and remained constant throughout the experiment. However, progesterone concentrations detected from the same wells decreased and were not attributed to lack of substrate availability (ORO Lipid staining was positive and not significantly reduced). The decrease in progesterone concentrations was first, not statistically significant when compared to 48 h time period and secondly, dropped to the same level as the control. Thus, assuming functionality of 3βHSD enzyme is optimal (indicated by increased intensity of staining) and there remained a drop in progesterone concentrations, it would suggest that the endogenous supply of pregnenolone substrate (precursor for progesterone production) was the limiting factor. It is possible that pregnenolone production by CYP11A1 was affected by either a direct action of CYN with the CYP11A1 enzyme, or by the inhibition of *de novo* protein synthesis of StAR the rate limiting enzyme for steroidogenesis (Stocco & Strauss 1998). If StAR is not available or functional then the translocation of cholesterol substrates, their presence detected by ORO Lipid stain in our assay, into the mitochondria would not

occur and thus the cholesterol substrates would remain un-utilised for steroidogenesis.

Further work is required to determine if 3βHSD enzyme activity was altered. CYN is a potent inhibitor of protein synthesis at the translational step of protein synthesis as determined in rabbit reticulocyte lysate translation system (Froscio et al. 2001). Changes in CYP11A1 or StAR expression may subsequently affect enzyme protein levels and progesterone production by 72 h *in vitro*, and is dependent on de *novo* protein synthesis (Stocco & Clark 1996). Decreases in 3βHSD activity due to decreased availability of substrate would not be detected in our assay system as the reaction mixture was supplemented with 3βHSD substrate. Further experiments are needed to determine if the substrate precursors for the enzymes producing progesterone and estradiol production are diminished and if the enzymes such as CYP11A1 were affected by CYN by direct interaction or protein synthesis inhibition.

Finally, as a comparison, cAMP-progesterone production in KGN cells was also investigated. Despite a decrease in KGN cell number after 72 h in culture in DMEM/F12 + 1mM cAMP, progesterone production continued to increase over the 72 h culture period. Decreases in progesterone concentration after CYN exposure were not correlated with loss of viability. Progesterone production by KGN was abolished after 48 h exposure to 1 and 3 µM CYN, despite the presence of cAMP. Cyclic AMP activates intracellular signalling systems favouring the expression of steroidogenic genes (Hanukoglu et al. 1990). The inhibition of progesterone production was unaccounted for by the loss of cell viable granulosa cells, also suggesting that protein expression or activity of key steroidogenic enzymes such as StAR or CYP11A1 may have been down regulated or inhibited in both KGN and granulosa cells (Arthur & Boyd 1974; Tsutsumi et al. 2008; Young et al. 2008). The effect of CYN on stimulated-progesterone production by KGN cells cannot be directly compared to granulosa cells, but rather they indicate that the steroidogenic rate limiting proteins play a major role in progesterone production despite sufficient stimulation with cAMP and no significant loss of cell viability. Furthermore the viability of KGN cells should also be determined with the MTT assay as this would

have indicated if CYN also affected the mitochondria of KGN cells. Thus the onset of cell death may be at an earlier time point and lower CYN concentration as observed in the granulosa cells.

A poor ovarian response to hyperstimulation *in vivo* results in a 2-fold decrease in progesterone production which subsequently results in decreased conception rate and development of granulosa cells with decreased steroid hormone production capabilities *in vitro* (Phy et al. 2002). This is likely to be attributed to a 17% decrease in StAR protein in granulosa-luteal cells (Phy et al. 2002), of which the StAR protein is the rate limiting step in steroid hormone production (Stocco & Strauss 1998). If granulosa cells do not have the capacity for maximal steroidogenesis (due to a poor ovarian response), then granulosa cells cultured *in vitro* may have either increased or decreased sensitivity upon exposure to a stimuli *in vitro* with respect to steroid hormone production. The establishment and maintenance of pregnancy is dependent on the presence of progesterone; if *in vivo* progesterone concentrations fall below 32 $nmol.L^{-1}$ then this indicates abnormal follicular growth, ovulation and steroid hormone production (Zonneveld et al. 1994). Thus granulosa cells will respond differently to an *in vitro* stimulus depending on their *in vivo* ovarian response. Furthermore granulosa-luteal cells require time in culture to desensitize from *in vivo* hyperstimulation which is inversely related to time in culture (Zosmer et al. 2002). In addition inflammatory cytokines are implicated in ovarian steroid hormone production (interlukin-1 is found in follicular fluid) and increases progesterone production by granulosa-luteal cells, with cAMP being the main messenger (Zosmer et al. 2002). Thus maximal progesterone production *in vitro* by granulosa-luteal cells is dependent on a good ovarian response, presence of StAR protein, desensitisation from hyperstimulation and adequate cAMP, whereas KGN cells require the presence of cAMP and do not require additional culture time to desensitize. The granulosa cells used in these experiments were also subdivided into healthy and unhealthy groups of which unhealthy women would be poor ovarian responders (Lobb & Younglai 2001). KGN cells were stimulated with cAMP, whereas GC were not and in addition to the aforementioned these are the main differences between KGN cells and GC response to CYN exposure *in vitro.*

There were differences in steroid hormone production between healthy and unhealthy granulosa cells after CYN exposure. Progesterone concentrations decreased below the critical threshold (10 $ng.mL^{-1}$, Figure 4-4) in the unhealthy cohort after 48 h exposure to 0.3 and 3.0 µM CYN. However the data from the unhealthy cohort is from a total of two repeats, thus not indicative of the wider population and the unhealthy granulosa cells may actually have the capability to maintain progesterone production above the critical threshold. In comparison the KGN cells required 72 h exposure to 1.0 µM CYN for progesterone production to be abolished, indicating that although KGN and GC respond differently to CYN, the outcome is similar - there is a risk of CYN decreasing progesterone production to the critical threshold which may affect pregnancy outcome (Phy et al. 2002; Zonneveld et al. 1994; Zosmer et al. 2002).

Hence, further investigations into protein synthesis inhibition or signalling pathways CYN targets in primary-derived granulosa cells and KGN may enhance understanding of the mechanism of CYN action on steroidogenesis.

4.8 Conclusion

Our data support cytotoxicity of CYN in primary granulosa cells as observed by Young et al. (2008). CYN cytotoxicity was unlikely to be mediated by CYP metabolites, primarily due to different CYP enzymes found in granulosa cells than mouse hepatocytes. Cytotoxicity was also delayed, attributable to different uptake mechanisms or inhibition of general protein synthesis. Decreases in progesterone concentrations were correlated to loss of viability, rather than availability of cholesterol lipids or loss of 3βHSD activity. In addition to the study by Young et al. (2008), estradiol concentrations decreased and were most likely attributed to the loss of cell numbers. The unhealthy granulosa cell cohort was more sensitive to cytotoxicity and had decreased steroid hormone concentrations in comparison to the healthy cohort. The unhealthy granulosa cells were from women characterised as having female infertility factors and were undergoing IVF, thus their increased sensitivity to toxins could be due to poor maturity and development *in vivo*, different metabolic processes and capabilities which target and activate similar signalling mechanisms of which CYN elicits its adverse effects. The differentiation

between healthy and unhealthy cells was important for two reasons; the first is that *in vitro* experiments can be tailored to investigate effects on the most sensitive cells and the mechanisms by which healthy cells are more robust. Secondly, it assists in decreasing inter-individual variability, aiding statistical analysis. Furthermore, the infertility factor will play a role and influence the development of GC within the ovarian follicle. The decrease in steroid hormone production suggests that CYN could target CYP pathways essential for steroidogenesis (Conti 2002). The following chapter aims to further investigate if CYN also inhibits protein synthesis in granulosa cells.

.

Chapter 5. Effects of Cylindrospermopsin on Human Granulosa Protein Synthesis *in vitro*

5.1 Introduction

In addition to the cytotoxic effects of cylindrospermopsin, which occur after 18 h exposure to 1 µM CYN in mouse primary hepatocytes, CYN is also a potent inhibitor of protein synthesis (Froscio et al. 2001; Froscio et al. 2003). Protein synthesis is inhibited after exposure to 0.5 µM CYN for 4 h, but this was unrelated to cytochrome P450 (CYP) metabolism-dependent cytotoxicity (Froscio et al. 2003). The two uptake mechanisms by which CYN is thought to enter cells, are the bile acid transport system and passive diffusion (Chong et al. 2002). In addition to CYN's hydrophilic properties which decrease its ability to easily permeate the cell membrane, CYN's molecular weight of 415 Da limits its diffusion. Hence, passive diffusion is a secondary and slower uptake mechanism (Chong et al. 2002). Froscio et al. (2009b) found that the type of cell and its location in the gastrointestinal tract may determine how sensitive it will be to CYN, due to differing metabolic activity and cellular uptake mechanisms. Recently, CYN uptake was found to be at least partially independent of energy dependent mechanisms, as cellular uptake occurred at 4°C and in a time-dependent manner, increasing by 6-fold after a 24 h period (Froscio et al. 2009). CYN uptake was relatively slow and likely to be dependent on the limited transport systems available in most cell lines in comparison to primary derived cells (Froscio et al. 2009).

Chong et al. (2002) found that CYN uptake by the bile acid transport system can also occur. Bile acids are synthesised from hydrophobic insoluble cholesterol molecules in which soluble molecules of bile acids are formed to maintain cholesterol homeostasis in the liver (Alrefai & Gill 2007). The movement of bile acids occurs rapidly into hepatocytes and is dependent on two processes: (1) Na^+ transport systems (Na^+-dependent taurocholic co-transporting peptide, NTCP) which are also modulated by cAMP, and (2) Na^+-independent organic anion

transporting peptide (OATP) transport system (conjugated forms of bile acids) (Alrefai & Gill 2007).

Granulosa cells can utilise various transport pathways and solute carriers to allow molecules to enter the intracellular environment. Molecules such as ascorbic acid are transported across the plasma membrane, dependent on active transportation via a Na^+ pump (Zreik et al. 1999), a solute carrier similar to the bile salt co-transport family (Hediger et al. 2004). Studies have shown that GC utilise Na^+ ion channels for other transport mechanisms (Alrefai & Gill 2007; Zreik et al. 1999). This may include the bile acid transport systems, utilising solute carriers to maintain homeostasis. Cholesterols are comprised of steroid structures and are synthesised in the liver via oxidation pathways (Smith et al. 2009). Cholesterol is also involved in the production of the sex steroids progesterone and estradiol indicating the ovarian follicle is abundant with cholesterol (Smith et al. 2009). Thus, bile acids are likely to be present in the follicular fluid and synthesised from granulosa cells (Smith et al. 2009), thus bile acid transport mechanisms are likely to be present in GC. Active CYN uptake by GC is likely, and once inside a cell, CYN is a potent inhibitor of protein synthesis (Froscio et al. 2003). Steroid hormone production by human primary-derived granulosa cells involves membrane hyperpolarisation and voltage dependent ion channels for Na^+ and Ca^{2+} (Kunz et al. 2007). In response to increased intracellular Ca^{2+} levels, which can be stimulated by Acetylcholine (Ach), K^+ channels are opened and result in membrane hyperpolarisation. Blockage of this channel results in a block of gonadotropin-stimulated steroid hormone production (Kunz et al. 2007).

Dysfunctions in steroid hormone biosynthesis result in impairment of ovarian function and infertility (Lin et al. 2009). Steroid biosynthesis is dependent on *de novo* protein synthesis of the steroidogenic proteins such as steroidogenic acute enzyme regulatory (StAR) and cytochrome P450 side-chain cleavage enzyme (CYP11A1) (Amsterdam et al. 1999; Breckwoldt et al. 1996; Stocco et al. 2001). Steroid biosynthesis is also dependent on gonadotropins which increase the level of intracellular cAMP (Ronen-Fuhrmann et al. 1998). The two rate-limiting steps in steroidogenesis (as detailed in section 1.1.3) are steroidogenic acute regulatory

enzyme (StAR) and CYP11A1 enzyme, which is the precursor to all steroid hormones (Breckwoldt et al. 1996; Clark et al. 1994; Devoto et al. 2002; Ronen-Fuhrmann et al. 1998; Stocco & Clark 1996). FSH and LH/hCG are the gonadotropins which regulate protein expression of StAR, CYP11A1 for steroid hormone production (Conti 2002; Huang et al. 2004; Sasson & Amsterdam 2002). The CYP11A1 enzyme is of particular interest as it may be a prime target of CYN toxicity either via direct interaction, production of toxic metabolites, or inhibition of protein synthesis. Many of the steroidogenic enzymes such as aromatase (production of estradiol) are affected at the mRNA level as a result of down-regulation of gene expression in granulosa cells and subsequent protein synthesis (Kagabu et al. 1999). Cycloheximide (CXH), which is a potent protein synthesis inhibitor in granulosa cells (Landefeld et al. 1997; Stocco & Clark 1996) was found to significantly reduce estradiol production at concentrations of 0.015 – 150 µg.mL^{-1}, which was not correlated to decreased cell viability.

The capability of luteinised and non-luteinised granulosa cells to undertake protein synthesis *in vitro* does not differ between the two cell states (Landefeld et al. 1997). Studies have shown that both stages of differentiation were capable of protein synthesis after *in vivo* exposure to 5000 mIU of hCG in female rats (Landefeld et al. 1997). Differentiation of granulosa cells and their responsiveness to further *in vitro* stimulation can occur anywhere between 24 h to 72 h post-isolation of granulosa cells (Landefeld et al. 1997; Tureck & Strauss 1982). Granulosa cells are allowed to adhere prior to stimulation with hCG, allowing cells to form a monolayer and desensitise from *in vivo* gonadotropin stimulation (Edgar et al. 1991). *In vitro* stimulation with hCG is known to increase CYP11A1 activity in rat granulosa cells, whereby progesterone production by granulosa cells was optimal after 8-14 days culture post-isolation (Voutilainen et al. 1986).

In order to determine protein synthesis, many laboratories measure ^{3}H Leucine incorporation (Kirchman et al. 1985; Nelson et al. 1978) and that of other radiolabelled amino acids such as uridine, guanosine or methionine sulphate, with their incorporation quantified by liquid scintillation counting (Heller et al. 1981), a well established assay by Lowry et al. (1951). Cyclosporine A, an

immunosuppressive drug (cyclosporin A, CsA), was found to inhibit estradiol production at high concentrations of 3 – 10 µg mL^{-1} as well as having cytotoxic effects and inhibition of protein synthesis, indicated by the incorporation of ^{3}H Leucine (Gore-Langton 1988). Thus, as steroidogenesis in GC can be dependent on *de novo* protein synthesis (Stocco & Clark 1996), ^{3}H Leucine incorporation is an appropriate assay to use in determining effects of CYN on protein synthesis in granulosa cells.

In our experiments, granulosa cells were cultured for 24 or 48 h prior to CYN exposure to determine if there would be a difference in the sensitivity of GC responding to hCG stimulation and whether changes in CYP11A1 protein levels would be correlated to steroid hormone biosynthesis. Furthermore, although 3βHSD mRNA levels vary throughout the luteal phase, protein levels of the enzyme remain in excess and functional, thus changes to 3βHSD gene expression are unlikely to cause decreases in progesterone production (Devoto et al. 2002; Duncan et al. 1999). Thus, it is important to determine whether protein synthesis, steroid hormone production and CYP11A1 protein levels in GC stimulated with hCG after 24 or 48 h pre-treatment adherence period will differ in response to CYN exposure in comparison to unstimulated granulosa cells.

5.2 Chapter Aims and Objectives

The investigations described in this chapter- whereby decreases in steroid hormone production as a result of CYN exposure will also be correlated to the inhibition of protein synthesis in human primary derived granulosa cells. The first step was to determine if the granulosa cell population represent healthy cells. The second step was to determine if CYN would affect protein synthesis in granulosa cells, and if so, at what concentration and time. This was followed by semi-quantitative detection of CYP11A1 (by western immunoblot-detection) in granulosa cells after exposure to the CYN concentration and exposure period found to inhibit protein synthesis in primary hepatocytes. This was subsequently related to any changes observed in steroid hormone biosynthesis. See Figure 5-1: Flow Diagram Chapter Objectives. The specific hypotheses were:

- Cylindrospermopsin will inhibit *in vitro* protein synthesis in primary-derived granulosa cells which is time and dose-dependent.

- Decreases in steroid hormone production will be relative to the decrease in protein synthesis inhibition after CYN exposure.

- Levels of CYP side chain cleavage enzyme will decrease after exposure to CYN with and without human chorionic gonadotropin stimulation as a result of protein synthesis inhibition.

- Decreases in steroid hormone production will be relative to CYP11A1 levels in granulosa cells.

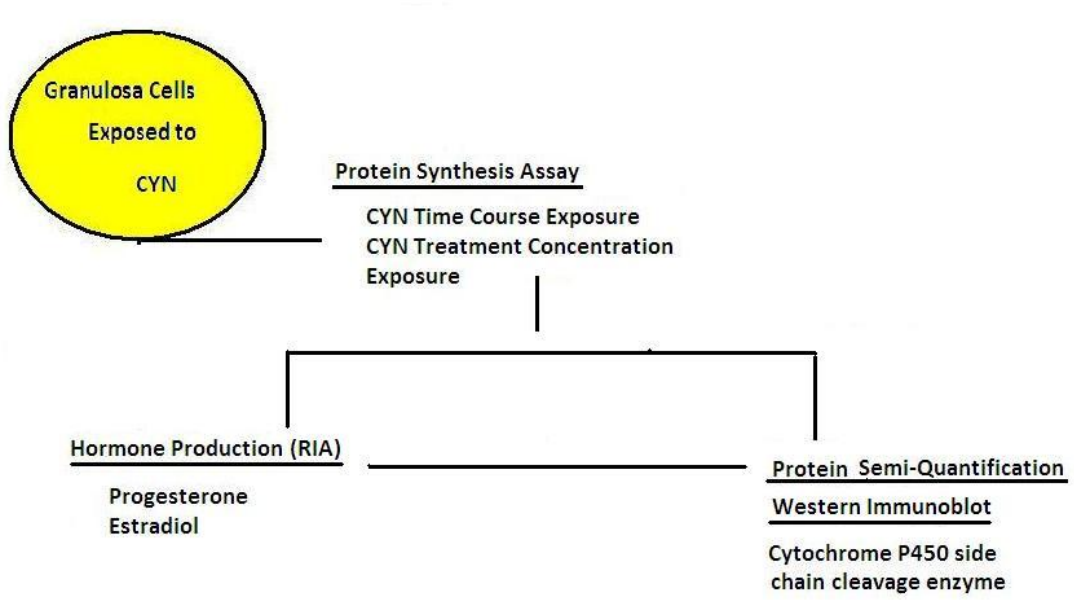


Figure 5-1: Flow Diagram Chapter Objectives

5.3 Materials & Methods

5.3.1 Isolation of Primary-derived Human Granulosa Cells

Refer to 2.3 Isolation of Human Granulosa Cells. The sources of primary cells were classified as cells used for "PSI and Westerns" under the "Experimental Endpoint" column in (Table 8-1) and include a total of n=13 (women 16-28). All cells were derived from either MIF patients or patients classified with unknown fertility factor but underwent ICSI. The granulosa cells used from patients classified with an unknown fertility factor refers to an unknown reason for which the couple was seeking fertility treatment. The relevance is that the unknown factor is not attributed to a female endocrine issue, and thus could be included in the study. Thus the reason for which the couple sought ART was unknown but likely to be a MIF due to ICSI.

5.3.2 Protein Synthesis Inhibition After Increased Exposure Time to CYN (Time Course Assay)

In order to determine at which time protein synthesis would be inhibited by granulosa cells exposed to 5 µM CYN, the following experiment was conducted. Granulosa cells were seeded into 6-well plates (10^5 cells/well) in duplicate wells (Nunc, inVitro Australia) in DMEM/F12 + 10% FCS + ITS (human insulin, 5 $\mu g.mL^{-1}$; Apo-transferrin, 5 $\mu g.mL^{-1}$ and sodium selenite 5 $ng.mL^{-1}$) in 1 mL final volume. Granulosa cells were allowed to adhere for 24 h prior to treatment. Media was replenished with treatments of either DMEM/F12+ 10% FCS ± 1 µCi [^{3}H] Leucine; DMEM/F12+ 10% FCS ± 5 µM CYN± 1 µCi [^{3}H] Leucine for 2 h, 4 h, 6 h or 24 h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. [^{3}H] Leucine incorporation was determined as detailed in Section 5.3.4 Protein Synthesis Inhibition Assays- ^{3}H Leucine Incorporation. Controls included DMEM/F12+ 10% FCS (Control) and DMEM/F12+ 10% FCS+1 µCi [^{3}H] Leucine (^{3}H Leucine Control).

5.3.3 Protein Synthesis Inhibition After Increased CYN Concentration (CYN Exposure Assays)

In order to determine if protein synthesis would decrease in GC in a dose-dependent manner with increasing CYN concentration, with or without hCG stimulation, after 6 h exposure, the following experiments were conducted.

5.3.3.1 [^{3}H] Leucine Incorporation without hCG stimulation

Granulosa cells were seeded into 6-well plates (10^5 cells/well) in duplicate wells (Nunc, inVitro Australia) in DMEM/F12 + 10% FCS+ ITS of 1 mL final volume. Granulosa cells were allowed to adhere for 24 h prior to treatment. Media was removed and treatments of DMEM/F12+ 10% FCS or DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine + CYN (0.1, 0.3, 1 or 3 µM) added for 6 h. Media were collected after treatment and stored at -20°C for steroid hormone analysis by RIA. [^{3}H] Leucine incorporation was determined as detailed in 5.3.4 Protein Synthesis Inhibition Assays- ^{3}H Leucine Incorporation.

5.3.3.2 [^{3}H] Leucine Incorporation with hCG stimulation

assay setup was repeated as for section 5.3.3.1 [^{3}H] Leucine Incorporation without hCG stimulation. Treatments consisted of

- DMEM/F12+ 10% FCS
- DMEM/F12+ 10% FCS + 1000 mIU.mL^{-1} hCG
- DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine + 1000 mIU.mL^{-1} hCG
- DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine + 1000 mIU.mL^{-1} hCG + CYN (0.1, 0.3, 1 or 3 µM)

for a period of 6 h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. [^{3}H] Leucine incorporation was determined as detailed in 5.3.4. Controls as for 5.3.3.1, with the addition of DMEM/F12+ 10% FCS+1 µCi [^{3}H] Leucine + 1000 mIU.mL^{-1} hCG (0 µM CYN + hCG), n=3.

5.3.4 Protein Synthesis Inhibition Assays- ^{3}H Leucine Incorporation

5.3.4.1 Quantification of [^{3}H] Leucine Incorporation

To quantify protein synthesis in human IVF-derived granulosa cells, the incorporation of [^{3}H]-Leucine into trichloracetic acid (TCA) precipitate was measured (Freshney 2005). Granulosa cells were isolated as described previously (Section 5.3.1 Isolation of Primary-derived Human Granulosa Cells) and allowed to adhere for a period of 24 h at a cell density of 4×10^{4} cells/well in DMEM/F12+ 10% FCS. Granulosa cells were incubated with 1 µCi [^{3}H]-Leucine (115.4 Ci/mmol, PerkinElmer Pty Ltd, Vic, Australia) per mL of medium. After the designated exposure period detailed in sections 5.3.2 and 5.3.3, the medium was removed and wells containing granulosa cells were washed with 2 mL of un-supplemented DMEM/F12 followed by 500 µL of ice-cold 10% TCA. Cells were collected by scraping using a rubber policeman, transferred into a sterile plastic tube and centrifuged at 5000 × *g* for 5 min. The supernatant was removed and a 10% TCA wash was repeated twice. The pellets were then digested with 250 µL of 0.3 M NaOH at 37°C for 1 h. Protein synthesis determination was performed by transferring 200 µL of the cell digest into Mini POLY-Q™ Vial (Polyethylene, Beckman Coulter, CA, USA) containing 5 mL of ReadySafe™ (Beckman Coulter, CA, USA) scintillation fluid. The samples were counted for 2 min in a Beckman LS 3801 scintillation counter (Beckman Coulter, CA, USA), deriving the counts per minute based upon a pre-defined quench curve using Beckman quench standards. The remaining 50 µL of the cell digest was used to quantify total genomic DNA (see: DNA Quantification).

5.3.5 DNA Quantification

The remaining 50 µL from the cell digest (section 5.3.4.1) was used as to quantify the amount of DNA. An accurate method for detecting and quantifying total genomic DNA from cultured granulosa cells (sections 5.3.2 & 5.3.3) by spectrophotometric analysis at 260 nm (Teare et al. 1997). This is because the amount in the sample is correlated to concentration of nucleic acid, based on the Beer-Lambert law (Teare et al. 1997).

5.3.6 Western Immunoblot: Cytochrome P450 side chain cleavage enzyme (CYP11A1)

Granulosa cells were seeded into 24-well plates (2×10^5concentration per well) and were allowed to adhere for either 24 or 48 h in DMEM/F12. Media were collected and replaced with treatment medium (DMEM/F12+ 10% FCS ± 100 mIU mL^{-1} hCG or DMEM/F12+ 10% FCS ± 3 µM CYN) for 6 h. Post-treatment, media was collected and stored at -20°C for RIA analysis of steroid hormones. GC monolayers were washed twice with PBS and harvested by cell scraping into 0.5 mL RIPA buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 10% Na-deoxycholate; 1 mM PMSF; 1 mM EDTA and a cocktail of protease inhibitors (Leupeptin/aprotinin/pepstatin, SIGMA). This was placed on ice for 15 min before the cell material was pelleted by centrifugation at 14000 × *g* for 5 min. The protein concentration in the supernatant was determined by Bradford assay (Bradford 1976). Samples were prepared (1:2) in loading buffer (3x Loading buffer: 0.5 M Tris-HCl, pH 6.8; 25% glycerol; 10% (w/v) sodium dodecyl sulfate (SDS) and 0.5% (w/v) bromophenol blue) and were heated for 5 min at 95°C; 5 µg protein per lane was electrophoresed in a 12% SDS-PAGE gel using standard procedures (Fatum et al. 2009) for 1 h at 150 V and transferred to a nitrocellulose membrane (BioRad) at 100 V for 1.5 h. A pre-stained molecular weight marker (MagicMark, Invitrogen) was used. Membranes were blocked overnight at 4°C with blocking buffer (40 mM Tris pH 7.4; 5% (w/v) skim milk powder; 0.1% (v/v) Tween20). Detection of CYP11A1 was achieved by the incubation of the membrane with a polyclonal anti-human CYP11A1 antibody (1:1300; abCam) in Tween buffer (40 mM Tris pH7.4; 0.1% (v/v) Tween20) for 1 h at 37°C. After incubation with the primary antibody, the membranes were rinsed 5 × with washing buffer (2 × 5 min; 1 × 10 min and 2 × 15 min, 20 mM Tris pH 7.4; 150 mM NaCl; 5% (w/v) skim milk powder; 0.1% (v/v) Tween20). Membranes were then incubated with a secondary anti-rabbit HRP-conjugate antibody (1:5000) for 1 h at 37°C. After incubation with the secondary antibody, membranes were rinsed 4 × with washing buffer (2 × 5 min and 2 × 15 min). Protein bands were detected with enhanced chemiluminescence HRP substrate and visualised using a cooled CCD camera (Fuijifilm LAS4000). Membranes were then stripped with stripping buffer (8.5 SDS-Reagents & Western Immunoblotting Buffers Stripping Buffer) at 50°C for 30 min, blocked as

previously described and re-probed with a monoclonal anti β-actin antibody raised in mouse (1:5000, SIGMA A3854, kindly donated by Rhys Hamon, Department of Medicine, Adelaide University) for 1 h at 37°C, membranes were then washed and incubated with secondary anti-mouse HRP conjugate antibody (1:5000) for 1 h at 37°C. After incubation with the secondary antibody, membranes were rinsed 4 × with washing buffer (2 × 5 min and 2 × 15 min). Detection and visualisation as described for CYP11A1 (refer to section 5.3.6).

5.3.7 Radioimmunoassay (RIA)

Media collected post treatment was analysed for hormone content. Refer to section 2.12 Radioimmunoassay. The [^{3}H]-Leucine did not interfere with the RIA, which is specific for ^{125}I isotope.

5.3.8 Statistical Analysis

Statistics Package for the Social Sciences (SPSS) version 15.0 for windows was used for statistical analyses and graphs. Differences between treatments and incubation periods analysed by one-way ANOVA; with post-hoc analysis carried out by Tukey or Dunnett's test. Statistical significance was accepted at $P < 0.05$ or where otherwise indicated.

5.4 Results

5.4.1 Characterisation of Primary-derived Human Granulosa Cells

The granulosa cells isolated for experimental use in this chapter (individually listed in Table 8-1 under "PSI and Westerns") were grouped under male infertility factor and unknown fertility factor (infertility could not be attributed to either female or male factors) and represent healthy cells. Although there was one IVF case, this woman was on her first ART treatment cycle and had a good ovarian response to the hyper-stimulation regime (Section 2.2.1). The clinical case notes did not attribute a male or female factor to the reason why the couple was seeking treatment for their infertility.

	Male Factor	Unknown	
ART Procedure	ICSI	IVF	ICSI
	10	1	2
Age of Patient at Isolation	33 ± 6	35	33 ± 0
Average Number of Treatment Cycle	5 ± 4	1	3 ± 2
Total FSH Administered (Units)	2794 ± 2547	2800	1763 ± 159
Maximum E2 pmol/L	9388 ± 10012	13575	8542 ± 8806
Number of Mature Follicles (>15mm)	8 ± 5	9	4 ± 1
Number of Oocytes Aspirated	11 ± 6	6	19 ± 18
Number of Embryos Transferred	1 ± 1	1	1 ± 1
Number of Pregnancy's	3	0	1
Number of Follicular Fluid Tubes Collected	7 ± 4	10	13 ± 11
Granulosa Cells Isolated (cells/mL)	1.3×10^6 ± 5.5×10^5	1.2×10^6	2.7×10^6 ± 2.1×10^6

Table 5-1: Ovarian Response Parameters for Male and Unknown Infertility Factor Cohorts

Women whose granulosa cells were used in this chapter were divided into two cohorts based on whether they had male infertility factor (MIF) or female infertility factor (FIF). If clinical data was not indicative of FIF or MIF then the patient's fertility factor was classified as Unknown. Ovarian response factors collected include; type of ART procedure (IVF/ICSI); age of patient at time of isolation; the number of previous treatment cycles, and total units of FSH administered; maximum plasma estradiol detected before oocyte aspiration, number of mature follicles produced, number of oocytes aspirated, number of embryos transferred and the number of women who became pregnant outcome; number of follicular fluid tubes collected, number of isolated GC/mL. Data represent mean ± standard deviation.

5.4.2 Effects of CYN on Protein Synthesis Inhibition in Primary-derived Human Granulosa Cell with Increased Exposure Time

Results were expressed as cpm.µg^{-1} DNA. A negative control (without ^{3}H-Leucine) was included for each experiment in order to determine background cpm. For CYN treated granulosa cells, [^{3}H]-Leucine was added at the same time as the toxin. The incorporation of the radiolabelled amino acid allowed the quantification of *de novo* protein synthesis (Duley et al. 1979). Maximum protein synthesis occurred after a 24 h culture period in control medium. Protein synthesis was significantly reduced by 61% (P <0.05) and 95% (P <0.005) after exposure to 5 µM CYN for 6 h and 24 h respectively in comparison to the control (Figure 5-2).

5.4.3 Effects of CYN on Steroid Hormone Production by Primary-derived Human Granulosa Cells with Increased Exposure Time

Steroid hormone analysis of the culture medium collected showed there were no statistically significant changes in either progesterone (Figure 5-3) or estradiol (Figure 5-4) levels relative to DNA content, after a total of 24 h exposure to 5 µM CYN. In the controls, both progesterone and estradiol concentrations were correlated to protein synthesis (Figure 5-2) and maximum levels were seen after 24 h (Figure 5-3 & Figure 5-4). However, protein synthesis after 24 h exposure to 5 µM was almost abolished relative to the amount of protein present. Hence, it appears that inhibition of protein synthesis does not directly translate to complete inhibition of steroid hormone production over a 24 h time-frame.

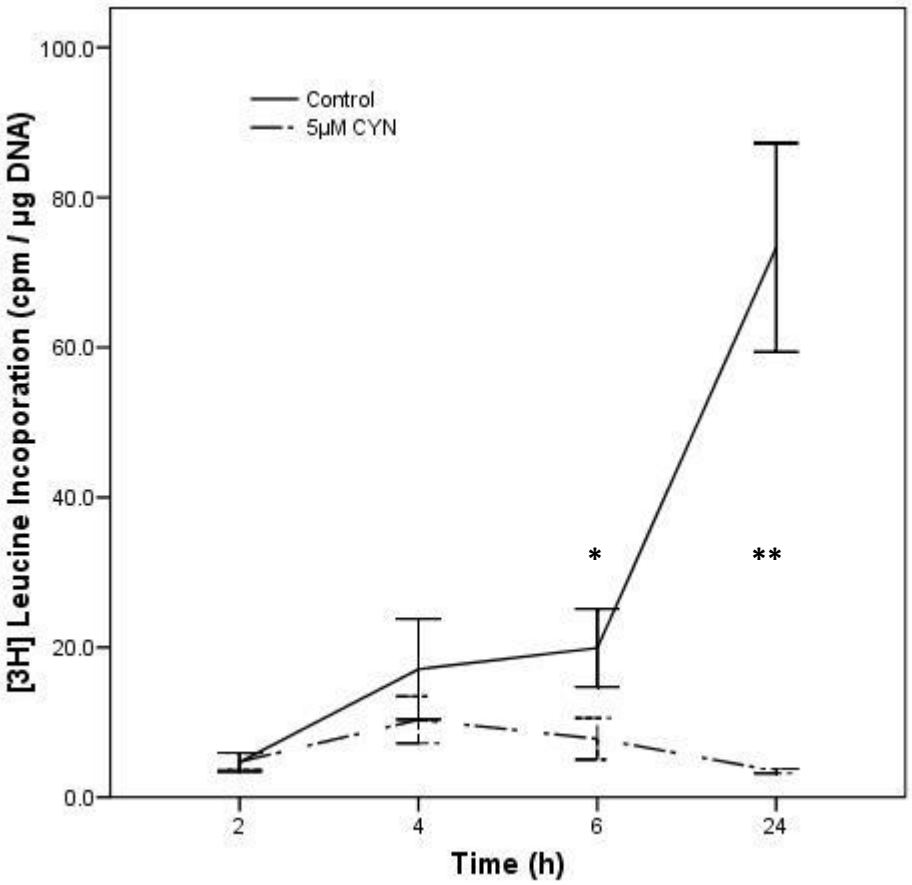


Figure 5-2: Inhibition of Protein Synthesis in Granulosa Cells

Granulosa cells (n=3 women) were seeded into 6-well plates (10^5 cells/well) in duplicate wells and were allowed to adhere for 24 h prior to treatment. Media were replenished with control (DMEM/F12+ 10% FCS + 1µCi [^{3}H] Leucine) or 5 µM CYN treatment (DMEM/F12+ 10% FCS + 5µM CYN + 1µCi [^{3}H] Leucine) for 2 h, 4 h, 6 h or 24 h. Cells were then collected for determination of protein synthesis as described in 5.3.4.1. Data values represent mean ± SEM, and significance was determined by Independent t-test and indicated by *P <0.05 and **P <0.005.

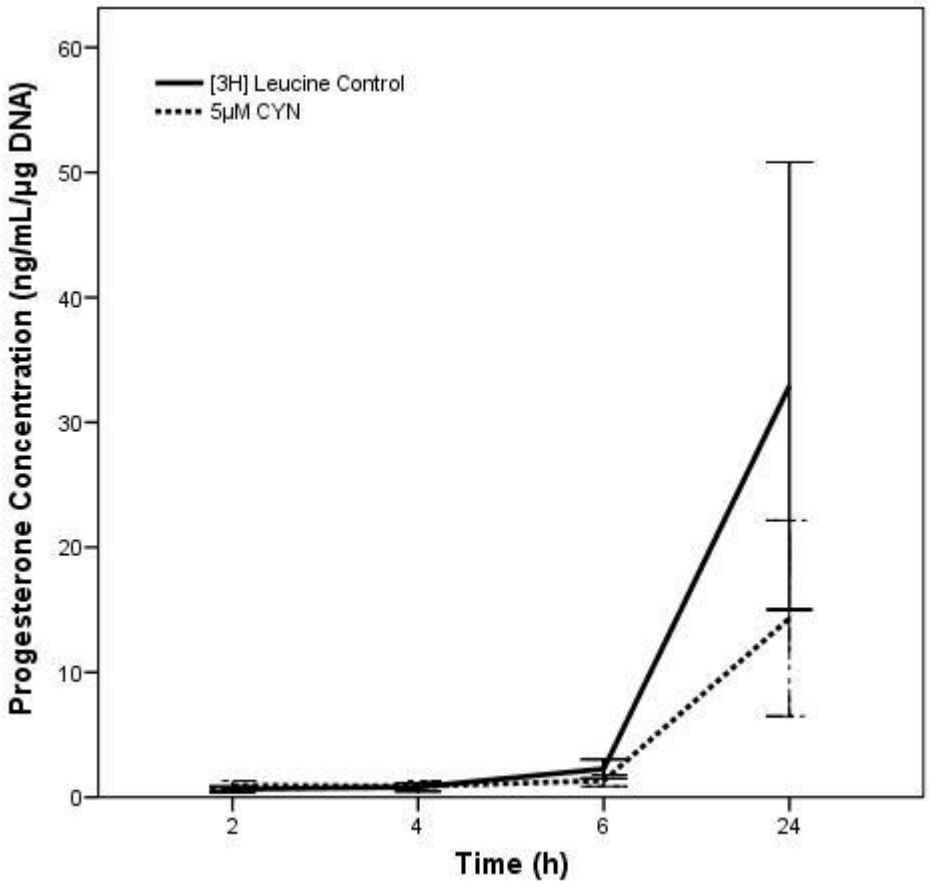


Figure 5-3: Progesterone Production by Granulosa Cells after Time Exposure to CYN

Granulosa cells (n=3 women) were seeded into 6-well plates (10^5 cells/well) in duplicate wells and were allowed to adhere for 24 h prior to treatment. Media were replenished with ^{3}H Leucine control (DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine) or 5µM CYN treatment (DMEM/F12+ 10% FCS + 5µM CYN + 1µCi [^{3}H] Leucine) for 2 h, 4 h, 6 h or 24 h. Media were collected after treatment and stored at -20°C for steroid hormone analysis by radioimmunoassay. Progesterone concentrations relative to amount of DNA (ng/mL/µg DNA) in duplicate wells of 3 separate assays were expressed as mean ± SEM.

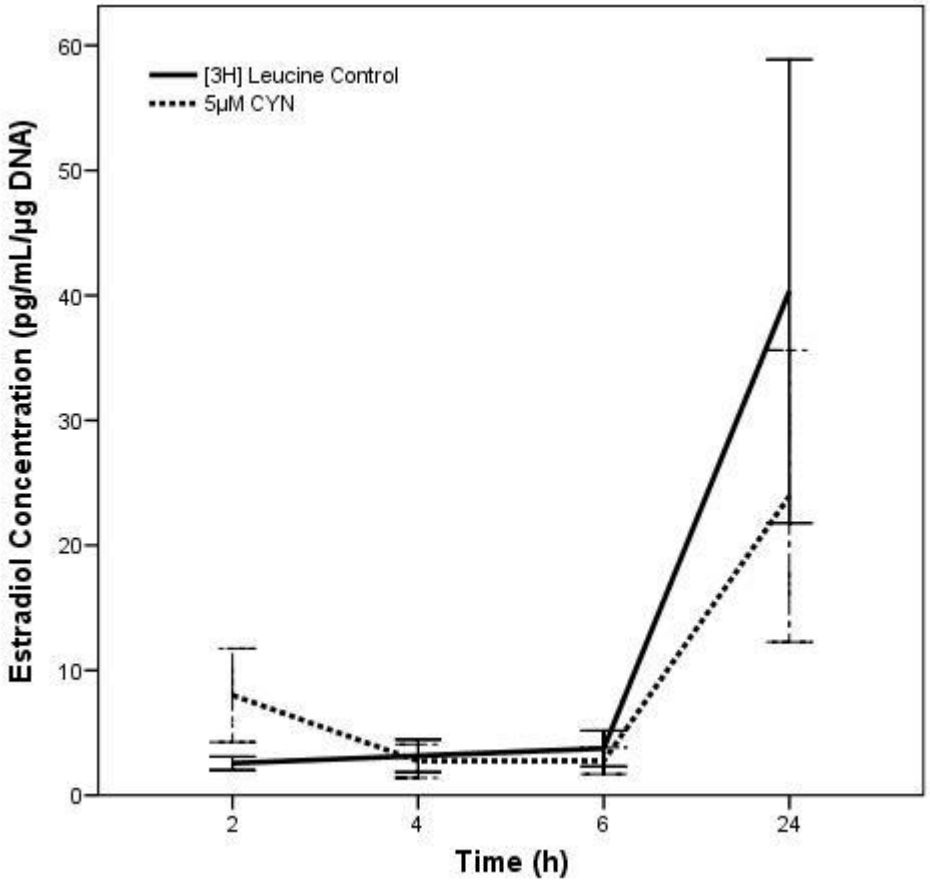


Figure 5-4: Estradiol Production by Granulosa Cells after Time Exposure to CYN

Granulosa cells (n=3 women) were seeded into 6-well plates (10^5 cells/well) in duplicate wells and were allowed to adhere for 24 h prior to treatment. Media were replenished with ^{3}H Leucine control (DMEM/F12+ 10% FCS + 1µCi [^{3}H] Leucine) or 5µM CYN treatment (DMEM/F12+ 10% FCS + 5µM CYN + 1µCi [^{3}H] Leucine) for 2 h, 4 h, 6 h or 24 h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. Estradiol concentrations expressed relative to amount of DNA (pg/mL/µg DNA) in duplicate wells of 3 separate assays are shown as mean ± SEM.

5.4.4 Effects of CYN on Protein Synthesis Inhibition In Primary-derived Human Granulosa Cell to Increasing CYN Concentrations

A 5 µM CYN concentration inhibited protein synthesis in GC after 6 h exposure (refer to section 5.4.2). Granulosa cells were then cultured for 6 h with increasing concentrations of CYN in the absence (Figure 5-5) or presence (Figure 5-6) of hCG stimulation to determine if the CYN-induced decrease in protein synthesis is dose-dependent and whether hCG modifies any effect of CYN. A decrease in protein synthesis occurred after 6 h exposure to the highest concentration of 3 µM CYN in comparison to control, in the unstimulated cells (P <0.05, Figure 5-5). There was no significant inhibition of protein synthesis by GC cultured with hCG after exposure to any of the CYN concentrations (Figure 5-6).

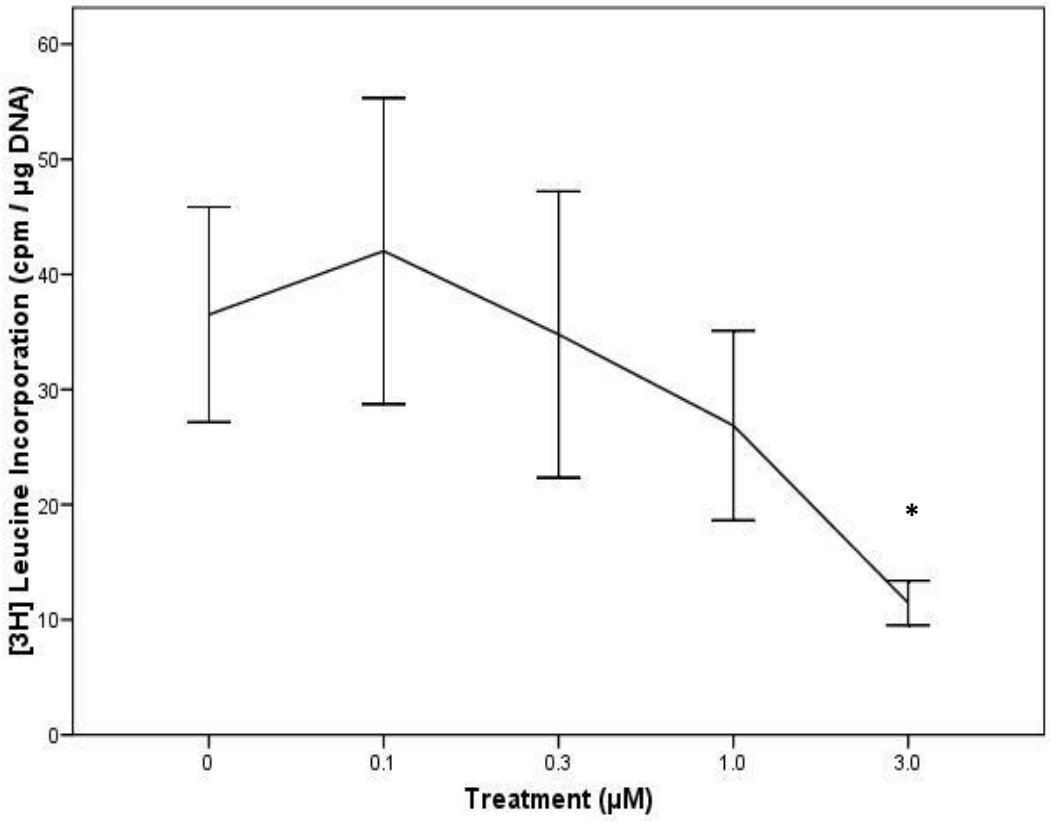


Figure 5-5: Protein Synthesis Inhibition in Granulosa Cells after Cylindrospermopsin Exposure for 6h

Granulosa cells (n=3) were exposed to the following treatments: control referred to as 0µM (DMEM/F12+ 10% FCS+ 1µCi [^{3}H] Leucine); 0.1-3.0 µM CYN treatments (DMEM/F12+ 10% FCS + 1µCi [^{3}H] Leucine + 1000mIU/mL hCG +CYN (0.1, 0.3, 1.0 or 3.0 µM) for 6h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. [^{3}H] Leucine incorporation was determined as detailed in 5.3.4. Data values represent mean ± SEM, and significance was determined by ANOVA and indicated by *P <0.05.

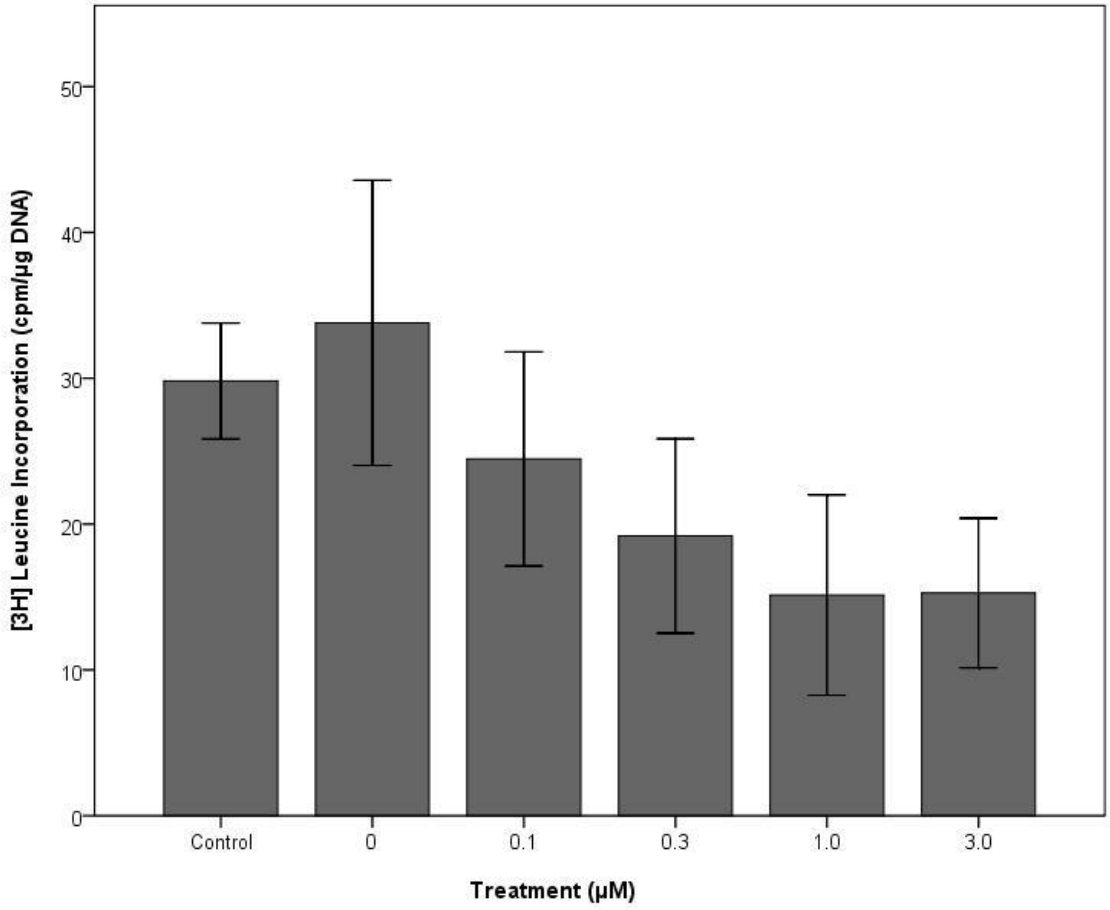


Figure 5-6: Protein Synthesis Inhibition after Exposure of hCG Stimulated Granulosa Cells to Cylindrospermopsin for 6h

10% FCS + 1µCi [^{3}H] Leucine; control with hCG referred to as 0µM CYN treatment (DMEM/F12+ 10% FCS + 1000 mIU/mL hCG + 1 µCi [^{3}H] Leucine); 0.1-3.0 µM CYN treatments (DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine + 1000 mIU/mL hCG +CYN (0.1, 0.3, 1.0 or 3.0 µM) for 6h. Media (n=3) was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. [^{3}H] Leucine incorporation was determined as detailed in 5.3.4. Bars represent mean ± SEM.

5.4.5 Effects of CYN on Steroid Hormone Production by Primary-derived Human Granulosa Cells to Increasing CYN Concentration

Protein synthesis by granulosa cells was inhibited by 6 h exposure to 3 µM CYN. There was no statistically significant inhibition of protein synthesis by granulosa cells in the presence of CYN and hCG (1000 $mIU.mL^{-1}$) (section 5.4.4, Figure 5-6). The effects of CYN on steroid hormone production with or without hCG stimulation were examined, (Figure 5-7 & Figure 5-8). Progesterone and estradiol concentrations were determined using radioimmunoassay and expressed relative to total genomic DNA. Granulosa cells stimulated with hCG in control wells significantly increased progesterone production (15.98 ± 4.3 $ng.mL^{-1}.\mu g^{-1}$ DNA) in comparison to un-stimulated granulosa cells (8.53 ± 1.5 $ng.mL^{-1}.\mu g^{-1}$ DNA). Progesterone production was not affected after 6 h exposure to increasing CYN concentrations ± hCG stimulation (Figure 5-7). Granulosa cells stimulated with hCG did not significantly increase estradiol synthesis in comparison to un-stimulated cells (Figure 5-8). In fact, estradiol produced by granulosa cells cultured in the presence of hCG in control wells was reduced and thus any effects due to CYN may have been masked (Figure 5-8). The decrease in estradiol concentrations (pg/mL/µg DNA) in unstimulated granulosa cells exposed to 6 h of CYN concentrations 0 – 3 µM appear to be dose-dependent, but not statistically significant (Figure 5-8).

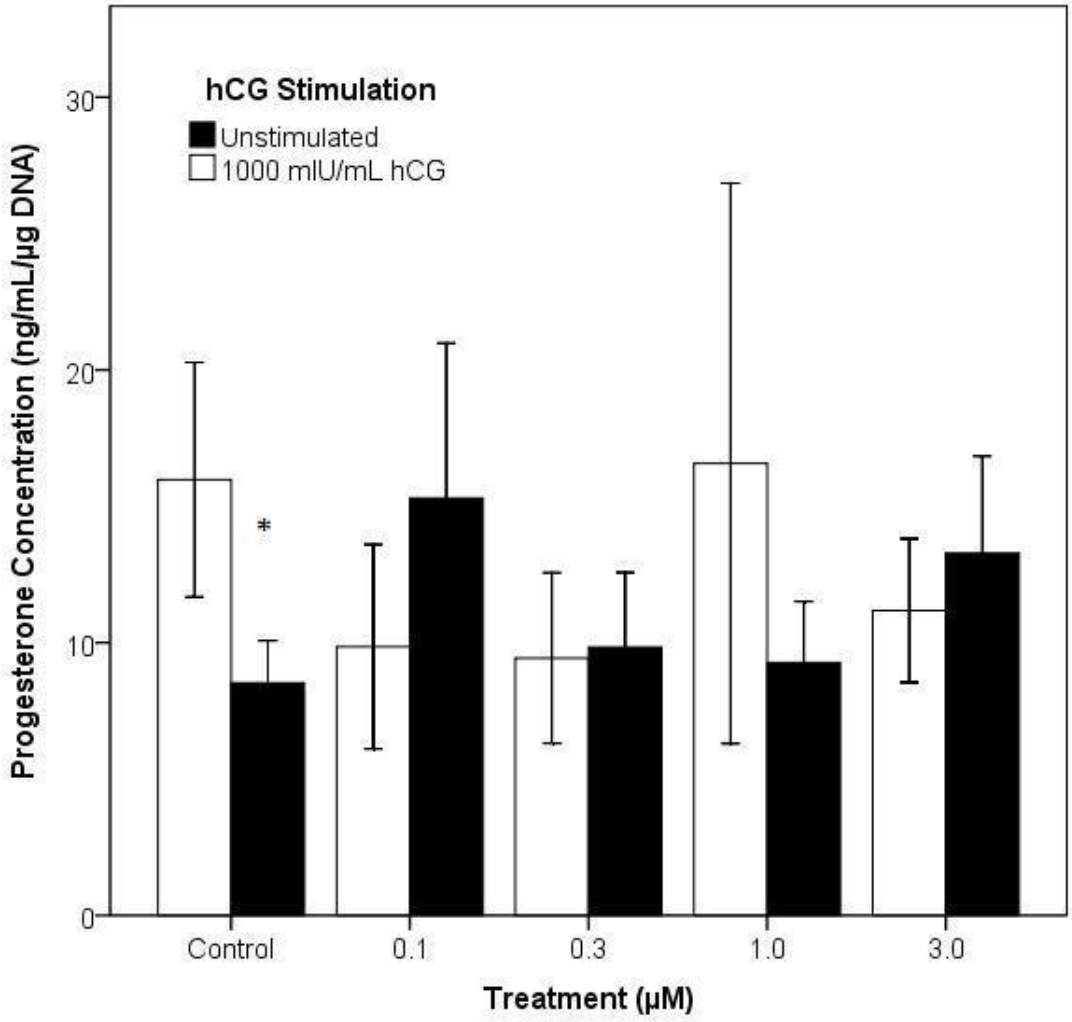


Figure 5-7: Progesterone Production by Granulosa Cells after CYN Exposure

Granulosa cells (n=3) were exposed the following treatments: control (DMEM/F12+ 10% FCS + 1000mIU/mL hCG + 1µCi [^{3}H] Leucine); 0.1-3.0 µM CYN treatments (DMEM/F12+ 10% FCS + 1 µCi [^{3}H] Leucine + 1000 mIU/mL hCG +CYN (0.1, 0.3, 1.0 or 3.0 µM) for 6h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. Progesterone concentrations relative to the amount of DNA (ng/mL/µg DNA) in duplicate wells of 3 separate assays was expressed as mean ± SEM. Significance indicated by *P <0.05 as determined by t-test.

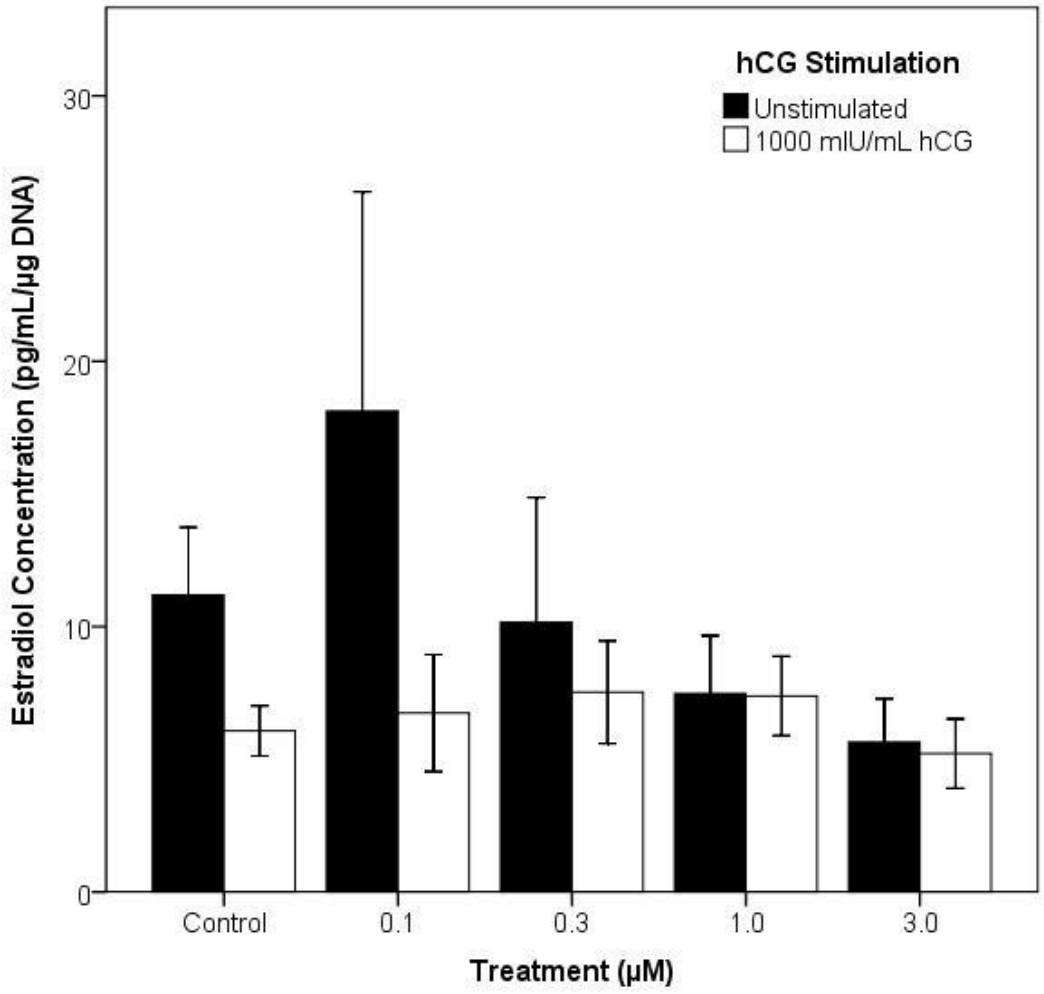


Figure 5-8: Estradiol Production by Granulosa Cells after CYN Exposure

Granulosa cells (n=3) were exposed the following treatments: control (DMEM/F12+ 10% FCS + 1000mIU/mL hCG + 1µCi [^{3}H] Leucine); 0.1-3.0 µM CYN treatments (DMEM/F12+ 10% FCS + 1µCi [^{3}H] Leucine + 1000mIU/mL hCG +CYN (0.1, 0.3, 1.0 or 3.0 µM) for 6h. Media was collected after treatment and stored at -20°C for steroid hormone analysis by RIA. Estradiol concentrations relative to the amount of DNA (pg/mL/µg DNA) in duplicate wells of 3 separate assays was expressed as mean ± SEM.

5.4.6 Effects of CYN on Semi-quantification of CYP11A1 Protein in Primary-derived Human Granulosa Cells

From these data, it appears that hCG increased granulosa cells sensitivity to protein synthesis inhibition, but there was no significant effect on steroid hormone production by 6 h exposure to 3 µM CYN. Studies suggest that primary-derived human granulosa cells regain sensitivity to hCG stimulation as a result of extended culture *in vitro* prior to treatment. Therefore, CYP11A1 protein levels and the correlation to steroid hormone production were examined after 24 or 48 h pre-treatment adherence periods. Granulosa cells were then exposed to 1000 $mIU.mL^{-1}$ hCG ± 3 µM CYN for 6 h after which CYP11A1 protein levels were detected by Western Immuno-blotting (Figure 5-9 A & B, section 5.3.6). A specific band of 53 kDa was observed, consistent with CYP11A1. The blot was stripped and re-probed with β-actin to act as a binding control. Semi-quantification of protein levels relative to β-actin show that CYP11A1 protein levels were highest in controls after 24 h adherence in DMEM/F12. This significantly decreased after 48 h adherence (P <0.05, Figure 5-10). After 24 h adherence, CYP11A1 protein levels significantly decreased after 6 h exposure to 3 µM CYN in comparison to 0 µM CYN control (P <0.05, Figure 5-10). CYN also appears to have caused a reduction in CYP11A1 protein after 48 h adherence, but this was not significant, probably due to the reduced levels in the 0 µM CYN treatment. After 24 h adherence followed by culture in hCG, CYP11A1 protein levels significantly decreased compared to the no hCG treatment, and were still lower after 48 h adherence (P <0.05, Figure 5-10). These effects of hCG were not altered by exposure to 3 µM CYN (Figure 5-10).

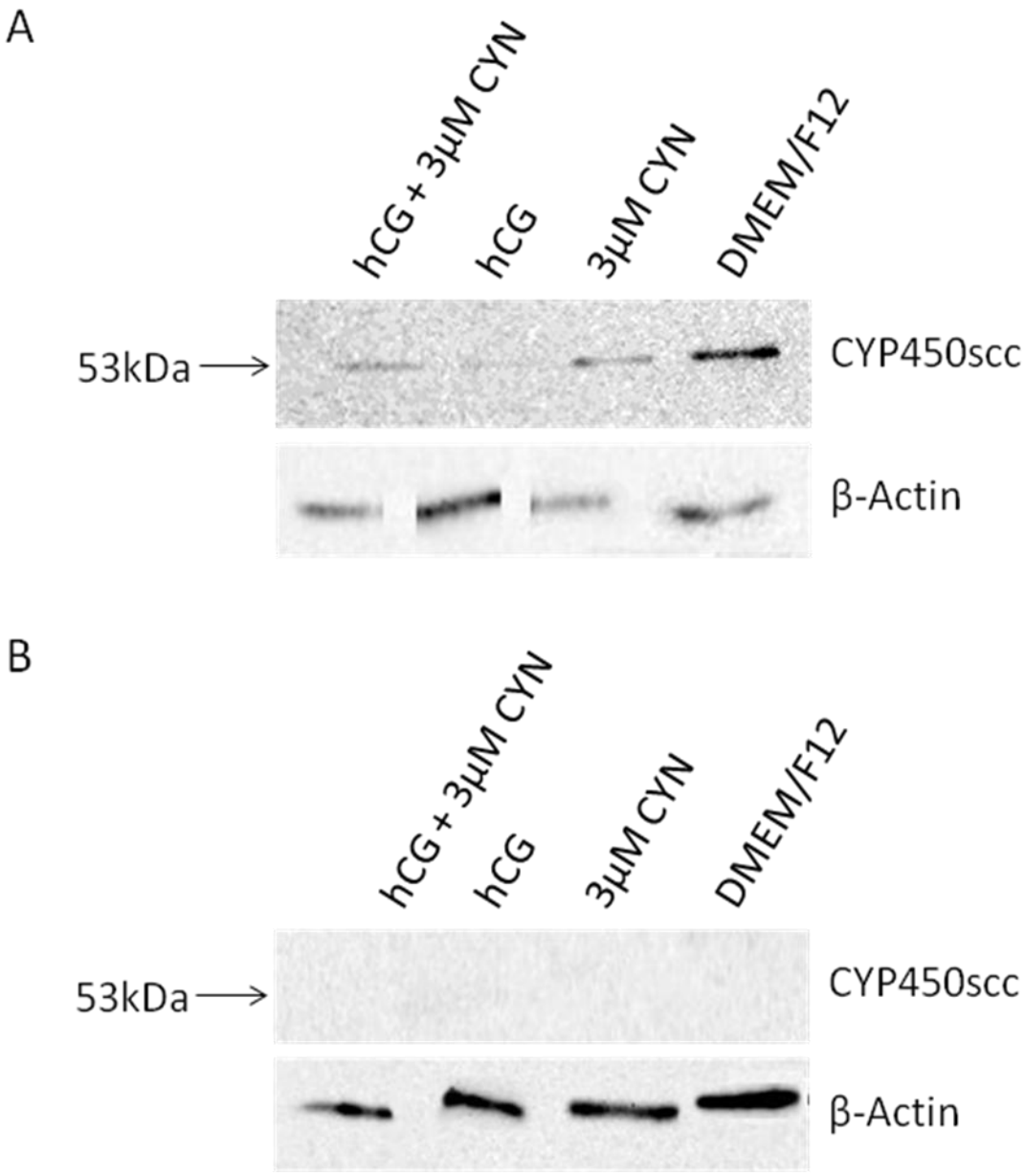


Figure 5-9: CYP11A1 Protein Detection in Granulosa Cells

Granulosa cells (n=3) were seeded into 24-well plates (2× 10^5concentration per well) and were allowed to adhere for either 24 h (A) or 48 h (B) in DMEM/F12 prior to treatment. Media post adherence time was collected and replenished with treatment medium DMEM/F12, 3 µM CYN, hCG or hCG + 3 µM CYN (DMEM/F12+ 10% FCS ± 1000mIU/mL hCG or DMEM/F12+ 10% FCS ± 3 µM CYN) for 6 h. Post-treatment, media was collected and stored at -20°C for RIA analysis of steroid hormones. Bands represent CYP11A1, 53kDa (1:750 of anti-human CYP11A1) and β-actin 43kDa.

The high variability in the 24 h adherence control group resulted in the decrease being non-significant in CYP11A1 protein levels after 48 h adherence. Similarly, the decrease in CYP11A1 protein after 24 h adherence with hCG stimulation was not significant when compared to control (Figure 5-10). In comparison to section 5.4.4, the presence of hCG either protected or masked the effect of CYN on granulosa cell CYP11A1 protein levels, as there was no difference between CYP11A1 protein levels in control wells and the CYN exposed wells in the 24 h adherence group (Figure 5-10). In un-stimulated granulosa cells exposed to 3 µM CYN there was a significant decrease in CYP11A1 protein levels in the 24 h adherence group when compared to the control (Figure 5-10).

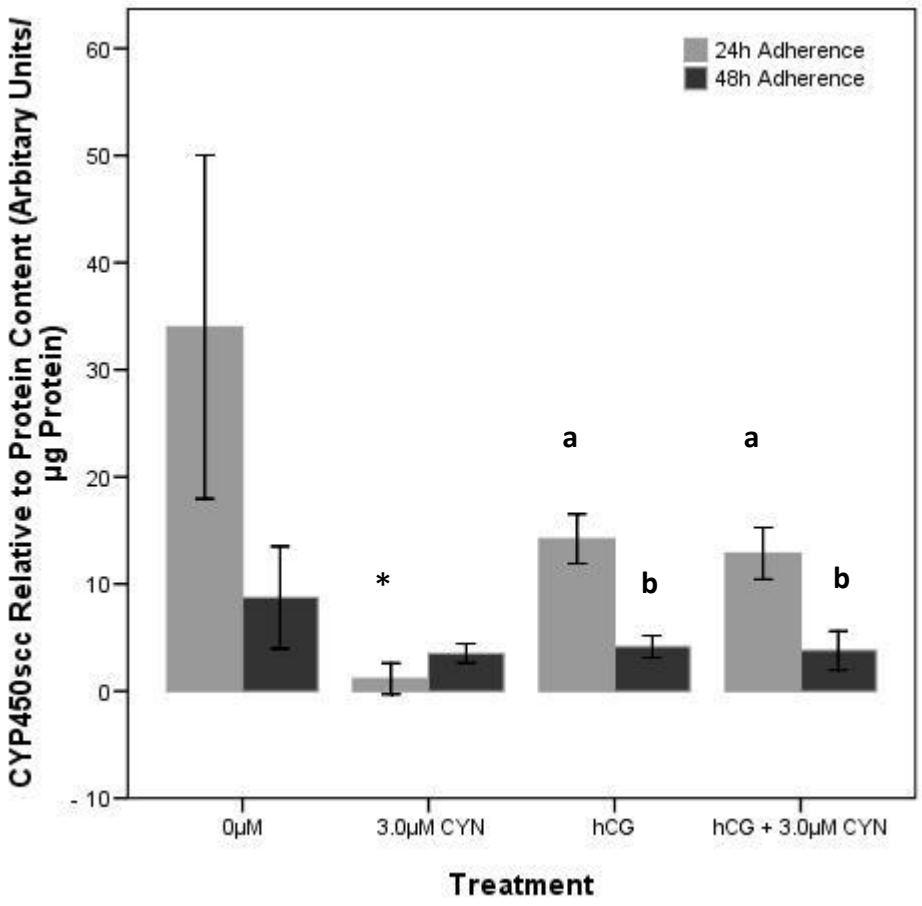


Figure 5-10: CYP11A1 relative to protein concentration

Granulosa cells (n=3) were seeded into 24-well plates (2× 10^5concentration per well) and were allowed to adhere for either for 24 h (A) and 48 h (B) in DMEM/F12 prior to treatment. Media post adherence time was collected and replenished with treatment medium DMEM/F12, 3µM CYN, hCG or hCG + 3µM CYN (DMEM/F12+ 10% FCS ± 1000mIU/mL hCG or DMEM/F12+ 10% FCS ± 3µM CYN) for 6h. Post-treatment, media was collected and stored at -20°C for RIA analysis of steroid hormones. Bars represent CYP11A1 protein band relative to total protein (Arbitrary Unit- Background/ mm^2/ µg protein) ± SEM, a and b *P* <0.05 are significantly between adherence periods, analysis carried out via Independent t-test. * *P* <0.05 was significantly to 0µM control with the same adherence. Analysis carried out via Independent One-way ANOVA.

5.4.7 Relationship between Steroid Hormone Production and CYP11A1 protein levels in Primary-derived Human Granulosa Cells after Exposure to CYN

It was observed that CYP11A1 protein levels change with time in culture prior to hCG stimulation and CYN exposure (section 5.4.6). This section investigates if the changes in CYP11A1 protein levels (24 or 48 h pre-treatment adherence) correlated to changes in steroid hormone production. Steroid hormones, progesterone and estradiol, were quantified from culture medium obtained after treatment exposure in section 5.4.6. The significant decrease in CYP11A1 protein levels after 6 h exposure to 3 µM CYN (24 h adherence) did not result in a significant change in either progesterone or estradiol production (Figure 5-11 & Figure 5-12). Progesterone production by granulosa cells allowed to adhere for 48 h followed by exposure to 0 or 3 µM CYN (Figure 5-11) increased after hCG stimulation (48 h adherence) and was unaffected by simultaneous exposure to 3 µM CYN. The increase in progesterone production was unrelated to CYP11A1 protein levels (Figure 5-11). These data indicate that increases in progesterone concentration may be independent of CYP11A1 protein levels and the rate of protein synthesis, at least over the 6 h time frame of these experiments.

Granulosa cells exposed to 3 µM CYN in the presence of hCG (24 h adherence) increased estradiol production relative to the amount of CYP11A1 protein levels and controls, but this was not observed after 48 h adherence (Figure 5-12).

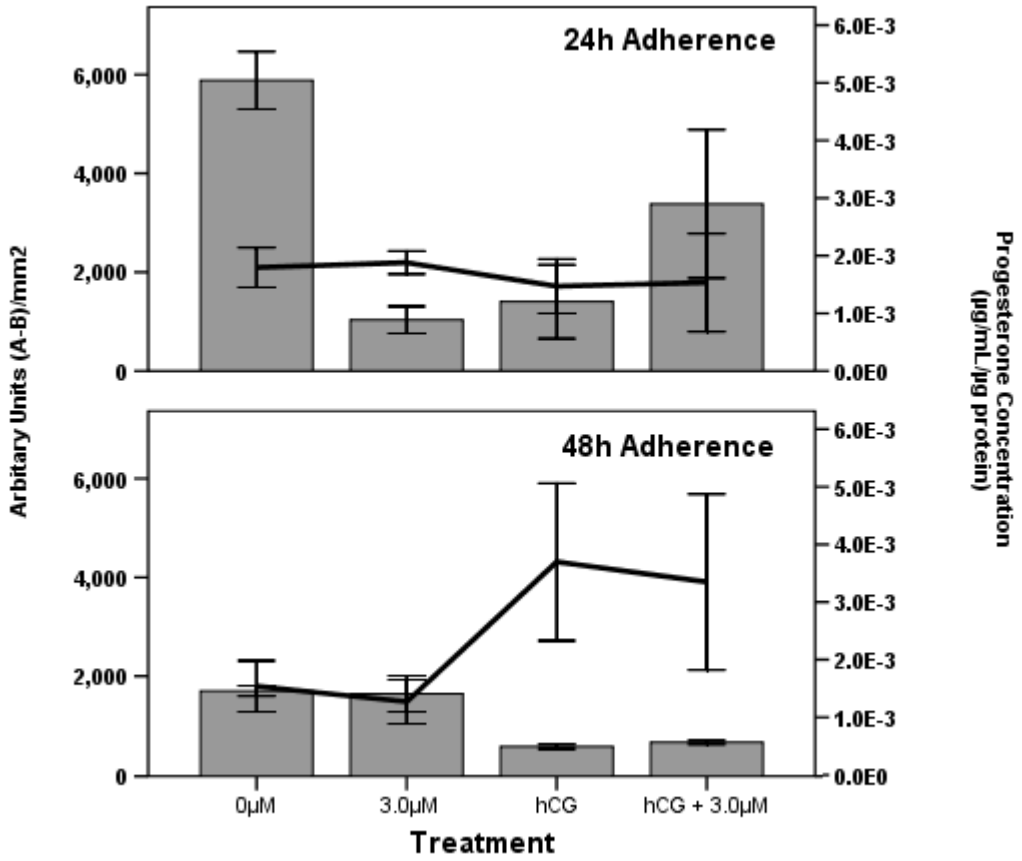


Figure 5-11: Progesterone Concentration (µg/mL/µg protein) relative to CYP11A1 levels in Granulosa Cells adhering for either 24 h or 48 h

Granulosa cells (n=3) were allowed to adhere for 24 h or 48 h prior to culture in DMEM/F12 (0 µM), 3µM CYN, hCG or hCG + 3µM CYN for 6 h. Bars represent CYP11A1 protein band relative to β-actin (Arbitrary Unit- Background/ mm^2) ± SEM. Line represents progesterone concentration (µg/mL/ µg protein) ± SEM.

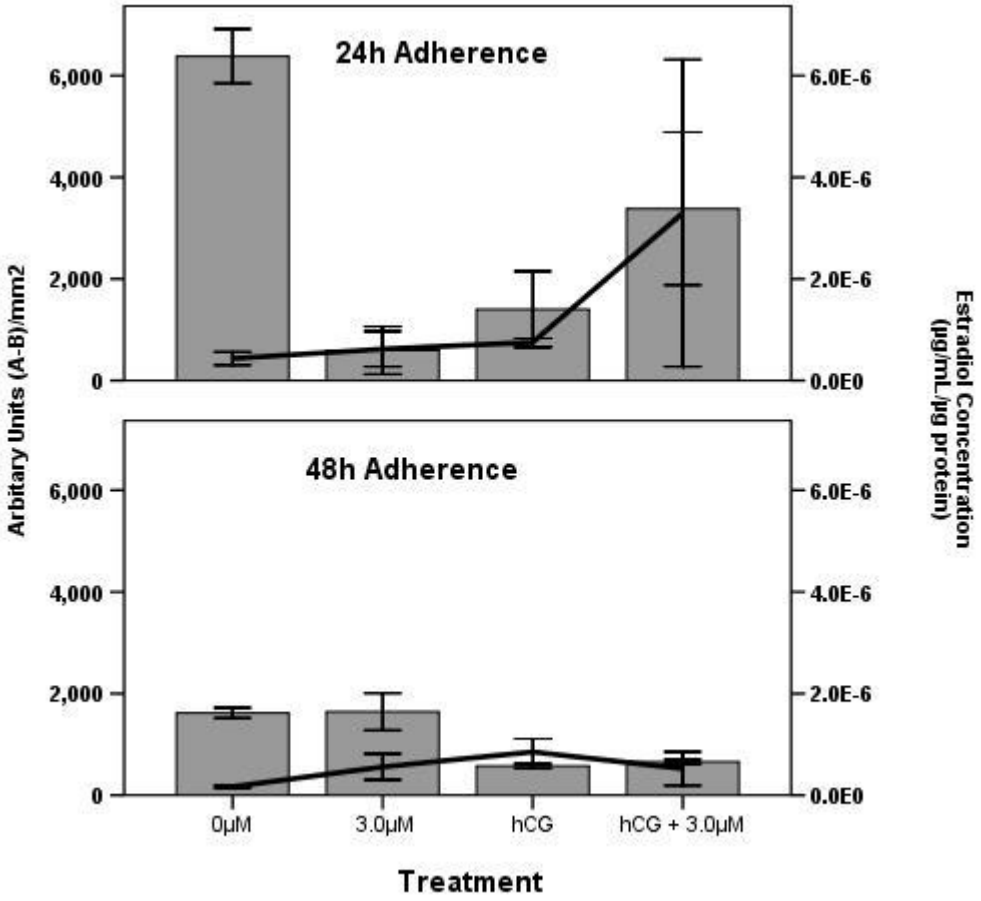


Figure 5-12: Estradiol Concentration (µg/mL/µg protein) relative to CYP11A1 levels in Granulosa Cells adhering for either 24 h or 48 h

Granulosa cells (n=3) were allowed to adhere for 24 h or 48 h prior to culture in DMEM/F12 (0 µM), 3 µM CYN, hCG or hCG + 3µM CYN for 6 h. Bars represent CYP11A1 protein band relative to β-actin (Arbitrary Unit- Background/ mm^2) ± SEM. Line represents estradiol concentration (µg/mL/ µg protein) ± SEM.

5.5 Discussion

Studies carried out in mouse hepatocytes found that exposure to 0.5 µM CYN for 4 h inhibited protein synthesis and that this was irreversible in mouse hepatocytes (Froscio et al. 2001; Froscio et al. 2003; Runnegar et al. 1994).

The GC used to study the effects of CYN on protein synthesis and CYP11A1 protein levels in this study, showed a 50% inhibition of protein synthesis after 6 h exposure to 5 µM CYN. This was a 10-fold higher concentration than was required to inhibit protein synthesis in primary mouse hepatocytes (Chong et al. 2002; Froscio et al. 2003; Runnegar et al. 1994), but the suppression occurred within a similar time frame. Protein synthesis was inhibited in GC prior to the onset of cytotoxicity as determined by MTT and Crystal Violet assays (which occurred after 48 h, section 4.4).

The decreases in steroid hormone production detected after 6 h exposure to 5 µM CYN were not statistically significant. Protein synthesis in granulosa cells was inhibited after 6 h exposure to 3 µM and the decrease in steroid hormone production was not of the same magnitude. Previous studies have also shown that decreases in steroid hormone production and protein synthesis in granulosa cells do not occur with the same magnitude (Stocco & Clark 1996). In Chapter 3, significant decreases in granulosa cell viability occurred after 48 h exposure to 0.1-3 µM CYN, while steroid hormone production was not affected until after 72 h exposure. The protein synthesis time-course experiments only covered a period of 24 h, since the yield of granulosa cells at isolation limited the amount of exposure periods which could be tested per experimental repeat. Hence, the experiments did not allow for the observation of similar decreases in steroid hormone production as observed in viability assays in Chapter 3. However, the available data suggests that steroid hormone production by granulosa cells were not directly affected by inhibition of protein synthesis, and that the decreases in steroid hormone production were a result of cytotoxicity.

Human chorionic gonadotropin (hCG) stimulates steroidogenesis and promotes the expression of CYP11A1 protein (Chaffin et al. 2000). In the study by Breckwoldt et

al. (1996) granulosa cells were cultured for 7 d to allow recovery from *in vivo* gonadotropin stimulation and regain sensitivity to further stimulation *in vitro*. Our data support the published literature in which the primary-derived granulosa cells required 24-72 h pre-treatment adherence time to differentiate and regain sensitivity to further gonadotropin stimulation. Granulosa cells used in the protein synthesis inhibition time-course and CYN dose-response experiments were responsive to hCG stimulation with significant increases in progesterone production in control wells (Figure 5-7). Progesterone concentrations after hCG stimulation (48 h adherence) increased (with a simultaneous decrease in estradiol concentrations) in comparison to hCG-stimulated cells after 24 h adherence (Figure 5-11) indicating a differentiated granulosa cell population.

In the CYN dose-response protein synthesis assays, a plateau in the inhibition of protein synthesis was reached after exposure to 1 µM CYN in the presence of hCG, whereas in unstimulated cells a dose-dependent decrease in protein synthesis was observed and significance reached after 6 h exposure to 3 µM CYN. The presence of hCG may have protected GC from protein synthesis inhibition after exposure to 3 µM CYN (Figure 5-10). However this is not certain, as hCG-stimulated cells at low CYN concentrations (0.1 and 0.3 µM CYN) decreased protein synthesis to levels lower than non-hCG stimulated granulosa cells. Young et al. (2008) found that granulosa cells exposed to 1µg.mL^{-1} of CYN (2.4 µM) had decreased hCG-stimulated progesterone production after 6 h exposure, with no effect on progesterone production in non-hCG responsive granulosa cells, which also seems to be the situation of our data. However, in order to determine whether cells were hCG non-responsive due to insufficient pre-treatment culture time, additional experiments where GC are cultured for 48 h prior to treatment need to be carried out for protein synthesis inhibition time-course studies.

After 48 h adherence the non-significant production of progesterone by the granulosa cells (cultured for in experiments to semi-quantify CYP11A1 protein levels) was not related to changes in CYP11A1 but rather due to the presence of hCG (hCG increases cAMP levels required for progesterone production which occurs during steroidogenesis refer to section 1.1.2). Hence granulosa cells require

a minimum of 24 h in culture prior to further *in vitro* gonadotropin stimulation cells, as aforementioned in this section, and further supports the literature that steroidogenesis is limited by StAR protein levels and not necessarily on CYP11A1 protein levels (Walsh et al. 2000). Thus further experiments investigating the levels of StAR protein are required.

The 3 µM CYN treatment did not have any effect on steroidogenesis after 6 h exposure. A minimum of 72 h exposure to 3 µM CYN was required to observe decreases in steroid hormone production (see Chapter 4, section 4.4.3). The non-significant decreases in steroid hormone production in protein synthesis assays were unlikely to be as a result of loss of cell viability due to PSI which is a slower process. Hence, loss of cell viability after 48 h would significantly decrease steroid hormone production by 72 h (see Chapter 4, section 4.4.3). *In vitro* culture of granulosa cells found CYP11A1 protein levels to be stable (Breckwoldt et al. 1996), and decreases in the protein levels of steroidogenic enzymes, such as 3βHSD are not necessarily immediately reflected by decreased steroid hormone production (Huang et al. 2004a). Studies have shown that although mRNA levels of 3βHSD and CYP11A1 can be up-regulated subsequent increases of protein levels of these two stable enzymes does not occur. Thus, changes in mRNA levels do not result in rapid changes to enzyme activity or protein level and hence mRNA levels are not a major factor in the control of steroid hormone production (Huang et al. 2004). Whereas acute response to hormonal stimulation (FSH, LH/hCG) results in stimulation of *de novo* protein synthesis, thus if PSI occurs, it may reduce the hormone-induced stimulation of steroidogenesis by inhibiting the translocation of cholesterol substrates into the inner mitochondrial membrane attributed to the presence of StAR and its functional activity (Stocco et al. 2005). Hence, the inhibition of protein synthesis may result of decreased ability for *de novo* StAR synthesis, and could decrease steroid hormone production.

Huang et al. (2004a) found that changes to protein levels of both CYP11A1 and 3βHSD are not necessarily reflected by immediate changes in enzyme activity or steroid hormone production and that protein levels cannot solely predict the effect on steroidogenic output, which was supported by our data, although limited by the

0-24 h exposure period examined. Whether the decreases in steroid hormone production were a result of decreased activity due to the targeted effects of CYN on the dehydrogenases or CYP enzymes needs to be determined. However, it is more likely that decreased steroid hormone production is related to onset of cell death.

Exposure to 5 µM CYN significantly inhibited protein synthesis and caused a decrease in steroid hormone biosynthesis, and so further experiments were carried out to observe if protein levels of CYP11A1 would also be significantly decreased. To determine if the decrease in steroid hormone production was a result of altered protein expression of CYP11A1, granulosa cells were cultured in presence of 3 µM CYN for 6 h with or without hCG stimulation after adherence periods of 24 h or 48 h. The results from our research showed the strongest detection of CYP11A1 after 24 h adherence without hCG or CYN exposure. Decreases in steroidogenesis could not be correlated to decreased protein levels of CYP11A1; this supported our investigations as CYP11A1 protein levels remained stable in culture (Breckwoldt et al. 1996). It was most likely that after 48 h adherence GC seem to require hCG stimulation to maintain progesterone production, supporting that desensitisation of GC to *in vivo* stimulation occurs between 24 and 72 h culture (Breckwoldt et al. 1996; Edgar et al. 1991; Landefeld et al. 1997).

5.6 Conclusion

The granulosa cells in this chapter were classified as healthy. Studies have shown that CYP11A1 protein levels and decreases in steroid hormone production are not correlated to healthy and unhealthy granulosa cells (Fatum et al. 2009). In summary, CYN-induced protein synthesis inhibition occurred at higher concentrations and required longer exposure periods in GC compared to mouse hepatocytes. This may be due to reduced uptake capability or metabolic activity of GC (Froscio et al. 2009). The role of hCG in protecting granulosa cells or increases the sensitivity of granulosa cells to CYN needs to be further investigated. Other studies have found that in the presence of hCG, CYP11A1 protein levels remained constant in prolonged culture of primary-derived granulosa cells despite apoptosis occurring simultaneously, this was not observed in our results (Breckwoldt et al. 1996). The effects of CYN on steroidogenesis could not be determined due to high

biological variability in addition to the selected time course was too short to detect significant effects particularly in Chapter 4 where significant inhibition of steroid hormone production occurred after 72 h. Further experiments are needed to determine if hCG and CYN function in unison to increase intracellular cAMP, possibly committing GC to either steroidogenesis, apoptosis or both in addition to inhibiting protein synthesis and if StAR protein levels or activity is affected (Amsterdam et al. 1999).

Chapter 6. The Effects of the Blue-Green Algal Toxin Cylindrospermopsin on Human Spermatozoa

Preliminary data suggested CYN was cytotoxic to cryopreserved and fresh spermatozoa as measured by the MTT assay, indicating an alternate, yet to be identified mechanism of CYN action. This chapter aims to further examine if the previous results from investigations carried on the effects of CYN on human mature spermatozoa are verifiable.

6.1 Introduction

A normal healthy semen sample should be a minimum 2mL in volume and consist of a minimum of 2×10^7 sperm.mL^{-1} with 50% having progressive motility and less than 25% abnormal morphology (WHO 1999). Human spermatozoa have cell membranes that are densely packed with phospholipids, which are highly susceptible to oxidation by toxins (Aitken et al. 1998; Claassens et al. 2000; De Jonge et al. 2003). The World Health Organisation (WHO) guideline for determining human spermatozoa viability recommends the Eosin Y Exclusion assay, on its own or in a two-step process with nigrosin, and this method is routinely used in examination of semen samples in IVF laboratories (Björndahl et al. 2004; Eliasson & Treichl 1971; WHO 1999). The MTT assay is dependent on the reduction of (3-[4,5-dimethyl (thiazole-2-yl-3,5-diphenyl] tetrazolium bromide) a yellow soluble tetrazolium dye in the mitochondria of live cells (Arnould et al. 1990; Carmichael et al. 1987; Mosmann 1983; Mueller et al. 2004; Petty et al. 1995; Young et al. 2005). Two independent studies have reported the metabolism of MTT by spermatozoa; the first involved observation of human spermatozoa under light microscopy to visualise the formazan crystals (Nasr-Esfahani et al. 2002). The second study showed that equine sperm also successfully metabolised the MTT salt (Aziz et al. 2005). As spermatozoa are densely packed with mitochondria to fuel their motility the MTT and MTS assays were trialled to determine if they could be used for human spermatozoa. The MTT and MTS assays are indirect measures of

glucose metabolism and oxidative phosphorylation required to generate the energy molecule ATP (Folgero et al. 1993; Murdoch & White 1967). Viable spermatozoa with functional mitochondria generate ATP, whereas the ATP from dead or dying cells is rapidly degenerated (Mueller et al. 2004; Petty et al. 1995). The tetrazolium-based assays and ATPLite assay (Perkin-Elemer) generate a control linear standard plot with the number of viable cells and formazan production or intensity of ATP-dependent luminescence, respectively (Mueller et al. 2004; Sevin et al. 1988).

Many drugs, chemicals and xenobiotics in our environment have been shown to directly affect the function and viability of human spermatozoa (Baumgartner et al. 2009; Fraser et al. 2006). A specific study on male patients seeking treatment for their infertility in south eastern Spain, have found that their infertility was attributed to occupational exposure to glues, solvents and silicones (Mendiola et al. 2008). Environmental xenobiotics and toxins have been shown to directly affect the function and viability of human spermatozoa (Baumgartner et al. 2009; Fraser et al. 2006). The viability of spermatozoa is commonly directly related to their motility; however non-motile spermatozoa can also be considered viable (Aitken & Baker 2006; Björndahl et al. 2004; Eliasson & Treichl 1971).

The cytotoxicity of CYN as detailed in section 1.7 suggests that it may have reproductive effects and currently there is no information available about the effects of CYN on male reproduction. CYN has the potential to reach spermatozoa as it has similar properties to nicotine, a volatile water-soluble alkaloid, which has been detected in the seminal fluid (Benowitz et al. 1982; Nakajima et al. 1998; Pacifici et al. 1995). The primary mechanism for the reduction of spermatozoa viability occurs when their membrane integrity is compromised via oxidation (Aitken et al. 2004; Pacifici et al. 1995; Wagner et al. 1990; Yousef et al. 2007). Mature spermatozoa lack transcriptional activity and active CYP enzymes, hence CYN-mediated inhibition of protein synthesis and the generation of CYP metabolites are unlikely in spermatozoa (Grootegoed et al. 2000; Hecht 1998). There is a possibility that CYN may affect viability of spermatozoa despite the inconclusive evidence to pinpoint the type of oxidative stress induced by CYN (Bain et al. 2007), and not

correlated with increased lipid peroxidation (Humpage et al. 2005; Runnegar et al. 1995).

6.2 Chapter Aims and Objectives

This study aims to establish methods appropriate for determining spermatozoa viability and investigating the effects of CYN on the viability of mature human spermatozoa. See Figure 6-1: Flow Diagram Chapter Objectives.

Hypotheses tested in this chapter were:

- Human mature spermatozoa are densely packed with mitochondria and as such will reduce the tetrazolium salt to produce formazan which is linearly related to spermatozoa number in the MTT and MTS assay. Thus this assay can be used as a viability assay.

- The primary energy molecule in human spermatozoa, ATP, will be quantified and linearly related to number of viable spermatozoa and so can be used as a viability assay.

- CYN will not adversely affect human mature spermatozoa as they lack active CYP enzymes and do not have active protein synthesis.

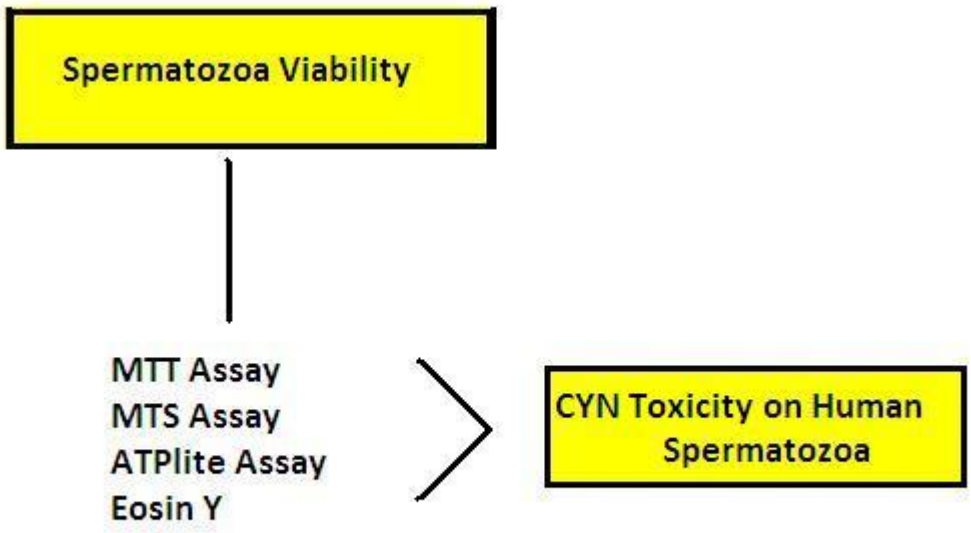


Figure 6-1: Flow Diagram Chapter Objectives

6.3 Materials and Methods

All chemicals and reagents used were obtained from SIGMA Aldrich and were of cell culture grade. Two types of human spermatozoa culture media were used; Quinns Advantage Medium with Hepes (QAHM's, SAGE Media In-Vitro Fertilisation Inc, CT, USA) and Hams F12 (SIGMA, St Louis, MO, USA). Hydrogen Peroxide (30% v/v, Aldrich, USA) was used as a positive control. Media were supplemented with 5% human serum albumin (HSA, Vitro-life). Media reagents are detailed in Reagents, Stock Solutions & Media Preparation.

6.3.1 Cylindrospermopsin

Refer to section 2.7 Cylindrospermopsin for details on the source of CYN. Serial dilutions were made in QAHM's supplemented with 5% HSA and to give final concentrations of 0.1-100 µM. Stock solutions were stored at -20°C in the dark. Refer to 2.7.1 Cylindrospermopsin Treatments, Table 2-5 for CYN treatment details.

6.3.2 Motility

Spermatozoa samples were subjected to a motility analysis (section 2.8.6) prior to use in experimental assays. Samples with less than 20% motile spermatozoa were not used in experiments.

6.3.3 Eosin Y Assay

A one-step Eosin-Y method was used to determine the number of viable spermatozoa remaining after exposure to CYN, section 2.8.5.

6.3.4 MTT and MTS Assay

Spermatozoa were added to a sterile 96-well plate (Nunc, In Vitro, Australia) at concentrations ranging from 0 to 5 × 10^5sperm/well in triplicate wells and incubated for 2 h or 24 h in either QAHM's + 5% HSA or HamsF12 medium + 5% HSA. MTT (3-[4, 5-dimethyl (thiazole-2-yl-3, 5-diphenyl] tetrazolium bromide), was added to give a final concentration of 0.5 mg.mL^{-1} in 100 µL total volume. If incubated with QAHM's medium, plates were left at room temperature in the laminar flow to maintain sterile conditions and pH of 7.4. If HamsF12 medium was

used, plates were placed in a humidified incubator at 37°C + 5% CO_2. The resultant formazan crystals were solubilised by the addition of 80 µL of 20% SDS in 0.02 M HCl and incubated in the dark at room temperature for 1 h. Optical density of formazan at a wavelength of 570 nm using an automatic plate reader (µQuant BioTEK Instruments Inc, VT, USA, with KCJunior Program). This was repeated for the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium; 0.32 $mg.mL^{-1}$) assay in both types of mediums following manufacturer's instructions (Promega 2003).

Incubation periods of 2, 4 or 24 h were examined. The formazan salt produced by MTS does not need solubilisation as is required for the MTT assay. Standard curve plates identical to that of the MTT assay were setup, the amount of MTS reduction and subsequent formazan production by spermatozoa was determined using the plate reader to measure absorbance at 490 nm. For both assays a linear relationship was taken to be confirmed if the R^2 value $\geq$ 0.98 correlating formazan production to spermatozoa concentration.

6.3.5 ATPLite Assay

The ATPLite assay utilises a luciferase system where ATP present within a sample supports the generation of luminescence which was quantified and linearly related to cell number. The assay was carried out as detailed in section 2.8.3 ATP Assay. In order to relate CPS to the number of live spermatozoa, a standard curve of 0 – 5×10^5 sperm/mL in triplicate wells was setup alongside treatments. A linear relationship between CPS and viable spermatozoa number was accepted at an R^2 value of greater than or equal to 0.98. The number of viable spermatozoa was expressed as 10^6 live $sperm.mL^{-1}$ as is the norm.

6.3.6 Collection and Preparation of Human Spermatozoa

Fresh and cryopreserved semen samples were donated (ethics approval 47/00) by men undergoing assisted reproductive technology at Flinders Reproductive Medicine Unit, Flinders Medical Centre, undergoing *in vitro* fertilisation (IVF) attributed to FIF. Cryopreserved samples (n=3 different men) were thawed at room temperature for 10 min, transferred to 5 mL sterile plastic tubes and cryoprotectant

media was removed by adding QAHM's medium + 5% HSA and centrifuging at 300 × *g* for 10 min. Spermatozoa were separated from the semen and motile spermatozoa were selected using PureCeption™ (SAGE BioPharma, In-Vitro Fertilisation Inc, CT, USA) silica bead discontinuous density gradient consisting of a 80% gradient layer overlaid with a 40% layer made in QAHM's + 5% HSA. Samples were loaded onto the column and centrifuged for 20 min at 300 × *g*. The non-viable spermatozoa layer at the 40%:80% interface was removed and the remaining pellet was collected and resuspended in QAHM's + 5% HSA, then sperm were centrifuged at 250 × *g* for 8 min to remove any residual PureCeption ™. Sperm viability and motility were evaluated. A minimum of 1×10^5 sperm/well were exposed to CYN. Fresh semen (total n=9 different men) was collected by masturbation into sterile containers and samples were allowed to liquefy at room temperature for 30 min prior to processing as for cryopreserved spermatozoa.

6.3.7 Selection of Culture Medium

In order to determine the optimum culture medium for human spermatozoa, 1×10^6 sperm/well were cultured in a 24-well plate for 1 or 24 h in QAHM's or Hams F12 + 5% HSA ± 1000 µg.mL^{-1} hydrogen peroxide (H_2O_2), which was used as a positive toxicity control. Spermatozoa in QAHM's medium were incubated at room temperature in the laminar flow and spermatozoa in HamsF12 were incubated at 37°C + 5% CO_2. Viable spermatozoa were determined by Eosin Y Exclusion assay (section 2.8.5).

6.3.8 Exposure of Fresh and Cryopreserved Human Spermatozoa to CYN

The effect of CYN on spermatozoa viability was investigated. Fresh and cryopreserved spermatozoa (n=3 respectively; total of 6 participants) were added to 24-well sterile plates in concentrations of 1×10^5 sperm/well (*InVitro*, Nunc Australia) and exposed to CYN (0, 0.1, 0.3, 1, 3 µM) or 1000 µg.mL^{-1} of H_2O_2 for 15 min, 90 min, 4 h, 6 h or 24 h. Each treatment was conducted in duplicate wells. Viability was determined by Eosin Y Exclusion assay (refer to section 2.8.5). In a separate experiment, fresh spermatozoa (n=3) were exposed to CYN (0.1, 0.3, 1. 3, 10 or 30 µM) for periods of 15 min, 4 h, 6 h or 24 h in triplicate wells of a 96-well luminescence plate (*InVitro* Nunc Australia), with a corresponding standard curve

of 0 to 5 × 10^5 spermatozoa/well and viability was determined by the ATPLite assay (PerkinElemer).

6.3.9 Statistical Analysis

Fresh spermatozoa viability determined by the ATP assay after various exposure periods to CYN was initially analysed by a one-way analysis of variance (one-way ANOVA); if analysis showed a significant interaction effect ($P \leq 0.05$) between CYN concentrations and exposure periods a follow-up analysis was conducted in which data were split into exposure period and viability in the CYN concentrations was analysed by a Tukey post-hoc test to compare means of different treatments against the control. *P* values $\leq$0.05 were accepted as significant. All viability data obtained from the Eosin Y exclusion assay were analysed by one-way ANOVA. *P* values $\leq$0.05 were accepted as significant. Linear regression was used to determine correlation between absorbance and spermatozoa number. Statistical Package for the Social Sciences (SPSS, v15.0) was used to undertake analyses. Data in graphs represent means ± 1 SEM.

6.4 Results

6.4.1 Comparison of Culture Media for Human Spermatozoa

Spermatozoa were cultured in both Hepes buffered QAHMs medium and sodium bicarbonate buffered HamsF12 medium for 1 or 24 h ± 1000 µg.mL^{-1} hydrogen peroxide, and number of viable spermatozoa were assessed by Eosin Y Exclusion assay. Spermatozoa viability was maintained for 24 h in QAHMs medium but declined significantly in HamsF12 (Figure 6-2, P <0.05). The exposure of spermatozoa to 1000 µg.mL^{-1} hydrogen peroxide for 1 h did not significantly reduce the numbers of viable spermatozoa in either medium, however after 24 h of exposure there was a significant decrease in viability in HamsF12 (P <0.05) and a similar but not statistically significant decrease in QAHMs when compared to their respective medium only controls.

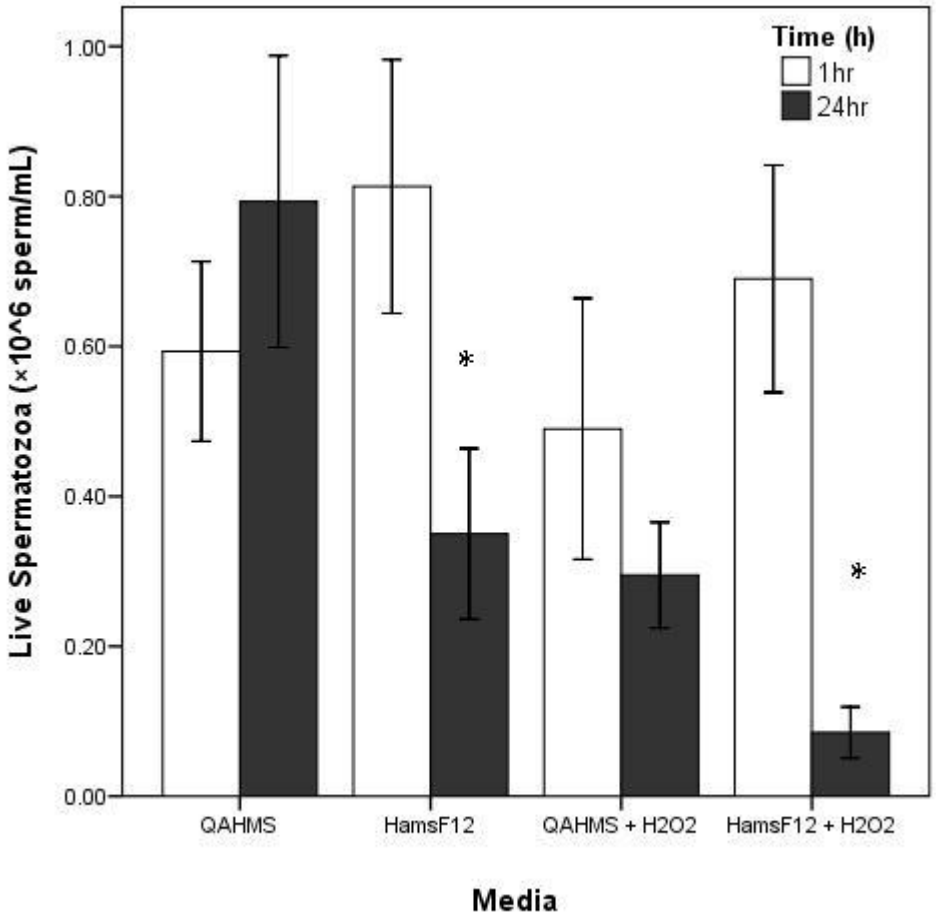


Figure 6-2: The Viability of Human Spermatozoa in Different Culture Media

Fresh human spermatozoa (n=3) were cultured in Hepes buffered (QAHMs) or sodium bicarbonate buffered media (HamsF12) media ± 1000 µg.mL^{-1} hydrogen peroxide for 1 h or 24 h. Live spermatozoa numbers were determined by the Eosin Y exclusion assay. Significance indicated by *P <0.05 as determined by one-way repeated measures ANOVA, bars represent mean ± 1 SEM.

6.4.2 Tetrazolium- Viability Assays for Assessment of Human Spermatozoa Viability

A concentration range of 0 to 1×10^5 spermatozoa incubated with 0.5 mg mL^{-1} MTT or 0.32 $mg.mL^{-1}$ MTS for 2 h did not generate a linear relationship between spermatozoa concentration and formazan production in either QAHMs (MTT, R^2=0.01; MTS, R^2=0.06) or HamsF12 (MTT, R^2=0.12; MTS, R^2=0.072) medium (Figure 6-3: upper and lower panels respectively). There was no difference in optical density after 2 h between wells containing spermatozoa and control wells containing no spermatozoa in either the MTT or MTS assay, possibly due to the high background (Figure 6-3, upper panel and lower panels respectively). In comparison to granulosa cells, the optical density in control wells of the MTT assay of HamsF12 and QAHMS media were similar. Increasing the incubation time with MTT or MTS to 24 h did not generate a linear relationship between spermatozoa concentration and optical density in QAHMs (MTT, r^2=0.10; MTS r^2=0.43) or HamsF12 (MTT, r^2=0.006; MTS, r^2=0.08) (Figure 6-4, upper and lower panels respectively).

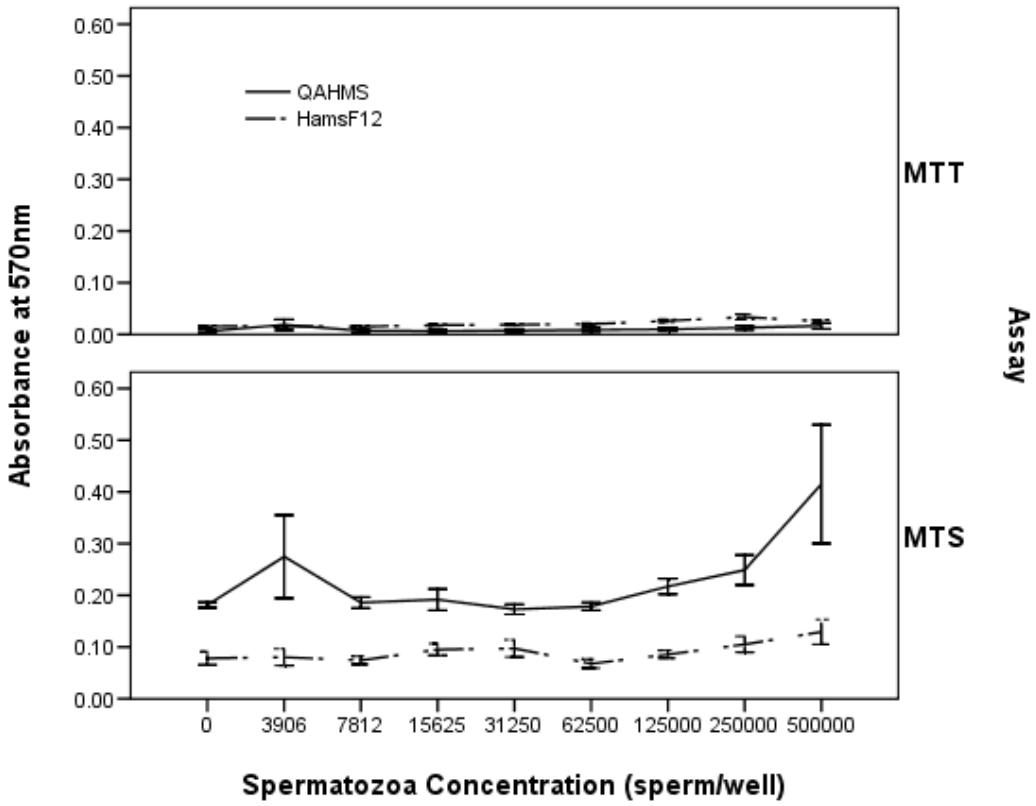


Figure 6-3: MTT and MTS conversion to formazan by Human Spermatozoa after 2 h

Fresh human spermatozoa (n=3) were cultured in Hepes buffered (QAHMs) or sodium bicarbonate buffered media (HAMs-F12) media with 0.5mg.mL^{-1} MTT (upper panel) or 0.32 mg.mL^{-1} MTS (lower panel) for 2 h. Spermatozoa incubated with MTT were lysed with 20% SDS in 0.02M HCl for 1hr in the dark at room temperature and absorbance of formazan at 570nm was plotted against spermatozoa number. Production of formazan production by spermatozoa incubated with MTS was assessed at 490nm, with no solubilisation step required. Error bars represent mean ± 1 SEM.

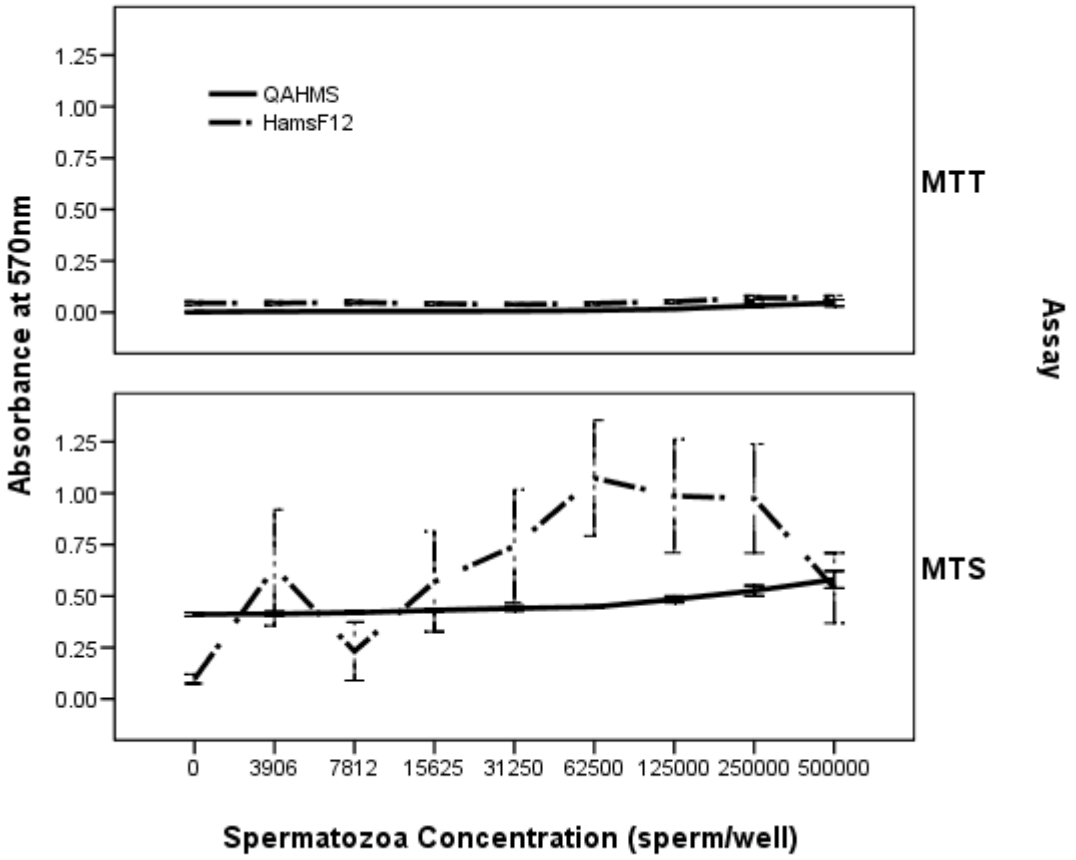


Figure 6-4: Conversion of MTT and MTS to formazan by human spermatozoa after 24 h

Fresh human spermatozoa (n=3) were cultured in Hepes buffered (QAHMs) or sodium bicarbonate buffered media (HAMs-F12) media with 0.5mg.mL^{-1} MTT (upper panel) or 0.32 mg.mL^{-1} MTS (lower panel) for 24 h. Spermatozoa incubated with MTT were lysed with 20% SDS in 0.02M HCl for 1hr in the dark at room temperature and absorbance of formazan at 570nm was plotted against spermatozoa number. Production of formazan by spermatozoa incubated with MTS was at determined 490nm, with no solubilisation step required. Error bars represent mean ± 1 SEM.

6.4.3 ATPLite Viability Assay for Assessment of Human Spermatozoa Viability

The ATPLite assay generated a linear relationship between luminescence (CPS) and number of spermatozoa (0 to 1×10^5 spermatozoa/well) cultured in QAHMs medium, R^2=0.98 (Figure 6-5).

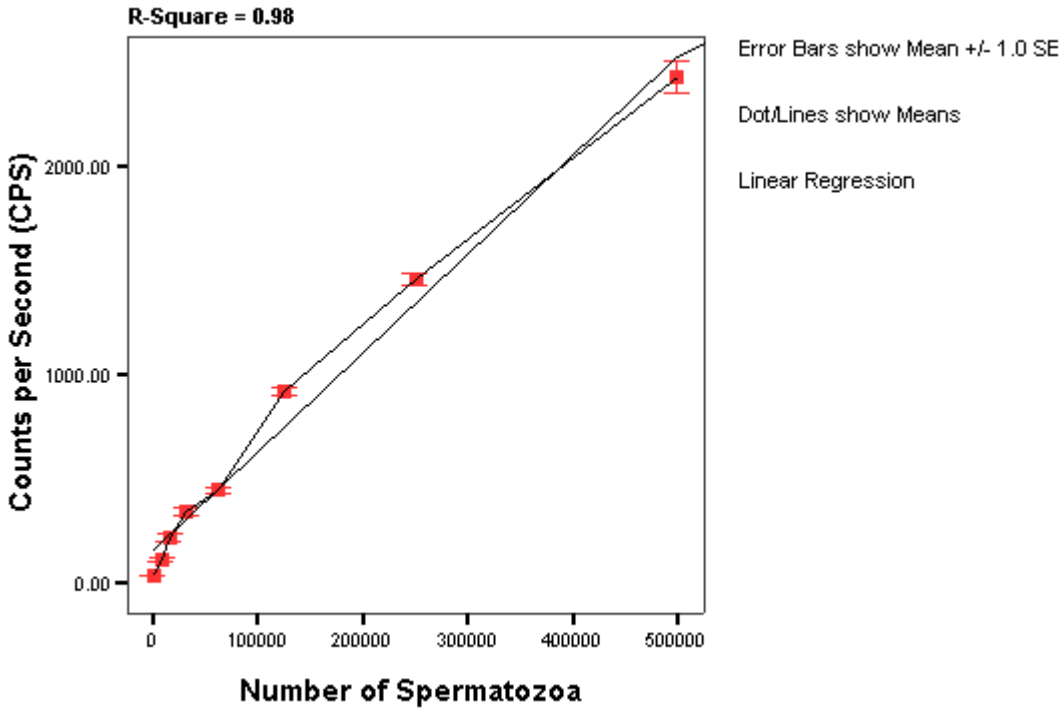


Figure 6-5: ATP Assay Standard Curve of Human Spermatozoa

Fresh human spermatozoa (n=3) were cultured in Hepes buffered (QAHMs) media, assay carried out as per manufacturer's instructions and luminescence (CPS) was plotted against number of spermatozoa. Error bars represent mean ± 1 SEM.

6.4.4 Effects of CYN on Cryopreserved and Fresh Human Spermatozoa

CYN concentrations of 0, 0.1, 0.3, 1 or 3 µM in QAHM's + 5% HSA had no significant effect on the viability of fresh (Figure 6-6, lower panel) or cryopreserved spermatozoa (Figure 6-6, upper panel) as assessed by the Eosin-Y exclusion assay. As expected, the H_2O_2 caused a significant decrease in viability after 24 h exposure (cryopreserved $P < 0.01$; fresh $P < 0.05$).

A significant decrease (27%) in the negative control, untreated spermatozoa viability ($P < 0.01$, Figure 6-7) were observed after 24 h exposure as determined by the ATP assay. The 1000 µg.mL^{-1} H_2O_2 positive control caused a decrease in spermatozoa viability ($P < 0.05$) as previously observed. However the ATP assay suggests a significant (88%) decrease in viable number of spermatozoa occurs by 15 min after exposure to H_2O_2. At the highest CYN concentration of 30 µM spermatozoa viability significantly decreased after 4 h exposure ($P < 0.01$, Figure 6-7).

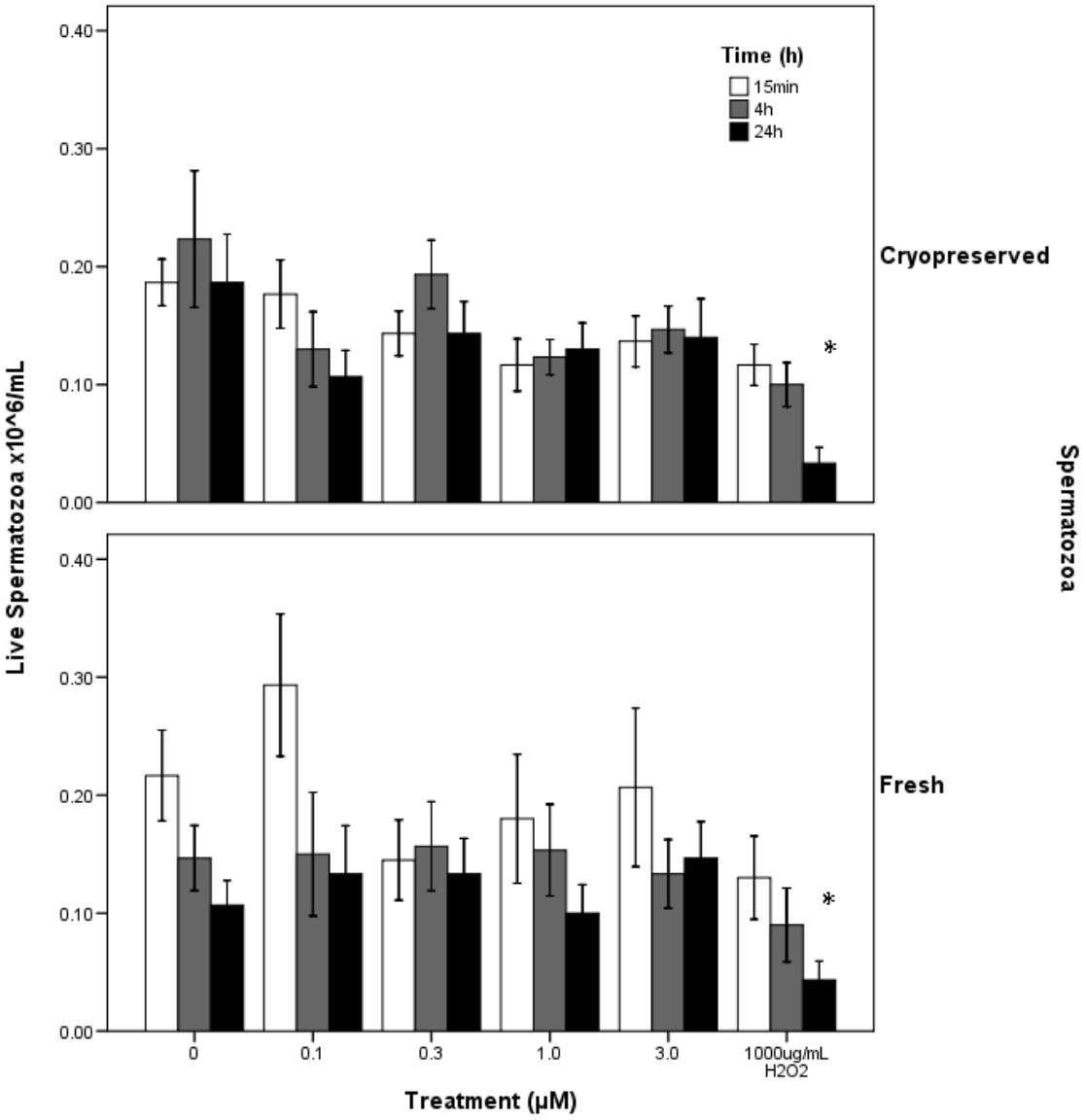


Figure 6-6: Effects of CYN on Cryopreserved and Fresh Spermatozoa Viability as Determined by the Eosin Y Assay

Fresh (upper panel, n=3) and Cryopreserved (lower panel, n=3) human spermatozoa were treated with 0, 0.1,0.3, 1.0, 3.0 µM CYN or 1000µg.mL^{-1} hydrogen peroxide in QAHM's + 5%HSA for 15 min, 4 h or 24 h. The numbers of viable spermatozoa were determined by the Eosin Y Exclusion assay. Error bars represent ± 1 SEM, significance indicated by *P <0.05 as determined by one-way repeated measures ANOVA.

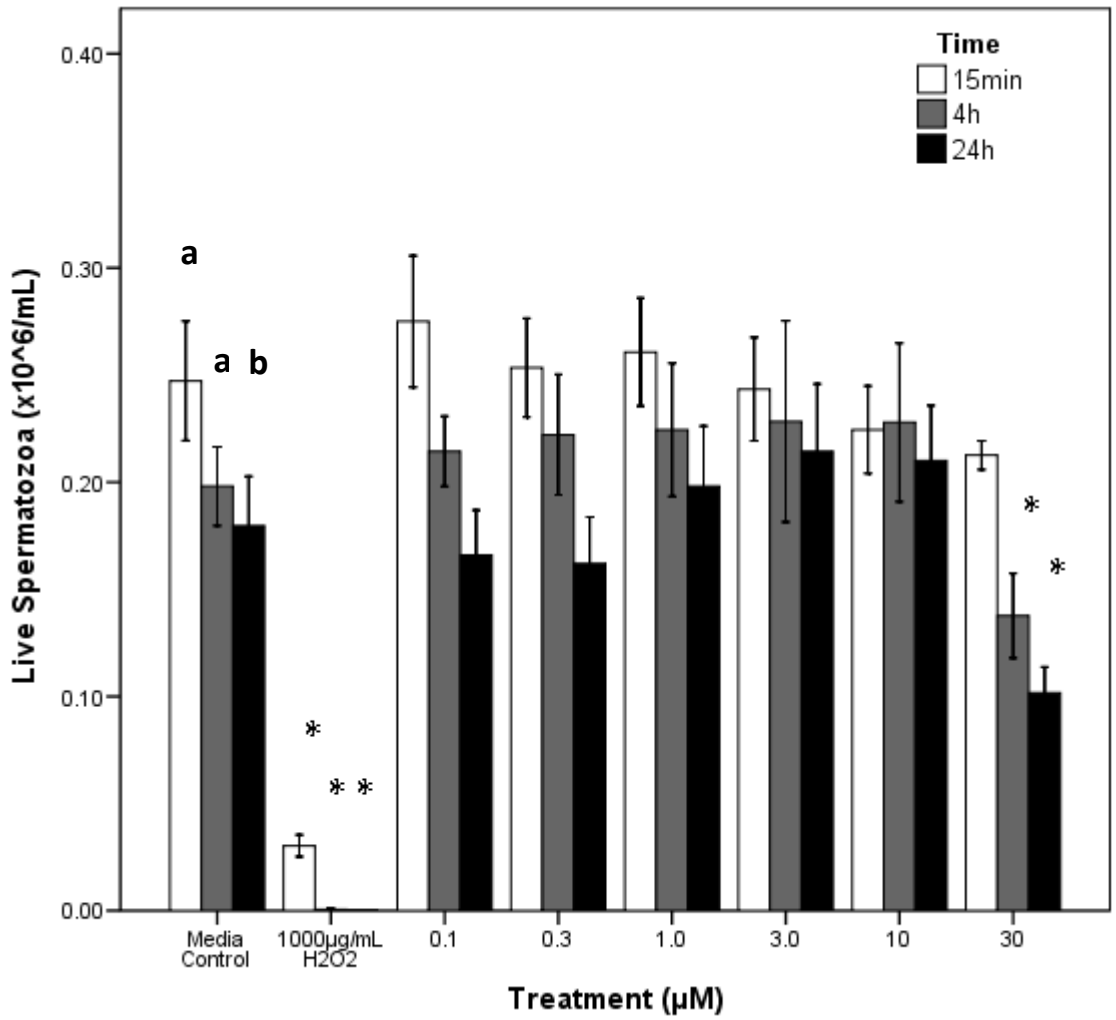


Figure 6-7: Effects of CYN on viability of fresh spermatozoa as Measured by ATP-Dependent Luciferase Luminescence

Fresh human spermatozoa (n=3) were treated with 0, 0.1,0.3, 1.0, 3.0, 10.0, 30.0 µM CYN or 1000 µg.mL^{-1} hydrogen peroxide in QAHM's + 5%HSA for 15min, 4 h or 24 h. The numbers of viable spermatozoa was determined by the ATPLite Luciferase assay. Error bars represent ± 1 SEM, significance indicated by *P <0.05 as determined by one-way between-groups ANOVA.

6.5 Discussion

Many studies examining spermatozoa have used HamsF10 as the culture medium which has an osmolarity of 278-311 mOsmol.L^{-1} when buffered with sodium bicarbonate. Other culture media reported include Biggers–Whitten–Whittingham (BWW), Human Tubal Fluid and Earls Essential Medium which have osmolarities ranging from 277-302 mOsmol.L^{-1}, all of which are within the optimal osmolarity range for human spermatozoa which is 220 – 380 mOsmol.L^{-1} (Acosta & Kruger 1996; Aitken et al. 1983; Hughes et al. 1998; Ibrahim et al. 1989; Morris et al. 2007; Murphey et al. 1986; Tomsu et al. 2002). However QAHM's medium (280 ± 10 mOsmol.L^{-1}) is the recommended culture medium for use in IVF culture (Patrizio et al. 2003). It was found that QAHM's medium maintained spermatozoa viability for 24 h of culture whereas viability declined in HamsF12 medium. QAHM's also appeared to protect against the oxidative effects of hydrogen peroxide more effectively than HamsF12. It has been found that the major factor that influences cell survival in defined medium is the presence of serum, such as human serum albumin (HSA) (Claassens et al. 2000; De Jonge et al. 2003; Rizzino et al. 1979). The maintenance of spermatozoa at room temperature reflects the *in vivo* conditions where mature spermatozoa are found in a natural environment of approximate temperatures resting at 26°C (Dunkel et al. 1997; Hossain et al. 2008). Thus the use of QAHM's medium in presence of HSA at room temperature is suitable for spermatozoa culture.

As spermatozoa are densely packed with mitochondria both the MTT and MTS assay were investigated to determine if the mitochondria within spermatozoa could reduce the tetrazolium salt to formazan, thus providing a correlation between OD and number of live spermatozoa. The inability to correlate absorbance of formazan to spermatozoa number after 2 and 24 h of culture with MTT in two buffered media types suggests that the tetrazolium salt could not reach the mitochondria within spermatozoa or was not metabolised under the conditions of our assay system. The MTT and MTS assays are dependent on the metabolism of the tetrazolium salt by succinate dehydrogenase, an enzyme which is part of the citric acid cycle (Folgero et al. 1993; Mosmann 1983) and hence these tetrazolium assays are indirect

measures of glucose metabolism (Folgero et al. 1993). The energy production (via oxidative phosphorylation) is fuelled by the presence of glucose and its metabolism; mature spermatozoa require energy for their motility and thus need to be actively metabolising glucose (utilizing oxygen) in order to generate ATP (Folgero et al. 1993; Orlando et al. 1982).

The reduction of MTT and MTS salts by the mitochondria is rapid and has been shown to occur within 1h by bovine and 2 h by human spermatozoa (Aziz 2006; Nasr-Esfahani et al. 2002). Studies by Aziz et al. (2005) and Nasr Esfahani et al. (2002) were able to optimise the MTT assay for equine and human sperm respectively. Nasr Esfahani et al. (2002) observed the production of formazan crystals in the mid piece of human spermatozoa using light microscopy at ×1000 magnification, whereas Bernas and Dobrucki (2000) used back scattered light confocal microscopy to detect the location of intracellular formazan production (Bernas & Dobrucki 2000). Aziz et al. (2006) were able to semi-quantify the formazan produced by bovine spermatozoa at 550 nm after 1 h at 37°C prepared in Hepes and 0.1% bovine serum albumin (BSA).

Studies have shown that the extracellular presence of succinate substrate did not increase ATP production by spermatozoa (Peterson & Freund 1970), nor increase rate of respiration (Folgero et al. 1993), and if MTT/MTS reduction is dependent on the succinate dehydrogenase enzymes, the ability of spermatozoa to metabolise the salts may not be efficient despite the high permeability of MTT (Mosmann 1983). Mitochondrial respiration is favoured at a pH of 7.0 (Murdoch & White 1967), an unlikely cause of why the MTT and MTS viability assays did not work in our culture system as spermatozoa were cultured in either Hepes (QAHMs) or sodium bicarbonate (HamsF12) buffered medium which maintain a pH of 7.0.

Secondly, it was unlikely that bacterial contamination may have occurred as the OD readings between control and spermatozoa containing wells of the MTS assay were similar. Despite the reduction of the MTS salt and production of formazan it was not linearly related to spermatozoa. The OD in spermatozoa containing wells were similar to control wells with no spermatozoa which may be an artefact of the assay where formazan is produced in the absence of cells as a result of media type

(Bruggisser et al. 2002; Debnam & Shearer 1997; Huang et al. 2004; Young et al. 2005).

Thirdly, the presence of serum albumin inhibits mitochondrial respiration, favouring anaerobic glycolysis and inhibition of respiration *in vitro* (Davidoff 1968). Aziz et al. (2006) used 0.1% BSA in their culture medium in which spermatozoa reduced MTT to formazan within 1 h, suggesting that 5% HSA used in our culture system may have inhibited respiration *in vitro* (Davidoff 1968; Huang et al. 2004). If spermatozoa in the presence of high concentrations of serum cannot metabolise the tetrazolium salt or inhibits respiration an electron transfer reagent may be required. The most commonly used is phenazine methosulfate (PMS), which is combined with the tetrazolium salts MTS and XTT (Goodwin et al. 1996; Vistica et al. 1991). However from our data the formazan produced in the MTS assay in spermatozoa containing wells were not significantly higher than control wells suggesting that the respiration in the presence of an electron transfer reagent was not the limiting factor.

The study by Davidoff (1968) found that fatty-acid free serum albumin in culture potentiated the inhibitory effects of phenethylbiguanide (guanidine derivative) on oxidation of pyruvate by the Krebs cycle. This is interesting and may have some relevance as CYN is a tricyclic guanidine (Ohtani et al. 1992), if the concentration of serum albumin were to be decreased, then the inhibitory effects to mitochondrial respiration may potentially be reflected by decreases in motility, decreased ATP levels (ATP assay) or decreased formazan production in the MTT or MTS assays (if they were to work with the decreased concentrations of serum albumin).

Two alternative methods, the Eosin Y Exclusion assay and the ATPLite assay, were used to determine the number of viable human spermatozoa. Our results show that ATP quantification (CPS) was linearly related to number of spermatozoa in QAHMs HEPE's buffered medium with an $R^2 > 0.98$ in QAHMs HEPE's buffered medium. The ATPLite assay differs from the tetrazolium based assays in that it does not require the uptake of a salt or its conversion to a product, but rather spermatozoa are lysed and amount of ATP released is quantified. The ATPLite assay has been found to be more reliable, sensitive and reproducible than the MTT

assay and other tetrazolium based assays (Cree & Andreotti 1997; Mueller et al. 2004; Petty et al. 1995). The Eosin Y Exclusion assay is a common and effective method for determining live spermatozoa based on the principle that live spermatozoa have structurally intact cell membranes whereas dead spermatozoa have disintegrating cell membranes and will take up the stain (Björndahl et al. 2004; Eliasson & Treichl 1971; WHO 1999). The ATPLite assay involves the quantification of ATP, generated by the mitochondria within spermatozoa, as a marker for cell viability and spermatozoa motility (Petty et al. 1995; Sevin et al. 1988; Vigue et al. 1992). The differences in Eosin Y Exclusion assay and the ATPLite assay in determining viability suggest that the ATPLite assay accurately determines the number of viable cells and is sensitive in assessing how the functionality and sub-lethal cell damage of spermatozoa is occurring after 1h of treatment (Cree & Andreotti 1997; Mueller et al. 2004). A decrease in ATP levels correlates to a decrease in motility and possibly fertilisation capability (Cree & Andreotti 1997; De Jonge et al. 2003; Vigue et al. 1992).

CYN concentrations of 0.1 – 3.0 µM were not cytotoxic to cryopreserved or fresh human spermatozoa by either the Eosin Y Exclusion assay or ATPLite assay. A time-dependent decrease in number of viable spermatozoa after exposure to 30 µM of CYN (equivalent to 12.5 $mg.L^{-1}$) was observed. A significant decrease in the number of viable spermatozoa was detected by the ATPLite assay after 4 h exposure to 30 µM. It is possible that very high concentrations of CYN are required to observe toxicity in spermatozoa, whereby the presence of albumin in culture medium may have masked the effects of CYN at lower concentrations (Claassens et al. 2000; De Jonge et al. 2003). The presence of albumin in the culture medium decreases the sensitivity of spermatozoa to toxins, as spermatozoa can bind toxic components but with preferential adsorption by albumin thus delaying or preventing expression of sperm toxicity (Claassens et al. 2000; De Jonge et al. 2003).

Guanidine derivatives are potent inhibitors of respiration in the mitochondria, the presence of albumin potentiates the inhibition by phenethylbiguanide, a biguanidine derivative (Davidoff 1968). Although not identical phenethylbiguanide and CYN (tricyclic guanidine) may share similarities in their affinity and or how they interact

with serum albumin *in vitro*, furthermore alkyl monoguanidines are specific inhibitors of NAD-linked mitochondrial oxidations (Davidoff 1968). Serum albumin also affects the mitochondria by binding and removing endogenous supply of free-fatty acids, which are also utilised during respiration. Hence if spermatozoa are preferentially adsorbing serum albumin in the medium, potentially decreasing respiration, which was supported by a decrease in energy production in the form of ATP (Figure 6-7, control wells) and the inability for tetrazolium salts to be reduced despite published literature suggesting otherwise, then the effects of CYN may be masked in mature spermatozoa *in vitro*. These data do not support the cytotoxic effects of CYN on both cryopreserved and fresh spermatozoa previously carried out in our laboratory, but show that a 24 h exposure to a concentration of 30 µM was required to observe significant decreases in ATP levels of fresh human spermatozoa as determined by the ATP assay, which is not unexpected as the mechanisms by which CYN induces cytotoxicity do not exist in mature human spermatozoa.

A decrease in ATP levels correlates to a decrease in motility and possibly fertilisation capability (Cree & Andreotti 1997; De Jonge et al. 2003; Vigue et al. 1992). Despite there being no significant decrease in ATP levels after exposure to the lower CYN concentrations, any decrease in ATP levels will adversely affect forward progressive motility and subsequent successful fertilisation capabilities of spermatozoa. As the motility of spermatozoa is dependent on ATP, the viability of spermatozoa in response to toxins *in vitro* can be investigated by determining the levels of ATP as useful end point to measure (Claassens et al. 2000). Semen samples with ATP concentrations of less than 40 pmol.10^6 sperm indicate diminishing sperm function and fertilisation capabilities (Vigue et al. 1992).

The presence of serum albumin also plays a protective role to mature spermatozoa decreasing their sensitivity to membrane oxidising agents; it is thought that the 17 disulfide bridges in serum albumin quenches free radicals preventing oxidative damage of the lipids of spermatozoa membrane (Claassens et al. 2000). Higher concentrations of CYN are required to cause significant effects on spermatozoa viability due to the mechanism by which CYN enters the intracellular environment. Although the hydrophilic properties of CYN make it difficult for it to readily

diffuse across the lipid bilayer of a cell membrane, CYN toxicity is dependent on transport mechanisms and or passive diffusion to enter the intracellular environment of a cell (Chong et al. 2002; Froscio et al. 2009) mechanisms which may not be present in mature spermatozoa.

Although the main mechanisms of CYN toxicity are CYP-independent protein synthesis inhibition and CYP metabolism dependent cytotoxicity (Froscio et al. 2001; Froscio et al. 2003; Humpage et al. 2005), protein synthesis in mature spermatozoa is limited to the mitochondria; mature spermatozoa also only contain mitochondrial ribosomes and no cytoplasmic ribosomes (Hecht 1998; Revelli et al. 1994). It is also unlikely that CYP metabolites are involved as mature spermatozoa do not contain these enzymes (Conley & Hinshelwood 2001). Exposure to CYN concentrations of 0 – 3 µM did not elicit a cytotoxic response in mature spermatozoa as observed in GC.

Spermatozoa membranes are densely packed with polyunsaturated phospholipids and lack reactive oxygen species (ROS) sequestering antioxidants and have reduced capabilities for DNA repair and response to oxidative damage (Aitken et al. 2004). Hence CYN-associated cytotoxicity in spermatozoa after 24 h exposure to 30 µM CYN was 50% of the control (which also had a significant decrease ATP levels) is unlikely to be solely attributed to increased lipid peroxidation, but rather due to an additive effect of decreased antioxidant defence mechanisms (Humpage et al. 2005; Runnegar et al. 1995) in the absence of seminal fluid (Aitken et al. 2004; Sanocka et al. 1997) and potential HSA effects on mitochondrial respiration (Davidoff 1968).

Oxidative damage is not limited to the phospholipids of the plasma membrane, DNA damage of spermatozoa can also be induced affecting the health of future generations as it occurs at the germ line level (Aitken & Baker 2006; Aitken et al. 2004; Pichini et al. 1994). Hydrogen peroxide is a potent oxidiser and has the capability of altering spermatozoa membrane integrity (Aitken et al. 1998). Both the number of live fresh and cryopreserved spermatozoa significantly decreased after 24 h exposure to hydrogen peroxide as assessed by the Eosin Y assay, supporting the hypothesis that hydrogen peroxide as a potent oxidiser of membrane

phospholipids (Aitken et al. 1998; Griveau & Lannou 1997). The ATPLite assay was much more sensitive than the Eosin Y Exclusion assay for detection of a loss of viability as a significant decrease in the number of viable spermatozoa was detected by 15 min of exposure to hydrogen peroxide, whereas the Eosin Y assay showed hydrogen peroxide to decrease viability after 24 h exposure in both cryopreserved and fresh spermatozoa. The decrease in ATP levels is reflective of metabolic changes within spermatozoa, and an indication of internal damage to energy supply. This may be damage to the mitochondria (Hecht 1998; Revelli et al. 1994). It may also be a reflection of ATP leakage from a damaged spermatozoa-plasma membrane. The possibility of CYN accumulating *in vivo* is a real risk as studies have showed that increased exposure to environmental tobacco smoke (passive smoking) was significantly correlated to an increase in nicotine (a volatile, water-soluble alkaloid) in seminal fluid, despite nicotine having a short half-life of 2 h and 80-90% is metabolised by the liver (Benowitz et al. 1982; Nakajima et al. 1998; Pacifici et al. 1995). Similarly a dose of 0.2 $mg.kg^{-1}$ (the approximate median lethal dose) was administered to male Quackenbush mice in which approximately 72% of CYN was excreted in the urine and faeces of after 12 h exposure (Norris et al. 2001).

Many studies have found that xenobiotics often do not affect the morphology of spermatozoa. However this does not preclude any effects on fertilising capabilities of spermatozoa within the female tract (Fraser et al. 2006). It has been previously determined that hydrocarbon concentrations can reach up to 100 $\mu g.kg^{-1}$ in human cervical mucus, where 1 $\mu g.kg^{-1}$ exerted inhibitory effects on spermatozoa functionality (Wagner et al. 1990). In addition, it is worth investigating the uptake mechanisms and transport of xenochemicals and similar compounds across spermatozoa membranes as they may adversely affect functionality at lower toxin concentrations despite no immediate effects on viability. Furthermore as non-motile spermatozoa can also be 'viable' it is important to determine viability by assessing DNA damage in spermatozoa using assays such as the COMET assay.

6.6 Conclusion

It is known that 'environmental factors, whatever the route, can clearly affect the development and function of the male reproductive tract' (Aitken et al. 2004). In conclusion the data presented here suggests that optimal conditions for examining the effects of CYN on spermatozoa were in QAHM's medium in conjunction with Eosin Y Exclusion assay or ATP quantification to measure the number of viable spermatozoa. The MTT and MTS tetrazolium based assays could not be optimised for determination of spermatozoa viability under our assay conditions, most likely attributed to the presence of 5% serum albumin. Further experiments with decreasing concentrations of serum in the culture medium can be investigated to determine if human spermatozoa can reduce the MTT and MTS salts to formazan and increase sensitivity to CYN.

There is a real possibility of CYN accumulating *in vivo* especially with exposure periods extending further than 24 h; it is unknown if CYN will accumulate *in vivo* and what the potential affect on the functionality, maturation and development of spermatozoa could be. This study presents the initial basis in which further spermatozoa functionality tests can be employed to build a deeper understanding of the potential risk of CYN on human male reproduction.

Chapter 7. General Discussion

CYN is a toxin produced by fresh water cyanobacteria and has been increasingly found in sub-tropical waters (Codd et al. 2005); furthermore the poisoning in Palm Island in 1979 was not solely attributed to CYN as Microcystins another cyanobacterial toxin was also present. What would be expected in a general cyanobacterial bloom is for the presence of many various types of toxins. Thus oral ingestion (one method of exposure) of a mixture of toxins may create the potential for them to act synergistically and affect reproductive cells. Many environmental contaminants such halogenated compounds, dioxins, pesticides and metals have been detected in human tissues and have been found in reproductive fluids such as seminal and follicular fluids (Edwards 1974; Hess et al. 1998; Wade et al. 2002; Wagner et al. 1990). Many of the studies that investigate the cytotoxic effects of mixed compounds (at concentrations which meet the TDI) have been *in vivo* mouse models in order to ascertain the damage that may occur as a result of the toxin being ingested. This then further allows the examination of organs and histological examination of tissues to determine degree of damage. Followed on from this, many *in vitro* studies can be carried out to determine intracellular mechanisms of action. The study by Wade et al. (2002) which investigated whether xenobiotic compounds work synergistically to increase overall toxicity, found that a mixture of compounds were unlikely to generate adverse effects on immune function or reproductive physiology. In contrast a study by Walsh et al. (2000) found that a mixture of pesticides did synergistically increase the overall inhibition of steroid hormone production but rather antagonistically decreasing overall inhibition of steroidogenesis to 50%. These types of studies are very valid and necessary when doing toxicity studies. However many of them are first dependent on establishing whether the toxin in question is toxic, in this thesis the potential of CYN to be a reproductive toxicant first needs to be established. The research aims of this thesis were to investigate the effects of cylindrospermopsin on human granulosa cells and mature spermatozoa from patients undergoing Assisted Reproductive Technology (ART). The research can be separated to individually discuss the effects on female

reproductive cells, male reproductive cells and then discuss as a whole. Patients seeking ART are either infertile or sub-fertile and thus the development and 'health' status particularly of granulosa cells is influenced by reproductive histories. This research also aims investigated if the granulosa cells which were classified as 'unhealthy' were more sensitive to CYN exposure than their 'healthy' classified granulosa counterparts.

Successful human reproduction requires normal functioning of reproductive cells. These cells include spermatozoa to fertilise the oocyte and granulosa cells which play a major role in production of steroid hormones vital for establishment and maintenance of pregnancy (Beckman et al. 1991; Breckwoldt et al. 1996; Chaffkin et al. 1993). The gonadotropins; FSH and LH/hCG control steroidogenesis by regulating StAR, CYP11A1 and 3βHSD enzymes at the mRNA and or protein level (Huang et al. 2004). Steroid hormone production is dependent on increased intracellular cAMP levels, translocation of cholesterol substrates into the mitochondria and subsequent conversion of cholesterol to pregnenolone by CYP11A1, the precursor to all steroid hormones produced (Breckwoldt et al. 1996; Clark et al. 1994; Conti 2002; Devoto et al. 2002; Ronen-Fuhrmann et al. 1998; Sasson & Amsterdam 2002; Stocco & Clark 1996). An essential criterion when using primary-derived cells for use in research is to ensure that they are healthy and capable of functioning optimally. This is difficult to determine but clinical parameters can be used as indicators. The ability to select healthy spermatozoa is relatively straightforward involving a one-step density gradient centrifugation dependent on motility of spermatozoa. This parallels the normal fertilisation selection processes *in vivo* where successful fertilisation is dependent on motile spermatozoa reaching the oocyte in the female reproductive tract. However, the selection of healthy granulosa cells is dependent on a multitude of factors including reproductive histories such as: polycystic ovary syndrome (PCOS), irregular menstrual cycle/amenorrhoea, or endometriosis which are considered to influence the development of granulosa cells as these conditions are influenced by the endocrine system which controls hormone regulation and development of ovarian cells. Oocyte growth and maturation is dependent on the communication feedback between oocyte and granulosa cells (Richards 1994; Russell & Robker 2007).

Furthermore this influences the development of GC *in vivo* and whether GC will have a "good" ovarian response characterised by the parameters discussed in section 2.2.1.

The majority of granulosa cells used in this body of research were isolated from women which represented "normal", healthy and fertile general population. A total of 55 women were included in this study, 35 cases were attributed to MIF (healthy), 5 cases FIF (unhealthy) with the remaining 15 attributed to unknown factors. Experiments that included granulosa cells which were classified as unhealthy were subdivided into healthy and unhealthy cohorts and further analysed to compare sensitivity of the cells in the two groups after CYN exposure.

7.1 *In vitro* Culture Conditions of Human Primary-derived Granulosa Cells

Prior to undertaking CYN exposure studies, the optimisation of culture conditions, viability assays and functionality assays was required.

Differentiated granulosa cells are characterised by increased steroid hormone production, particularly progesterone which is reflected by metabolic changes. Not all granulosa cells respond to hCG administered *in vivo* by differentiating into granulosa-lutein cells, this creates a heterogeneous population of cells with varying steroidogenesis capabilities (Breckwoldt et al. 1996). Thus upon isolation granulosa which have yet to luteinise (~20%) require a 'pre-treatment' period in culture to allow the cells to differentiate a process which can range between 24 h and up to 7 d (Landefeld et al. 1997; Schipper et al. 1993). Thus a 24 h pre-treatment culture period was investigated to determine if it was sufficient for primary-derived granulosa to attach to the culture vessel and differentiate into granulosa-lutein cells.

The optimal period of pre-treatment culture and granulosa cell density to ensure that steroid hormone production and viability of cells was maintained *in vitro* was a 24 h pre-treatment adherence period using 20×10^3 cell/well. These two factors were determined from our optimisation studies and are supported by published literature (Breckwoldt et al. 1996; Chaffkin et al. 1993; Gerlier & Thomasset 1986; Ho et al. 2004; Macklon & Fauser 1998; Quinn et al. 2006; Sasson & Amsterdam 2002;

Steuerwald 2007; Zlotkin et al. 1986). The 20×10^3 cells/well density produced optimal progesterone production, minimal hypertrophy of cells reflected by stable numbers of viable cells over a 72 h period in control culture medium and the maximum number of experimental combinations could be carried out in 96-well culture plates.

It was hypothesised that the absorbance produced by the reduction of tetrazolium salt (MTT) to formazan or staining of granulosa cells with Crystal Violet would be proportional to the number of live and viable cells.

The reduction of MTT tetrazolium salt to formazan was based on the premise that cells which have active mitochondria are capable of metabolising the MTT (Mosmann 1983). In addition to energy production for cell maintenance, granulosa cell function also depends on the mitochondria in order for steroidogenesis to occur (Fatum et al. 2009). Determining granulosa cell viability by assessing mitochondrial activity using the MTT assay is a particularly important end point; furthermore sub-optimal functionality will also reflect capability of steroid hormone production. Thus the MTT assay is sensitive enough to detect early changes in the viability of GC. This creates difficulty in expressing hormone production relative to viable cell number determined by MTT assay, hence the need to assess viability via two distinct mechanisms simultaneously to increase reliability of results when determining cytotoxic effects (Amsterdam et al. 1999; Arthur & Boyd 1974; Fotakis & Timbrell 2006; Hansen et al. 1989). The Crystal Violet assay is a simple and rapid assay which determines the number of cells that are still attached after treatment exposure.

In our results both assays had OD_{570nm} that were either strongly linearly or exponentially associated to number of cells. Thus the MTT and Crystal Violet assay's both accurately determined viability and were reproducible, supporting that of published literature (Best et al. 1995; Fotakis & Timbrell 2006; Hansen et al. 1989; Lambert et al. 2000; Mosmann 1983). The linear regression was used to estimate the viability of granulosa cells after CYN exposure; as the strong correlation to cell number was within the linear region of 2,500 to 40×10^3 cells/well required of the experimental model.

As hypothesised granulosa cells which are in a state of differentiation will be positive to ORO lipid stain indicating presence of cholesterol substrate. Our results show that after 24 h pre-treatment culture period cholesterol substrates were detected in granulosa cells using the optimal staining parameters of ORO stain 2 by Kinkel et al. (2004) and Stoklosowa et al. (1978), for 15 min at room temperature.

Furthermore the 20×10^3 cell/well density is also within the sensitivity and linear range of both the MTT and Crystal Violet assays and thus changes to viability can be detected as can changes to steroid hormone production. Furthermore this model for *in vitro* culture of GC in our experiments is similar to other GC toxicity models established in the literature (Breckwoldt et al. 1996; Sasson & Amsterdam 2002; Young et al. 2008).

7.2 Cytotoxic Effects of CYN on Human Primary-derived Granulosa Cells *in vitro*

As the human ovaries are highly vascularised the contents of the follicular fluid are comprised of secretions from granulosa cells and plasma exudates due to the permeability of the blood-follicle membrane (membrane separating theca and granulosa cells) to components of the plasma; thus the granulosa cell and the oocyte within the follicle are exposed to components of the circulatory system similar to other tissue and cell types (Edwards 1974; Hess et al. 1998). Thus there is a real risk of granulosa cell exposure to the blue green algal toxin cylindrospermopsin (Young et al. 2008). Furthermore hydrocarbons, drugs and environmental toxins have been found to enter the human semen; of particular interest is nicotine, an alkaloid with similar properties to CYN (Benowitz et al. 1982; Nakajima et al. 1998; Pacifici et al. 1995).

The primary objective of this research was to investigate the cytotoxicity of CYN on primary-derived human granulosa cells in which it was hypothesised that CYN would be cytotoxic in a time and dose dependent manner.

CYN is primarily hepatotoxic, with cytotoxicity occurring by 18 h at 1.0 µM CYN (LDH assay) in primary mouse hepatocytes and at 0.1 µM CYN (MTT assay) after 72 h exposure in rat hepatocytes (Chong et al. 2002; Froscio et al. 2003). More

recently a study was carried out by Young et al. (2008) investigating the effects of CYN on the viability and steroid hormone production by human primary-derived granulosa cells *in vitro*. Results showed CYN to be cytotoxic (viability was assessed by the MTT assay) and to cause a decrease in hCG-stimulated progesterone production. Toxicity was observed after 24 h exposure to 1 $\mu g.mL^{-1}$ CYN (~2.4 µM). In our results CYN was cytotoxic after 48 h exposure to 1.0 µM (MTT assay) and 3.0 µM (Crystal Violet assay). Young et al. (2008) also showed that 48 h exposure to 1.0 µM CYN caused GC cytotoxicity (MTT assay). It is possible that primary-derived granulosa cells are less sensitive to cytotoxic effects of CYN than primary rat and mouse hepatocytes. This may be attributed to the higher concentration of CYP- CYN metabolising enzymes found in hepatocytes specifically targeted to eliminating foreign compounds and toxins, or different uptake mechanisms between species and cells between primary-derived and immortalised cell line (Froscio et al. 2009; Froscio et al. 2009; Froscio et al. 2003; Payne & Hales 2004). Alternatively cytotoxicity may be indirectly caused by inhibition of protein synthesis which is a slower mechanism of toxicity.

It was also hypothesised that CYN would decrease basal granulosa cell steroid hormone production reflected by the percent of cholesterol staining and or decrease in 3βHSD activity. Steroid hormone production after exposure to CYN was quantified by radioimmunoassay and results were comparable to Young et al. (2008). Young et al. (2008) found basal progesterone production decreased after 24 h exposure to 0.0625 $\mu g.mL^{-1}$(~0.3 µM) CYN and that a concentration of 1 $\mu g.mL^{-1}$ CYN (~2.4 µM) decreased hCG-stimulated progesterone production after 6 h exposure. In this study progesterone production decreased after 72 h exposure to 0.1 µM CYN and was attributable to the loss of viability. Similarly, estradiol concentrations in combined data significantly decreased after 72 h exposure to all CYN concentrations tested of which only 1.0 and 3.0 µM were attributable to loss of viability. The consequence of the inhibition of estradiol production is the arrest in the development and maturation of granulosa cells (Devoto et al. 2002).

Steroidogenesis depends on the availability of cholesterol substrates (positive Oil Red O (ORO) lipid stain) of which the rate of steroidogenesis is dependent on the

intracellular availability of cholesterol substrates and activity of enzymes involved in steroidogenesis (Bar-Ami 1994). Furthermore the decrease in steroid hormone production was not entirely as a result of decreased cholesterol substrate which occurred after 24 h exposure to 3.0 µM CYN, yet the decrease in cholesterol substrate availability cannot be ruled out as a contributing factor to decreased steroid hormone production.

Progesterone production is dependent on 3βhydroxysteroid dehydrogenase enzyme activity (3βHSD), section 1.1.1. The decreases in progesterone production occurred after 72 h exposure to 0.1 µM CYN. In our results the decreases in progesterone concentrations were a result of decreased viability rather than 3βHSD activity or cholesterol availability, which is unlikely to be caused by a decrease in substrate availability at 3.0 µM CYN. The presence of cholesterol lipids reflected the presence of an endogenous supply of substrate for steroid hormone production, and the presence of 3βHSD activity suggests that live cells remaining after CYN exposure maintain properties for steroidogenic functionality (Bar-Ami 1994; Fischer & Kahn 1972). Furthermore our results did not support the decrease in progesterone production found by Young et al. (2008) to be attributed to a loss of 3βHSD functionality or substrate availability. Further work is required to determine if enzyme activity was altered.

The decreased steroid hormone production observed by Young et al. (2008) only occurred in hCG responsive granulosa cells. The non- hCG responsive granulosa cells in the study by Young et al. (2008) indicate either an un-luteinised granulosa cell population or granulosa cells that have yet to desensitise from *in vivo* gonadotropin stimulation and are unable to respond to further stimulation without a pre-treatment culture period *in vitro*, which can be up to 7 d (Landefeld et al. 1997; Schipper et al. 1993). However it can also indicate differences between granulosa cells based on their 'health' status (as described in section 4.1) in which responsive cells are considered to have luteinised and likely to reflect healthy cells.

It was hypothesised that differences in the sensitivity to cytotoxic effects of CYN would be observed between 'healthy' and 'unhealthy' granulosa cells. The data from Chapter 4 were from combined women of healthy and unhealthy granulosa

cells and differences in sensitivity to the cytotoxic effects of CYN were apparent between these groups. It was found that granulosa cell viability and steroid hormone production in the unhealthy cohort were more sensitive to the cytotoxic effects of CYN at lower concentrations but not an earlier time point. Granulosa cells which are healthy would be expected to have optimal mitochondrial capabilities and steroidogenesis, dependent on CYP enzymes hence 'healthy' GC would be expected to be more sensitive which was not the case most likely attributed to their decreased capabilities for cellular defence mechanisms (Fatum et al. 2009; Russell & Robker 2007).

7.3 Cytotoxic Effects of CYN on Granulosa Tumour Cell Line -The KGN Cells

Differences have been found to exist in the cellular uptake of CYN between cell lines and primary cells (Froscio et al. 2009). Primary-derived human granulosa cells are a good model to work with as they are the closest in vitro reflection of the *in vivo* physiological state. However, the granulosa tumour cell line, KGN provides an opportunity to compare results and possibly identify mechanisms that control the response in primary derived cells, with minimal biological variability and are particularly useful when primary-derived granulosa cells are less readily available (Macklon & Fauser 1998; Minehata et al. 2007; Nishi et al. 2001). It was hypothesised that CYN would adversely affect KGN cells at a similar concentration and exposure period to primary-derived granulosa cells. In our study the proliferation of KGN cells was both time and dose-dependently inhibited after 48 h exposure to 3.0 µM CYN. In comparison to primary-derived granulosa cells loss of KGN viability as determined by the Crystal Violet assay were similar. Indicating that CYN toxicity in granulosa cells is delayed in comparison to mouse hepatocytes for reasons aforementioned in section 7.2. However cAMP- stimulated progesterone production in KGN cells was significantly inhibited after 48 h exposure to 0.1 µM CYN suggesting that steroidogenesis and mitochondrial functioning was affected prior to onset of cell death. If the MTT assay was carried out as the viability assay may have detected an earlier loss of viability which may have reflected the significant decreases in progesterone production at 0.1 µM CYN.

7.4 Effects of CYN on Human Primary-derived Granulosa Cell Protein Synthesis *in vitro*.

It was hypothesised that CYN would inhibit protein synthesis in a time and dose dependent manner causing subsequent decreases in steroid hormone production. Cylindrospermopsin is a potent inhibitor of protein synthesis (PSI) which occurs after 4 h exposure to 0.5 µM CYN and is unrelated to cytochrome P450 (CYP) metabolism dependent cytotoxicity (Froscio et al. 2003). The capability of luteinised and non-luteinised granulosa cells to undertake protein synthesis *in vitro* does not differ between the two cell states (Landefeld et al. 1997).

As hypothesised CYN inhibited protein synthesis after 6 h exposure to 5 µM. It occurred within a similar time frame as observed in mouse hepatocytes (4 h). However protein synthesis inhibition occurred at a concentration 10-fold higher than primary mouse hepatocytes (0.5 µM CYN), supporting the difference in sensitivity and uptake mechanisms between type of cells (Froscio et al. 2009). The earlier onset of cytotoxicity in hepatocytes is attributable to uptake mechanisms which may involve the bile acid transport pathway, passive diffusion and the generation of bioactive CYP metabolites (Chong et al. 2002; Froscio et al. 2001; Froscio et al. 2003). CYP metabolite induced cytotoxicity and protein synthesis inhibition occur independently (Froscio et al. 2001; Froscio et al. 2003). Whereas in granulosa cells, CYP metabolism dependent cytotoxicity remains to be elucidated, what can be extrapolated from the data was granulosa cells (at 5 µM after 6 h exposure) were 10-fold less sensitive to PSI than hepatocytes (0.5 µM after 4 h exposure) but the uptake into cells occurred within a similar time frame and continued over a 24 h period where PSI was signficantly inhibited in GC. Froscio et al. (2009) found that in the presence of a cell membrane, CYN uptake was slower but continued to enter the cell over a 24 h period. This may suggest that CYN is utilising similar uptake mechanisms to enter cells, such as the bile acid transport system (Chong et al. 2002) as bile acids have been detected in the ovarian follicle (Smith et al. 2009).

Although no statistically significant decreases in steroid hormone production were observed in direct relation to inhibition of protein synthesis. The significant

decreases in steroid hormones which occurred after 72 h exposure were correlated to loss of cell viability (Chapter 4), whereas CYN exposure periods in the protein synthesis inhibition assays were only for 24 h, hence loss of viability and steroid hormone production may have been attributed to decreased protein synthesis but is not apparent until after 72 h of *in vitro* culture. Steroid hormone production is dependent on *de novo* protein synthesis, thus the acute exposure period and decreases in protein synthesis may have contributed to the significant decreases in steroid hormone production in protein synthesis assays and cytotoxicity of GC (Chapter 4). The protein synthesis time course assay ranged from 0 to 24 h which was selected to gauge the onset of protein synthesis and compare to protein synthesis inhibition observed in primary mouse hepatocytes studies carried out by Froscio et al. (2003).

In vitro hCG stimulates the expression of CYP11A1 protein promoting steroidogenesis (Chaffin et al. 2000; Voutilainen et al. 1986). Granulosa cells were cultured for 24 h or 48 h prior to CYN exposure to investigate if there would be a difference in the sensitivity of GC responding to hCG stimulation and whether changes in CYP11A1 protein levels would be correlated to steroid hormone production. The results showed the strongest detection of CYP11A1 after 24 h adherence with no hCG stimulation or CYN exposure. The decrease in protein synthesis was not directly reflected in decreased CYP11A1 levels in neither hCG-stimulated nor non-hCG stimulated granulosa cells. Granulosa cells were responsive to hCG stimulation after 48 h adherence supporting that desensitisation of GC to *in vivo* stimulation occurs after 2 d in culture (Breckwoldt et al. 1996; Edgar et al. 1991). The data further supports that granulosa cells were in a state of differentiation and could be responsive to hCG stimulation despite the increase in progesterone production not being statistically significant, in section 5.4.7. Studies have shown that decreases in steroidogenic enzyme mRNA levels are not immediately reflected in protein levels, this may explain the delay in inhibition of steroid hormone production observed in our results (Huang et al. 2004). Furthermore acute steroidogenesis is dependent on the rate limiting enzyme StAR and its *de novo* synthesis, therefore if PSI is causing decreased steroid hormone production it is more likely to be attributed to StAR protein levels, than those of

CYP11A1. The PSI experiments carried out indicate general inhibition of protein synthesis by CYN.

An extended exposure period to reflect that of cytotoxicity assays is required in order to correlate CYP11A1 enzyme levels and steroid hormone production to determine if the decreases observed in protein synthesis assays are correlated to decreases in steroid hormone after CYN exposure. The semi-quantification of CYP11A1 protein levels does not provide an indication about enzyme activity or if the active site has been modified and thus specific effects of CYN on CYP11A1 protein cannot be elucidated.

7.5 Effects of CYN on Mature Human Spermatozoa

It was hypothesised that mature human spermatozoa would reduce the MTT and or MTS tetrazolium salts as the axenomes of spermatozoa are densely packed with mitochondria. Although the MTT assay was applicable for determining viability of granulosa cells we could not adapt the MTT or MTS assay to determine viability of human spermatozoa, as previously reported by the other studies. (Aziz et al. 2005; Nasr-Esfahani et al. 2002). It is possible that the MTT and MTS salts could not reach the mitochondria within spermatozoa or were not metabolised under the conditions of our assay system despite conducting the MTT assay under similar culture conditions to that of the successful studies. The presence of serum albumin, known to inhibit mitochondrial respiration (Davidoff 1968; Huang et al. 2004), of which our culture medium comprised of 5% human serum albumin may have inhibited the reduction of MTT by mitochondrial succinate dehydrogenases. Aziz et al. (2006) cultured their spermatozoa in Hepes containing 0.1% of bovine serum albumin.

As hypothesised ATP concentrations were quantified and linearly related to the number of viable spermatozoa. The Eosin Y and ATPLite assay were alternative methods of determining viability providing a rapid, sensitive and accurate determination of live number of spermatozoa and an indication of spermatozoa functionality by determining membrane integrity and energy production respectively.

The mechanisms by which CYN mediates its toxicity include CYP metabolites and protein synthesis inhibitions, both of which are not actively present in spermatozoa, thus it was hypothesised that CYN concentrations of 0 - 10 µM CYN were not cytotoxic to spermatozoa. Despite studies showing that oxidative stress was not a significant factor in the toxicity of CYN (Humpage et al. 2005), spermatozoa membranes are highly susceptible to oxidative stress. Whether the 30 µM CYN concentration was a level to induce a chain reaction of oxidative damage or directly oxidised lipids is unclear. The 30 µM CYN caused what was interpreted as a loss of spermatozoa viability by compromising membrane integrity and decrease of intracellular ATP levels (which degenerates rapidly) as determined by both Eosin Y and ATP assay respectively. The ATPLite assay was much more sensitive than the Eosin Y Exclusion assay for detection of a loss of viability. The decrease in ATP levels is reflective of metabolic changes within spermatozoa, and an indication of internal damage to energy supply. This may be damage to the mitochondria or protein synthesis occurring at the mitochondrial level (Hecht 1998; Revelli et al. 1994).

7.6 Conclusion

In conclusion the mitochondrial dehydrogenase enzymes of granulosa cells were capable of reducing the MTT tetrazolium salt to formazan producing absorbance that was proportional to cell number. Furthermore the granulosa cells stained with the Crystal Violet dye and produced an absorbance that was proportional to cell number. It was determined that primary-derived granulosa cells require a minimum of 24 h adherence to attach and exhibit characteristics of a differentiated population capable of steroidogenesis, indicated by increased progesterone production. The differentiated state of primary-derived granulosa cells that exhibit characteristics of granulosa-lutein cells are reflected by the presence of cholesterol substrates, increased progesterone production and hCG responsiveness- the latter by which depends on a cell's ability to first desensitise to *in vivo* hyperstimulation. The results indicate that GC required a minimum of 24 h pre-treatment culture period; however data from hCG experiments suggest 48 h is required for desensitisation to occur. The granulosa-lutein cells were identified by positive Oil Red O lipid staining for the presence of cholesterol substrates required for steroidogenesis.

Cylindrospermopsin was cytotoxic to granulosa cells at 1.0 µM CYN after 48 h reducing granulosa cell viability comparable to that observed by Young et al. (2008). Furthermore CYN decreased basal progesterone and estradiol production by primary-derived granulosa cells after 72 h exposure to 0.1 µM CYN in both unhealthy and healthy granulosa cells. Changes to steroid hormone production were not a direct result of decreased cholesterol substrate availability which occurred after 24 h exposure to 3.0 µM. In addition the decrease in progesterone production was not as a result of decreased 3βHSD enzyme activity staining which remained unaffected throughout CYN treatment. This research introduced the need to incorporate the reproductive histories of women as an important factor when selecting the granulosa cells to use for *in vitro* research. Granulosa cells classified as unhealthy were more sensitive to the cytotoxic effects of CYN in comparison to their healthy counterparts. Cylindrospermopsin was also cytotoxic to the granulosa cell line, KGN cells in which the proliferation of KGN granulosa cells ceased within the time frame required for cell number to double. In addition there was a decrease in cell viability after exposure to CYN at similar concentrations to those that were cytotoxic to primary GC. Cylindrospermopsin decreased cAMP-stimulated progesterone production in KGN cells after 72 h exposure to 0.1 µM CYN and inhibited cAMP-stimulated progesterone production after 48 h exposure to 1.0 µM CYN.

Protein synthesis in primary-derived granulosa cells was inhibited by 6 h exposure to 5.0 µM CYN, within a similar time-frame as primary-derived hepatocytes but at a 10-fold higher concentration. The subsequent decreases in steroid hormone production were not correlated to significant decreases in protein synthesis inhibition after a total 24 h CYN exposure. The protein levels of the CYP11A1 enzyme decreased after exposure to CYN in the absence of hCG stimulation after 24 h adherence. Loss of CYP11A1 protein levels after CYN exposure may have been prevented by the presence of hCG, with no effect on CYP11A1 protein levels after 48 h adherence in the presence of hCG and CYN. Progesterone production was found to be unrelated to CYP11A1 protein levels, in contrast to estradiol concentrations after 24 h adherence.

Human mature spermatozoa could not reduce the tetrazolium salt to produce formazan such that no linear relationship could be established relating absorbance to spermatozoa numbers in either the MTT or MTS assay. Thus the MTT and MTS assays could not be used as viability assays. However upon lysis of spermatozoa, ATP concentrations measured by the luminescence signal generated by the ATPLite assay conditions was linearly related to number of viable spermatozoa and was used as a viability assay, in addition to the Eosin Y assay routinely used to determine viability of spermatozoa in ART laboratories. CYN concentrations up to 10 µM did not adversely affect human mature spermatozoa. However exposure to 30 µM decreased ATP levels which is related to viability of spermatozoa.

Finally, CYN was cytotoxic to granulosa cells both primary-derived and the KGN cell line. However granulosa cells were less sensitive than mouse hepatocytes. CYN uptake by granulosa cells may utilise the bile acid transport pathway in addition to passive diffusion, with cytotoxicity occurring as a result of protein synthesis inhibition and not CYP metabolites. Decreases in steroid hormone production were likely to be caused by protein synthesis inhibition of the steroidogenic rate limiting enzyme StAR and also loss of viability. Granulosa cells were the reproductive cells used to investigate the affects of CYN as a reproductive toxicant in the female system; however the equivalent Sertoli cells from the male reproductive system were not used but rather mature human spermatozoa. The Leydig cells are found adjacent to the seminiferous tubules, surround the Sertoli cells, and secrete testosterone (also under the influence of LH) essential for the production of mature spermatozoa. The Leydig cells are also susceptible to CYN toxicity through similar mechanisms to granulosa cells. However mature human spermatozoa were used primarily due to preliminary data obtained from our laboratory which suggested that CYN was cytotoxic to mature spermatozoa and significantly reduced their viability within 1 h. This researched aimed to further elucidate those claims. Although granulosa cells are diploid cells and spermatozoa are haploid cells, both of these cell types are crucial for the establishment and maintenance of pregnancy. CYN was found not to be cytotoxic to spermatozoa which remained viable compared to the hydrogen peroxide positive control. Human mature spermatozoa are unlikely to be affected by CYN as they lack the primary mechanisms by which

CYN mediates its effects. Thus the development of spermatozoa within the Sertoli cells (site of spermatogenesis) of the testes or testosterone production by the Leydig cells would be the next steps in determining CYN's role as a reproductive toxicant. Furthermore it would also be necessary to investigate the affect of growth and differentiation of immature granulosa cells after exposure to CYN. These subsequent research investigations would parallel the development of key reproductive cells in both male and female reproductive systems which are required in the establishment and maintenance of pregnancy within ethical boundaries.

7.7 Future Directions

The reality is cylindrospermopsin is not the only cyanobacterial toxin to be found in Australian waters. In more temperate waters, microcystins, anatoxins and nodularins can also be found and are potent cyanotoxins. Although *C.raciborskii* is observed mainly in tropical areas it is also increasingly found in temperate Australian waters and hence a mixture of cyanotoxins is likely to occur in freshwaters. Hence investigations in to the toxicity of a mixture of cyanotoxins need to be carried out as their combined effects on human reproductive cells at low concentrations which mimic a natural occurrence will eventually be required. Extensive research needs to be directed towards cylindrospermopsin toxicity due to its potency as a hepatotoxin and potential carcinogenic effects *in vivo* and *in vitro*. Recent studies have begun to investigate the carcinogenetic, genotoxicity, and reproductive effects of CYN; however these have been in murine models. Thus this research identifies the potential risk to human reproduction. The combination of cell death, decrease in steroid hormone and protein synthesis inhibition in primary GC and KGN cells requires further investigation to determine mode of cell death, whether cell death is occurring via apoptosis or as a response to increased cAMP levels. Toxins have the ability to reach and pass through the reproductive systems in both males and females. A toxin such as nicotine, which has similar properties to CYN have been demonstrated to reach the reproductive cells and hence indicates that CYN may potentially be cytotoxic and adversely affect functionality of these cells. Further toxicity assays over longer exposure periods need to be undertaken, whilst investigating the various uptake mechanisms by which CYN enters granulosa cells, if CYP inhibitors play a minor role in cytotoxicity and if steroidogenic

enzymes are affected by the general inhibition of protein synthesis. Decreases in mRNA levels are not immediately reflected in protein levels and thus the delay in effects on steroid hormone production should be further investigated by quantifying steroid hormone substrate precursors to aromatase, CYP11A1 and 3βHSD steroidogenic enzymes to assess if there is decreased functional activity as a result of exposure to cylindrospermopsin. By decreasing the concentrations of human serum albumin in the spermatozoa culture medium, it may be possible to determine if the presence of serum albumin inhibited the reduction of tetrazolium salts to formazan. Despite no adverse effects to membrane integrity of spermatozoa after acute exposure to CYN *in vitro* there is a chance of chronic exposure within the female reproductive tract where spermatozoa can survive for up to 4 days. The potential of decreased ATP concentrations in vitro may reflect decreases in motility and functionality in vivo which need to be further investigated.

CYN has the potential to adversely affect the seeds of tomorrow by accidental exposure through the one element we cannot live without- water.

Chapter 8. Appendices

8.1 Clinical Data from Entire Cohort of Women

Experimental End Point	Woman ID	Age of Patient	Previous Pregnancy?	Years of Infertility	BMI	Smoker
Optimisation	29	41	No	Infertility <1 year	20	Non-Smoker
Optimisation	30	32	No	Unknown	unknown	Unknown
Optimisation	32	33	No	Infertility <1 year	20	Non-Smoker
Optimisation	33	34	No	Infertility 2-5 years	unknown	Non-Smoker
Optimisation	34	35	No	Infertility <1 year	unknown	Unknown
Optimisation	35	44	No	Unknown	unknown	Unknown
Optimisation	36	28	No	Infertility <1 year	unknown	Unknown
Optimisation	37	32	No	Unknown	unknown	Unknown
Optimisation	39	42	No	Unknown	unknown	Unknown
Optimisation	41	39	No	Infertility 2-5 years	unknown	Unknown
Optimisation	42	39	No	Infertility > 6 years	31	Non-Smoker
Optimisation	44	36	No	Infertility 2-5 years	23	Non-Smoker
Optimisation	45	33	No	Infertility <1 year	unknown	Non-Smoker
Optimisation	46	31	No	Infertility 2-5 years	30	Smoker
Optimisation	47	30	No	Infertility <1 year	unknown	Non-Smoker
Optimisation	48	34	No	Infertility 2-5 years	unknown	Non-Smoker
Optimisation	49	43	No	Infertility 2-5 years	28	Non-Smoker
Optimisation	50	56	No	Infertility 2-5 years	unknown	Non-Smoker
Optimisation	51	29	No	Infertility 2-5 years	25	Non-Smoker
Optimisation	52	36	No	Infertility <1 year	unknown	Unknown
Optimisation	54	48	No	Infertility 2-5 years	unknown	Unknown
Optimisation	55	44	No	Infertility 2-5 years	20	Non-Smoker
Optimisation	31	36	Yes	Infertility <1 year	unknown	Non-Smoker
Optimisation	38	29	Yes	Infertility <1 year	28	Non-Smoker
Optimisation	40	35	Yes	Infertility 2-5 years	unknown	Non-Smoker
Optimisation	43	40	Yes	Unknown	31	Non-Smoker
Optimisation	53	34	Yes	Infertility 2-5 years	unknown	Non-Smoker
Optimisation	11	41	yes	Infertility <1 year	34	Non-Smoker
Optimisation	13	36	yes	Infertility <1 year	unknown	Unknown
CYN and GC	1	29	no	Infertility 2-5 years	27	Non-Smoker
CYN and GC	6	34	no	Infertility <1 year	18	Non-Smoker
CYN and GC	7	41	no	Infertility <1 year	24	Non-Smoker

CYN and GC	9	31	no	Infertility 2-5 years	unknown	Non-Smoker
CYN and GC	10	33	no	Unknown	unknown	Unknown
CYN and GC	12	38	no	Unknown	33	Non-Smoker
CYN and GC	14	27	no	Unknown	25	Unknown
CYN and GC	15	36	no	Unknown	23	Non-Smoker
CYN and GC	2	32	yes	Infertility 2-5 years	unknown	Unknown
CYN and GC	3	32	yes	Infertility 2-5 years	43	Non-Smoker
CYN and GC	4	37	yes	Infertility 2-5 years	18	Smoker
CYN and GC	5	33	yes	Infertility <1 year	29	Non-Smoker
CYN and GC	8	33	yes	Infertility > 6 years	22	Non-Smoker
PSI & Westerns	16	38	no	Infertility <1 year	25	Non-Smoker
PSI & Westerns	17	33	no	Infertility 2-5 years	unknown	Non-Smoker
PSI & Westerns	18	33	no	Infertility 2-5 years	unknown	Non-Smoker
PSI & Westerns	19	30	no	Infertility <1 year	29	Non-Smoker
PSI & Westerns	20	35	no	Infertility <1 year	29	Non-Smoker
PSI & Westerns	21	27	no	Unknown	35	Non-Smoker
PSI & Westerns	22	25	no	Infertility <1 year	20	Non-Smoker
PSI & Westerns	23	35	no	Unknown	unknown	Unknown
PSI & Westerns	24	36	no	Infertility 2-5 years	28	Non-Smoker
PSI & Westerns	25	28	no	Infertility 2-5 years	25	Non-Smoker
PSI & Westerns	26	43	no	Unknown	38	Smoker
PSI & Westerns	27	33	no	Infertility > 6 years	22	Smoker
PSI & Westerns	28	31	no	Infertility 2-5 years	29	Smoker

Table 8-1: ART Demographic and Clinical Variables of all women in Cohort

Experimental End Point	Woman ID	Previous Number of ART Cycles	Infertility Factor	Infertility Description	ART Procedure	Basal FSH (IU/L)	Total FSH Administered (units)	Maximum Plasma E_2 (pmol/L)
Optimisation	29	2	Both	Endometriosis & Spermatozoa	ICSI	6.00	1,300	11,153
Optimisation	30	1	Unknown	Normal Parameters	IVF	unknown	1,900	22,521
Optimisation	32	3	Male Factor	Spermatozoa	ICSI	7.00	1,700	4,522
Optimisation	33	1	Male Factor	Spermatozoa	IVF	9.00	1,250	3,341
Optimisation	34	1	Unknown	Unknown	IVF	unknown	2,000	8,504
Optimisation	35	8	Male Factor	Un Specified	ICSI	unknown	2,250	8,054
Optimisation	36	1	Male Factor	Un Specified	ICSI	unknown	1,100	6,419
Optimisation	37	1	Male Factor	Un Specified	ICSI	unknown	1,575	6,689
Optimisation	39	4	Unknown	Unknown	IVF	unknown	3,600	10,138
Optimisation	41	3	Male Factor	Un Specified	ICSI	unknown	2,400	2,321
Optimisation	42	3	Unknown	Unknown	IVF	8.00	4,900	4,552
Optimisation	44	2	Female Factor	Tubal Ligation	IVF	7.00	3,000	3,471
Optimisation	45	5	Both	Endometriosis & Normal Sperm Parameters	ICSI	8.00	3,180	6,689
Optimisation	46	1	Male Factor	Spermatozoa	ICSI	6.00	1,400	8,516
Optimisation	47	1	Male Factor	Spermatozoa	ICSI	14.00	1,800	1,892
Optimisation	48	1	Male	Donor Sperm	IVF	5.00	1,275	4,129

			Factor					
Optimisation	49	2	Female Factor	Endometriosis & Normal Sperm Parameters	ICSI	5.00	2,213	14,790
Optimisation	50	2	Male Factor	Spermatozoa & Ovarian Cyst	ICSI	7.00	2,400	8,722
Optimisation	51	3	Male Factor	Spermatozoa	ICSI	8.00	1,200	13,747
Optimisation	52	1	Unknown	Un Specified	IVF	unknown	900	5,085
Optimisation	54	8	Male Factor	Un Specified	ICSI	unknown	4,050	15,320
Optimisation	55	2	Male Factor	Spermatozoa	IVF	25.00	5,850	5,254
Optimisation	31	3	Unknown	Unknown	IVF	9.00	3,500	8,944
Optimisation	38	2	Male Factor	Spermatozoa & History of Pregnancy	ICSI	5.00	1,237	8,105
Optimisation	40	6	Male Factor	Donor Sperm & History of Pregnancy	ICSI	5.00	1,800	5,503
Optimisation	43	8	Male Factor	Spermatozoa	IVF	10.00	4,050	5,052
Optimisation	53	9	Female Factor	Endometriosis/Tubal Ligation/Ectopic/History of Pregnancy	ICSI	15.00	6,300	2,551
Optimisation	11	1	Male Factor	Spermatozoa/History of Pregnancy	ICSI	5.00	2,200	20,686
Optimisation	13	1	Male Factor	Spermatozoa/History of Pregnancy	ICSI	unknown	1,350	2,332

CYN and GC	1	1	Male Factor	Spermatozoa	ICSI	10.00	1,850	4,479
CYN and GC	6	1	Unknown	Normal Parameters/Anorexia	IVF	5.00	650	5,367
CYN and GC	7	1	Female Factor	Tubal Ligation	IVF	7.80	2,900	8,173
CYN and GC	9	6	Male Factor	Spermatozoa/Type II Diabetes (female)	ICSI	10.00	4,462	4,249
CYN and GC	10	1	Unknown	Normal Parameters	IVF	unknown	1,575	3,199
CYN and GC	12	2	Male Factor	Spermatozoa	ICSI	6.00	2,050	18,961
CYN and GC	14	4	Male Factor	Spermatozoa	ICSI	5.00	937	4,293
CYN and GC	15	1	Male Factor	Spermatozoa	ICSI	14.00	1,800	6,416
CYN and GC	2	5	Both	Ectopic Preg/Endometriosis/History of Pregnancy	ICSI	10.00	3,500	3,664
CYN and GC	3	6	Male Factor	Spermatozoa/History of Pregnancy	ICSI	5.00	3,500	3,664
CYN and GC	4	3	Both	Ectopic Preg/History of Pregnancy	ICSI	10.00	2,500	4,602
CYN and GC	5	1	Female Factor	Ectopic Pregnancy	IVF	unknown	1,300	9,869
CYN and GC	8	5	Male Factor	Spermatozoa/History of Pregnancy	ICSI	6.00	1,500	15,699

PSI & Westerns	16	5	Male Factor	Spermatozoa	ICSI	10.00	9,450	6,256
PSI & Westerns	17	4	Unknown	Normal Parameters	ICSI	5.00	1,875	2,315
PSI & Westerns	18	1	Unknown	Normal Parameters	ICSI	5.00	1,650	14,768
PSI & Westerns	19	4	Male Factor	Spermatozoa	ICSI	6.00	1,500	32,476
PSI & Westerns	20	1	Unknown	Normal Parameters	IVF	8.00	2,800	13,575
PSI & Westerns	21	1	Male Factor	Spermatozoa	ICSI	6.00	3,375	5,068
PSI & Westerns	22	3	Male Factor	Un Specified	ICSI	4.00	1,650	22,971
PSI & Westerns	23	5	Male Factor	Spermatozoa	ICSI	unknown	1,800	4,628
PSI & Westerns	24	2	Male Factor	Un Specified	ICSI	5.00	2,025	4,087
PSI & Westerns	25	7	Male Factor	Spermatozoa	ICSI	5.00	975	4,987
PSI & Westerns	26	3	Male Factor	Un Specified	ICSI	6.00	4,200	3,805
PSI & Westerns	27	15	Male Factor	Un Specified	ICSI	7.00	1,012	7,314
PSI & Westerns	28	3	Male Factor	Spermatozoa	ICSI	7.00	1,950	2,285

Table 8-1: ART Demographic and Clinical Variables cont

Experimental End Point	Woman ID	Follicular Fluid Tubes Collected	Number of Mature Follicles	Number Oocytes Aspirated	Granulosa Cells Isolated (cells/mL)	Pregnancy
Optimisation	29	3	6.00	11.00	1.7E+05	No
Optimisation	30	8	6.00	14.00	3.9E+05	No
Optimisation	32	4	4.00	6.00	4.0E+05	Yes
Optimisation	33	3	3.00	8.00	3.2E+05	Yes
Optimisation	34	8	2.00	24.00	7.9E+05	No
Optimisation	35	5	2.00	7.00	4.0E+05	No
Optimisation	36	6	2.00	11.00	7.9E+05	Yes
Optimisation	37	5	5.00	11.00	1.2E+06	No
Optimisation	39	5	5.00	3.00	3.9E+05	No
Optimisation	41	4	3.00	1.00	5.6E+05	No
Optimisation	42	4	5.00	7.00	3.2E+05	Yes
Optimisation	44	3	1.00	8.00	8.0E+05	No
Optimisation	45	7	5.00	9.00	3.0E+05	No
Optimisation	46	11	7.00	23.00	6.1E+05	Yes
Optimisation	47	3	3.00	2.00	9.6E+04	Yes
Optimisation	48	4	3.00	6.00	8.0E+05	Yes
Optimisation	49	16	8.00	30.00	4.2E+05	No
Optimisation	50	6	6.00	10.00	6.1E+05	No
Optimisation	51	7	4.00	14.00	5.9E+05	Yes
Optimisation	52	5	8.00	30.00	1.2E+06	No
Optimisation	54	2	2.00	14.00	5.6E+04	Yes
Optimisation	55	2	3.00	7.00	4.6E+05	No
Optimisation	31	6	7.00	5.00	2.8E+05	No
Optimisation	38	7	7.00	16.00	7.3E+05	No
Optimisation	40	6	5.00	18.00	7.3E+05	Yes
Optimisation	43	4	3.00	4.00	2.2E+05	No
Optimisation	53	4	4.00	6.00	5.5E+05	Yes
Optimisation	11	9	8.00	15.00	6.9E+05	Yes
Optimisation	13	2	2.00	4.00	3.4E+05	No
CYN and GC	1	6	5.00	7.00	7.6E+05	No
CYN and GC	6	5	8.00	8.00	6.5E+05	Yes
CYN and GC	7	6	6.00	8.00	8.4E+05	No
CYN and GC	9	7	7.00	10.00	3.2E+05	No
CYN and GC	10	7	5.00	5.00	3.1E+05	No
CYN and GC	12	9	7.00	21.00	6.4E+05	No
CYN and GC	14	8	5.00	14.00	3.7E+05	No
CYN and GC	15	7	5.00	12.00	1.6E+06	No
CYN and GC	2	5	3.00	7.00	6.1E+05	No
CYN and GC	3	5	4.00	7.00	6.1E+05	No
CYN and GC	4	4	5.00	6.00	6.0E+05	No
CYN and GC	5	6	2.00	13.00	1.1E+06	No
CYN and GC	8	10	10.00	12.00	1.6E+06	No
PSI & Westerns	16	5	3.00	6.00	9.4E+05	No

PSI & Westerns	17	5	3.00	6.00	1.2E+06	No
PSI & Westerns	18	21	5.00	32.00	4.2E+06	Yes
PSI & Westerns	19	17	12.00	14.00	7.9E+05	No
PSI & Westerns	20	10	9.00	6.00	1.2E+06	No
PSI & Westerns	21	8	10.00	14.00	2.2E+06	No
PSI & Westerns	22	12	19.00	22.00	1.8E+06	No
PSI & Westerns	23	5	7.00	11.00	9.0E+05	Yes
PSI & Westerns	24	8	4.00	7.00	1.8E+06	No
PSI & Westerns	25	7	12.00	12.00	1.8E+06	No
PSI & Westerns	26	3	2.00	3.00	8.8E+05	Yes
PSI & Westerns	27	6	6.00	14.00	1.3E+06	Yes
PSI & Westerns	28	3	3.00	4.00	6.4E+05	No

Table 8-1: ART Demographic and Clinical Variables cont

Table details individual clinical data. Total ART cases N=55; woman ID (note this is ID for identification within thesis); previous pregnancy (number of pregnancies prior to infertility treatment resulting in live births); Years of infertility (number of years patient has been clinically diagnosed as in/subfertile); BMI= body mass index (kg/m^2); smoker; Number of previous cycles (number of previous ART cycles undertaken); infertility factor (female or male infertility factor); infertility description (indicators of why in/subfertility is an issue); ART procedure (*in vitro* fertilisation or intracytoplasmic sperm injection); Basal FSH (IU/L) blood sample taken prior to commencement of treatment cycle; E_2 = maximum plasma estradiol concentrations (pmol/L); Number of Mature Follicles aspirated per woman for each cycle; Number of Oocytes aspirated refers to the total number of oocytes collected from cycle; Number of Follicular Fluid Tubes refers to number of tubes of follicular fluid collected from each woman on day of oocyte retrieval; Number of GC isolated refers to GC yield from density gradient centrifugation

8.2 Reagents, Stock Solutions & Media Preparation

8.2.1 Reagents

Chemical/ Product	Source
Acetic Acid-glacial	Aldrich Chemicals, St Louis, MI, USA
Albumin from Bovine Serum Fraction V minimum 96% lyophilized powder	Sigma-Aldrich, St Louis, MI, USA
Ammonium Persulphate	BioRad, Vic Australia
Arcylamide Bis (30%)	BioRad, Vic Australia
Crystal Violet	Aldrich Chemicals, St Louis, MI, USA
DMEM/HAMS F12 (with Hepes, glutamine & no sodium bicarbonate)	Invitrogen, Australia
Donkey anti-Sheep/Goat (DAG)	Chemicon (Millipore), Australia
Estradiol ^{125}I Tracer	MP Biomedicals, Australia
Fetal Calf Serum	(GIBCO) Invitrogen, Australia
Formalin	Sigma-Aldrich, St Louis, MI, USA
Glycine	Sigma-Aldrich, St Louis, MI, USA
Hydrochloric Acid(12M):	Aldrich Chemicals, St Louis, MI, USA
Insulin	Sigma-Aldrich, St Louis, MI, USA
KCl	Sigma-Aldrich, St Louis, MI, USA
Lymphoprep (Ficoll-Hypaque)	GE Healthcare, Australia

Methanol	Aldrich Chemicals, St Louis, MI, USA
Neutral Red (1% v/v)	Department of Anatomical Physiology & Pathology, Flinders Medical Centre
PEG (Polyethylene glycol)	(BDH) Merck Pty Ltd, Australia
Penicillin/Streptomycin	Invitrogen, Australia
Primary antibody (Sheep polyclonal to Progesterone)	MP Biomedicals, Australia
Progesterone	Sigma-Aldrich, St Loui, MI, USA
Progesterone I^{125} Tracer	MP Biomedicals, Australia
Sheep Anti-rabbit Serum (SAR)	Chemicon (Millipore), Australia
Skim Milk	Diploma- Local Supermarket
Sodium Bicarbonate (NaH_2CO_3)	Pfizer, Australia
KH_2PO_4	Sigma-Aldrich, St Louis, MI, USA
Merthiolate (Thimerosal)	Sigma-Aldrich, St Louis, MI, USA
$Na_2H_2PO_4$	Sigma-Aldrich, St Louis, MI, USA
Na_2HPO_4 (disodium hydrogen orthophosphate)	Sigma-Aldrich, St Louis, MI, USA
NaCl	Sigma-Aldrich, St Louis, MI, USA
$NaH_2PO_4.2H_2O$ (sodium dihydrogen orthophosphate)	Sigma-Aldrich, St Louis, MI, USA
Sodium Bicarbonate ($NaHCO_3$)	Sigma-Aldrich, St Louis, MI, USA
Sodium dodecyl sulphate	Sigma-Aldrich, St Louis, MI, USA
Sodium Selenite	Sigma-Aldrich, St Louis, MI, USA

TEMED	Sigma-Aldrich, St Louis, MI, USA
Thiazolyl Blue Tetrazolium (MTT):	Sigma-Aldrich, St Louis, MI, USA
Transferrin	Sigma-Aldrich, St Louis, MI, USA
Trizma Base	Sigma-Aldrich, St Louis, MI, USA
Tryp/EDTA (0.5g/L porcine trypsin/ 0.2g/L EDTA	Sigma-Aldrich, St Louis, MI, USA
Trypan Blue	Sigma-Aldrich, St Louis, MI, USA
Tween 20	Sigma-Aldrich, St Louis, MI, USA

8.2.2 Media

Granulosa Cell Base Medium

Preparation of wash medium (DMEM/F-12) with no foetal bovine serum (FBS) for a 5L volume:

- 60 g DMEM/HAMS F12 powder (Gibco BRL, Invitrogen Life Sciences, Mount Waverly, Vic., Australia) (12 $g.L^{-1}$ final concentration)
- 6 g of Sodium Bicarbonate $NaHCO_3$
- Streptomycin (SIGMA, Australia 5000 $\mu g.mL^{-1}$)
- Penicillin (SIGMA, Australia 5000$IU.mL^{-1}$)

Wash medium DMEM/F12 was filter sterilised Nitrogen 4.0 gas cylinder. A vacuum tank and medium tank from were used. 1000ml irrigation bottles were used to make up to 5L. The solution's pH was set to 7.4. The media was then sterilised.

Prior to use, wash media was further filter sterilised using a 250mL filter steriliser using 0.22 µm filter.

Spermatozoa QAHM's Medium

Media was purchased pre-prepared:

QAHM's®, SAGE Media In-Vitro Fertilisation Inc, CT, USA

Spermatozoa Hams F12 Medium

Preparation of Hams F12 medium (with 25mM HEPES, 1.176g/L Sodium Bicarbonate and 5% Human Serum Albumin) for a 500mL volume:

- 5.35g HAMS F12 powder (10.64 g/L modified with L-Glutamine and without Sodium Bicarbonate MP Biomedicals, Australia)dissolved in 450mL MQ water

- 2.98g HEPES
- 7mL of Sodium Bicarbonate $NaHCO_3$ (8.4% v/v)
- Adjusted volume to 500mL

Media was filter sterilised using 500mL filter with 0.2µM filter (Millipore, Australia). The solution's pH was set to 7.4 using potassium hydroxide.

For use in culture Human Serum Albumin added to final concentration of 5%. Media was equilibrated in humidified incubator with 5% CO_2 prior to use.

Full Granulosa Cell Medium

- Fetal Bovine Serum at 10% (Gibco BRL, Invitrogen Life Sciences, Mount Waverly, Vic., Australia) of final volume
- 1ml of 100 × ITS stock (Insulin 0.5 $mg.mL^{-1}$, Transferrin 0.5 $mg.mL^{-1}$, Selenium 0.5 $mg.mL^{-1}$, Invitrogen Life Technologies, Mount Waverly, Australia)
- 89 mL 'Granulosa Base Medium' DMEM/F12

These are then filter sterilised using a 250 mL filter steriliser prior to use and can be stored at -20°C until use.

8.2.3 Stock Solutions

100 × Insulin/Transferrin/Selenium (ITS) Stock

Dissolve 20 µl selenium, 20 mg transferrin and 2 mL insulin in 40 mL DMEM/F12, filter sterilise (0.2 µm), aliquot into 1.5 mL eppendorfs. Stored at -20°C.

Heat Inactivation of Fetal Calf Serum

Heat 500 mL bottle of FCS at 56°C for 30 min. Aliquot into 10 mL tubes (aseptically). Store at -20°C

10 × Phosphate Buffered Saline (PBS)

Reagents/Product	Grams/Litre
NaCl	**80**
KCl	**2**
$Na_2H_2PO_4$	**14.4**
KH_2PO_4	**2.4**

Dissolve salts in 900 mL M.Q. H_2O
Adjust pH to 7.4
Make up to 1 L with M.Q. H_2O
Store at room temperature

10 × MTT (5 mg/mL)

Dissolve 500 mg in 100 mL 1× PBS
Filter Sterilise
Aliquot into 1 mL
Store at -20^{o}C

Trypan Blue

Dissolve 0.9 g sodium chloride in 90 mL M.Q. H_2O (salt solution)
Dissolved 0.2 g Trypan Blue in salt solution
Made up to 100 mL
Filtered
Stored at room temperature

20% Sodium dodecyl solution in 0.2 M HCl

Reagents/Product	Concentration
SDS	**20% (w/v)**
HCl	**0.2 M**

Dissolve 20 g of SDS in 100 mL 0.2 M HCl (with gentle heating)
Store at room temperature

Crystal violet

Reagents/Product	Volume
0.5% (w/v) Crystal Violet	**0.5 g**
50% Methanol (1:1 methanol: M.Q.H_2O)	**100 mL**

Dissolve Crystal Violet in 50% methanol.
Store at room temperature

33% Acetic acid (100mL)

Mix 33 mL of acetic acid with 67 mL of M.Q.H_2O. Store at room temperature.

Sperm diluent

Reagents/Product	Volume
0.05%(w/v)Sodium Bicarbonate ($NaHCO_3$)	**0.5 g**
0.01%(v/v)Formalin/Formaldehyde	**100 mL**

Dissolve 5 g $NaHCO_3$ 80 mL M.Q.H_2O, add 1 mL Formalin
Make up to 100 mL
Autoclave
Store at room temperature

8.3 Calculations and Conversions

Spermatozoa Conversion Table

Dilution	25 squares	10 squares	5 squares
undiluted	Divide by 100	-	-
1:2	Divide by 50	Divide by 20	Divide by 10
1:5	Divide by 20	Divide by 8	Divide by 4
1:10	Divide by 10	Divide by 4	Divide by 2
1:20	Divide by 5	Divide by 2	Divide by 1
1:50	Divide by 2	Divide by 0.8	Divide by 0.4

Figure 8-1: Calculation of Live Spermatozoa Concentration (sperm/mL)

When determining spermatozoa concentration, if there is a high concentration of spermatozoa then less number of squares are counted as described in 2.8.5 Eosin Y Assay, thus calculations are adjusted according to the number of squares counted.

Mix a 40 µL sample of spermatozoa sample with 20 µL of Eosin Y for 30sec.

Add 20 µL of Sperm diluent, giving a total of 1:2

Count the number of pink and white spermatozoa in 10 µL aliquot on haemocytometer (in central 25 square) until total spermatozoa counted is 200.

Hence if all 25 squares counted at 1:2 dilution and obtained 20 pink (dead) sperm and 40 white (live) sperm then the concentration of live sperm is (40/50)= 0.8 x 10^6 sperm/mL

MTT/MTS/Crystal Violet Assay Standard Curve and Determination of Number of Viable Cells

The standard curve was derived by creating a graph which had a Y-axis representing the absorbance obtained and an X-axis representing the cells per well concentration. The average absorbance obtained for the six-replicate wells per cell concentration was graphed. A linear regression trendline was added to the graph from which the R-squared coefficient (R^2) was displayed. The R-squared value is an indicator from 0 to 1 that shows how closely the estimated values for the trendline correspond to the actual data. The trendline was said to be reliable when its R-squared value was greater than that of 0.98 as it produced the most linear standard plot (Young et al 2004).

The linear region of the plot was used to calculate the relationship between optical density and cell number by using the form of

$$y = mx + c$$

where "y" was equal to the optical density, "m" was equal to gradient of the straight line and "c" was equal to the intersection with the Y-axis.

This equation was used to calculate the number of viable cells in each well after the cells had undergone their respective treatments.

8.4 ImageJ Analysis

Images taken on BX50 Brightfield Microscope were scored for ORO Lipid Staining and 3βHSD staining using ImageJ software (Wright Cell Imaging Facility, University Health Network Research, Toronto, Canada):

ImageJ Analysis software was downloaded from:
http://www.uhnres.utoronto.ca/facilities/wcif/fdownload.html

1. Image was opened and "Plugins" was selected from the toolbar menu
2. The "particle analysis" option was selected
3. The cell counter option in "particle analysis" was selected.
4. A new window of the same image was opened, with additional buttons at the bottom of the window. This was called the counter window of (image name). An additional window, the results window, was used to tabulate the counts generated.
5. At the bottom of the counter window four buttons each assigned with a colour, red, green, blue and yellow, and a fifth button is labelled results.
6. When analysing ORO images
    a. The red button was assigned to cells that did not stain with ORO and thus are referred to as ORO negative cells.
    b. The green button was assigned to cells that did stain with ORO and thus are referred to as ORO positive cells.
7. When analysing main 3βHSD images:
    a. Negative 3βHSD large cells (red button, Table 2-6)
    b. Light stained 3βHSD large cells (green button, Table 2-6)
    c. Intense stained 3βHSD large cells (blue button, Table 2-6)
    d. Small Dense Cells (yellow button, Table 2-6)

    NB a separate set of images were analysed for Negative staining (red button); very light staining (green button) or light staining (blue button)
8. How to use the colour coded buttons:
    a. If the red button was pressed then any cells in the image selected (for example an ORO negative cell with no visible staining of ORO droplets in cells) then the cell will have a red dot placed on it, this will then be simultaneously recorded in the results window.
    b. After "red button" selection was completed, the next coloured button would follow in which the results will be simultaneously recorded in the results window.
9. When the whole image has been scored then the results were tallied, providing an average of all red, green, blue and yellow counts in selected in the image. This is done by pressing the results button in the counter window.
10. Data was then saved as an Microsoft Excel file (.xls)
11. This was repeated for 4 images (repeat) per treatment per time point for each woman.
12. Data was then exported to SPSS for statistical analysis.

8.5 SDS-Reagents & Western Immunoblotting Buffers

Below details the buffers and reagents used in semi-quantification of CYP11A1 protein.

Separating Gel (12%)

Reagent	Volume
M.Q H_2O	3.4 mL
Solution A Gel Buffer (pH8.8)	2.5 mL
10%SDS	0.1 mL
Acrylamide Bis (30%)	4.0 mL
10%APS	50 µl
TEMED	5 µl

Stacking Gel (4%)

Reagent	Volume
M.Q H_2O	6.1 mL
Solution B Gel Buffer (pH6.8)	2.5 mL
10%SDS	0.1mL
Acrylamide Bis (30%)	1.3 mL
10%APS	50 µL
TEMED	10 µL

3x Loading Buffer

Reagents	Amount
M.Q H_2O	3.55 mL
0.5M Tris-HCl, pH6.8	1.25 mL

Glycerol	**2.5 mL**
10% (w/v) SDS	**2 mL**
0.5% (w/v) bromophenol blue	**0.2 mL**

Mix 475 µL loading Buffer + 25 µL of 2-mercaptoethanol prior to mixing with sample

Solution A Gel Buffer (1.5M Tris-HCl, pH 8.8)

Reagents	Amount
Tris-Base	**90.86 g**
M.Q H_2O	**400 mL**

Adjust to pH 8.8 with HCl.
Bring 500 mL with M.Q H_2O
Store at 4^{o}C.

Solution B Gel Buffer (0.5M Tris-HCl, pH 6.8)

Reagents	Amount
Tris-Base	**30.29 g**
M.Q H_2O	**400 mL**

Adjust pH 6.8 with HCl.
Bring to 500 mL with M.Q H2O
Store at 4^{o}C.

10% Ammonium Persulphate

Reagents	Amount
Ammonium Persulphate	**100 mg**
M.Q H_2O	**1 mL**

Running Buffer:

Reagent	Amount
Tris Base(15 g/L)	**30.3 g**
Glycine(72 g/L)	**144.0 g**
SDS(5 g/L)	**10.0 g**
M.Q H_2O	**1 L**

Transfer Buffer

Reagent	Amount
25 mM Tris Base	3.03 g
192 mM Glycine	14.4 g
20% (v/v) Methanol	200 mL
M.Q H_2O	800 mL

Do not adjust the pH
Store at 4^{o}C.

Blocking Buffer

Reagent	Amount
40 mM Tris	2.422 g
5% (w/v) Skim Milk	25 g
0.1% (v/v) Tween 20	0.5 mL
M.Q H_2O	500 mL

Dissolve powders in M.Q H_2O
Adjust pH 7.4 with concentrated HCl,
Add the Tween20
Store at 4^{o}C (maximum 1day).

Wash Buffer

Reagents	Amount
20 mM Tris Base	**2.42 g**
150 mM NaCl	**8.77 g**
5% (w/v) Skim Milk Powder(g)	**50 g**
0.1% (v/v) Tween20	**1 mL**

Dissolve powders in M.Q H_2O
Adjust pH 7.4 with concentrated HCl
Add the Tween20
Store at 4^{o}C

10 x Tris-Tween Buffer

Reagent	Amount
40 mM Tris Base	48.46 g
0.1% (v/v Tween 20	10 mL
M.Q H_2O	1 L

Dissolve powders in M.Q H_2O
Adjust pH 7.4 with concentrated HCl,
Add the Tween20
Store at 4^{o}C

Ponceau S Stain

Reagent	Amount
0.2% (w/v) Ponceau S	**20 g**
10% acetic acid	**10 mL**

Dissolve Ponceau S in 10% acetic acid

Store at room temperature.

10% SDS:

Reagents/Product	Amount
10% (w/v)SDS	**10 g**
M.Q H_2O	**90 mL**

Dissolve powders in M.Q H_2O
Store at room temperature

Stripping Buffer

Reagents/Product	Amount
100 mM B-mercaptoethanol	**698 µL**
2% w/v Sodium Dodecyl Sulphate (SDS)	**2.0 g**
62.5 mM Tris-HCl (pH: 6.7)	**0.757 g**

Dissolve Tris-HCl in 80 mL M.Q. H_2O
Adjust pH to 6.7 with NaOH
Add 2.0g SDS
Make up to 100 mL with M.Q. H_2O
Add 698 µ: of β-mercaptoethanol
Store room temperature

8.6 Radioimmunoassay Reagents

8.6.1 Common Reagents

Normal Rabbit Serum (NRS)

Final concentration 1:100
Store at 4^{o}C

PEG (Polyethylene glycol)

Dissolve 164 g in 1 L M.Q.H_2O
Store at 4^{o}C

NaFam Buffer

Reagents/Product	Amount
$NaH_2PO_4.2H_2O$ (sodium dihydrogen orthophosphate)	1.86 g
Na_2HPO_4 (disodium hydrogen orthophosphate)	4.581 g
Merthiolate (Thimerosal)	0.24 g
NaCl	6 g
EDTA (ethlenediamine-tetraacetic acid)	3.274 g
RO H_2O	1 L
BSA (albumin from Bovine Serum)	2.5 g

Dissolve all reagents except the BSA in 1 L M.Q.H_2O
Adjust pH to 7.4 with NaOH.
Add 2.5 g of BSA to 1 L of NaFam buffer
Store at room temperature

BioRad Quality Control 1, 2 and 3

Hormone	Quality Control	Mean	Range
Progesterone	QC 1	0.94 ng/mL	0.38-1.50 ng/mL
	QC 2	6.54 ng/mL	4.25-8.3 ng/mL
	QC 3	16.7 ng/mL	10.9-22.5 ng/mL
Estradiol	QC 1	83.2 pg/mL	22.5-144 pg/mL
	QC 2	189 pg/mL	66.2-312 pg/mL
	QC 3	416 pg/mL	191-641 pg/mL

Table 8-2: BioRad Assay Quality Control Concentrations

The BioRad Quality controls (QC 1, 2 and 3) were used for both progesterone and estradiol radio immunoassays. To ensure that the standard curve produced from either progesterone or estradiol standards used are reliable the concentrations of progesterone and estradiol for each of the QC need to match the above levels.

8.6.2 Progesterone Reagents

Progesterone ^{125}I Tracer (1.1 ml/vial 10µCi)

Determine counts/min
Dilute to 10,000 counts/ 100 µL/ sample tube
Store at 4^{o}C

Progesterone Standards Range

Standards of 0,1,2,5,50,500,1000, and 2000 ng/mL made from Progesterone (see Reagents, Stock Solutions & Media Preparation)

Make up in DMEM/F12 + 10%FCS + ITS
Store at 4^{o}C

Progesterone Primary Antibody

Primary antibody (Rabbit polyclonal to Progesterone)
Dilute to final concentration of 1:5000 per 100 µL/sample tube in NaFam buffer
Store at 4^{o}C

Sheep Anti-rabbit Serum (SAR- 18-36 mg/mL protein)

Dilute 1:30 per 100 µL/sample tube in NaFam buffer
Store at 4^{o}C

Progesterone RIA Experimental Flow Diagram

Tube #	Woman	Time Period	Tube Contents	Step 1 Add Assay Buffer	Step 2-3 Add Standard/Sample	Step 4 Add 125I Progesterone	Step 5 Add Progesterone Antibody	Step 6	Step 7-9 Add NRS	Step 7-9 Add SAR	Step 7-9 Add PEG	Step 10	Step 11-12
1,2	-	-	Total Counts	-	-	100ul	-	Vortex, incubate for 60min at 37C in heated waterbath	-	-	-	Vortex, incubate for 30min at room temperature	Centrifuge at 3000rpm, 4C for 30min, aspirate supernantent. Count the residue for 1min using protocol 7
3,4	-	-	Blank (NSB)	200ul	25ul		100ul of 1:8000		100ul	100ul	200ul		
5,6	-	-	std 0ng/ml	100ul									
7,8	-	-	std 1ng/ml										
9,10	-	-	std 2ng/ml										
11,12	-	-	std 5ng/ml										
13,14	-	-	std 50ng/ml										
15,16	-	-	std 100ng/ml										
17,18	-	-	std 500ng/ml										
19,20	-	-	std 1000ng/ml										
21,22			std 2000ng/ml										
23,24	-	-	BioRad QC1										
25,26	-	-	BioRad QC2										
27,28	-	-	BioRad QC3										
29	1	24	Media										
30													
31													

Figure 8-2: Progesterone RIA Experimental Flow Diagram

The progesterone RIA experimental flow diagram is a step by step outline of the order in which reagents were added to each tube to determine progesterone concentration in samples. Each sample was identified with a tube number. Assay controls: total counts (progesterone tracer only), Non-specific binding tubes (NSB tubes without primary antibody), progesterone standards (0-2000 ng/mL) and BioRad QC1-3 controls were assayed in duplicate. Sample tubes were assayed in triplicate. Time period refers to which post-treatment media was collected from. Flow diagram then shows the order (steps 1 through to 12) in which reagents were added, respective volumes, incubation periods and technique involved.

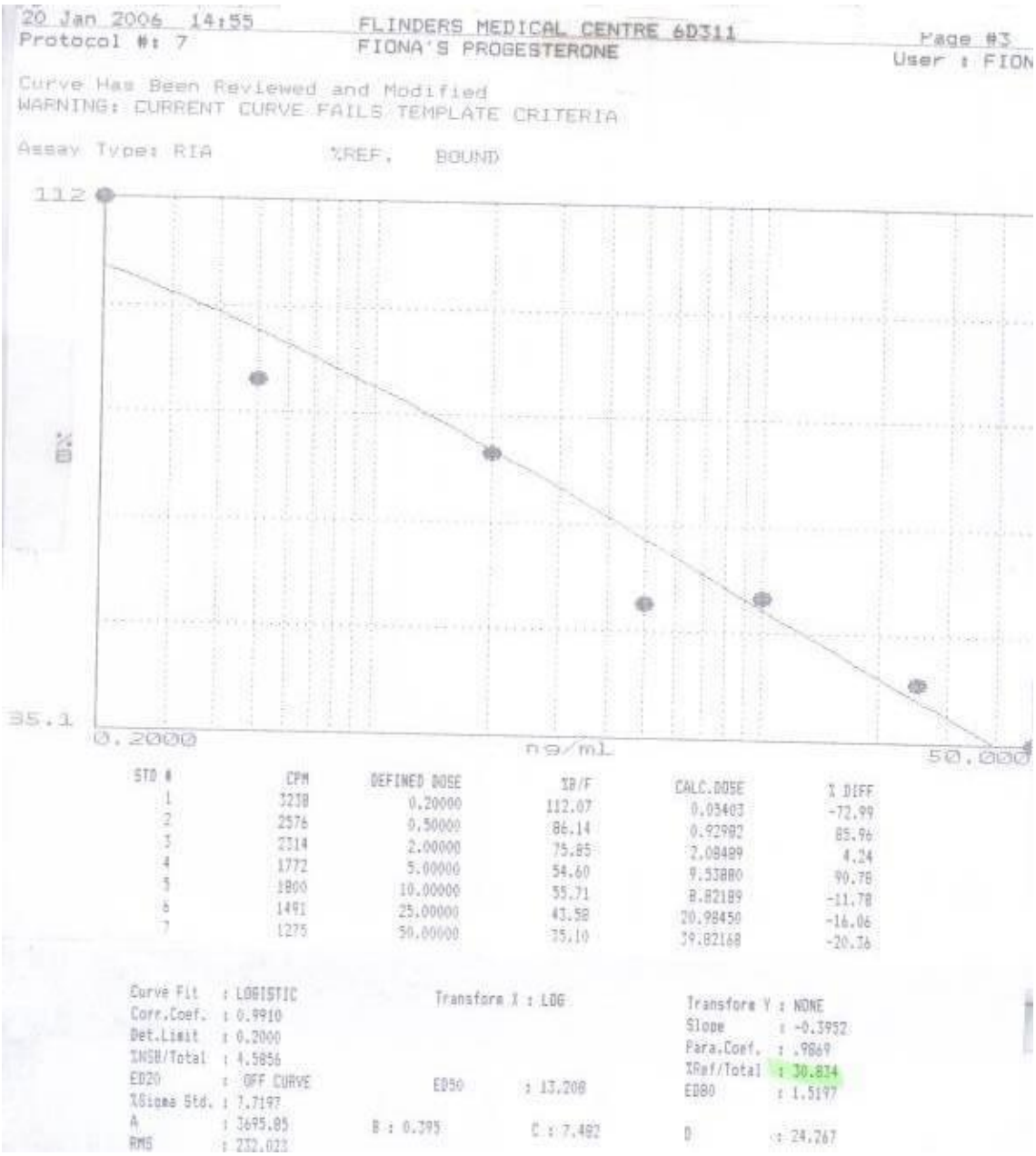


Figure 8-3: Progesterone RIA Standard Curve

X-axis represents concentration of progesterone standards (ng.mL^{-1}) with the y-axis representing % of bound progesterone tracer, hence as progesterone concentration in sample or standard increases the amount of progesterone ^{125}I tracer bound in the fraction decreases. The %Ref/Total refers to the binding of progesterone ^{125}I tracer that can bind from the total amount present. Values should not exceed 50.0%. The %NSB/total is non-specific binding of total amount of total progesterone present in the form of ^{125}I tracer; this value should remain below 10%.

8.6.3 Estradiol Reagents

Estradiol ^{125}I Tracer (1.1 ml/vial 10 µCi)

Determine counts/min
Dilute to 10,000 counts/ 100 µL/sample tube
Store at 4°C

Estradiol Standards

0, 20, 50, 250, 750, and 2000 pg/mL (stock of 4 x 10^6 pg/mL)
Make up in DMEM/F12 + 10% FCS + ITS
Store at 4°C

Primary antibody (Goat polyclonal to 17β estradiol)

Dilute to final concentration of 1:100,000 per 100 µL/sample tube in NaFam buffer
Store at 4°C

Secondary antibody (Donkey anti-sheep, 36 mg/mL total protein)

Dilute 1:30 per 100 µL/sample tube in NaFam buffer
Store at 4°C

Estradiol RIA Experimental Flow Diagram

				Step 1	Step 2-3	Step 4	Step 5	Step 6	Step 7-9			Step 10	Step 11-12
Tube #	Woman	Time Period	Tube Contents	Add Assay Buffer	Add Standard/Sample	Add 125I Progesterone	Add Progesterone Antibody		Add NRS	Add SAR	Add PEG		
1,2	-	-	Total Counts	-	-	100uL	-	shake 20-30 sec, incubate for 18-24hrs at 4-8C in cold room 6E310	-	-	-	Vortex, incubate for 30min at room temperature	Centrifuge at 3000rpm, 4C for 30min, aspirate supernantent. Count the residue for 1min using protocol 7
3,4	-	-	Blank (NSB)	200uL	25ul		-		100uL	100uL	200uLl		
5,6	-	-	Std 0ng/ml	100uL			100uL						
7,8	-	-	std 20pg/mL										
9,10	-	-	std 50pg/mL										
11,12	-	-	std250pg/ml										
13,14	-	-	std 750pg/ml										
15,16	-	-	std 2000 pg/ml										
17-18	-	-	BioRad QC1										
19-20	-	-	BioRad QC2										
21-22	-	-	BioRad QC3										
23													
24		24	Media										
25													

Figure 8-4: Estradiol RIA Experimental Flow Diagram

The Estradiol RIA experimental flow diagram is a step by step outline of the order in which reagents were added to each tube to determine estradiol concentration in samples. Each sample was identified with a tube number. Assay controls: total counts (estradiol tracer only), Non-specific binding tubes (NSB tubes without primary antibody), estradiol standards (0-2000 pg/mL) and BioRad QC1-3 controls were assayed in duplicate. Sample tubes were assayed in triplicate. Time period refers to which post-treatment media was collected from. Flow diagram then shows the order (steps 1 through to 12) in which reagents were added, respective volumes, incubation periods and technique involved.

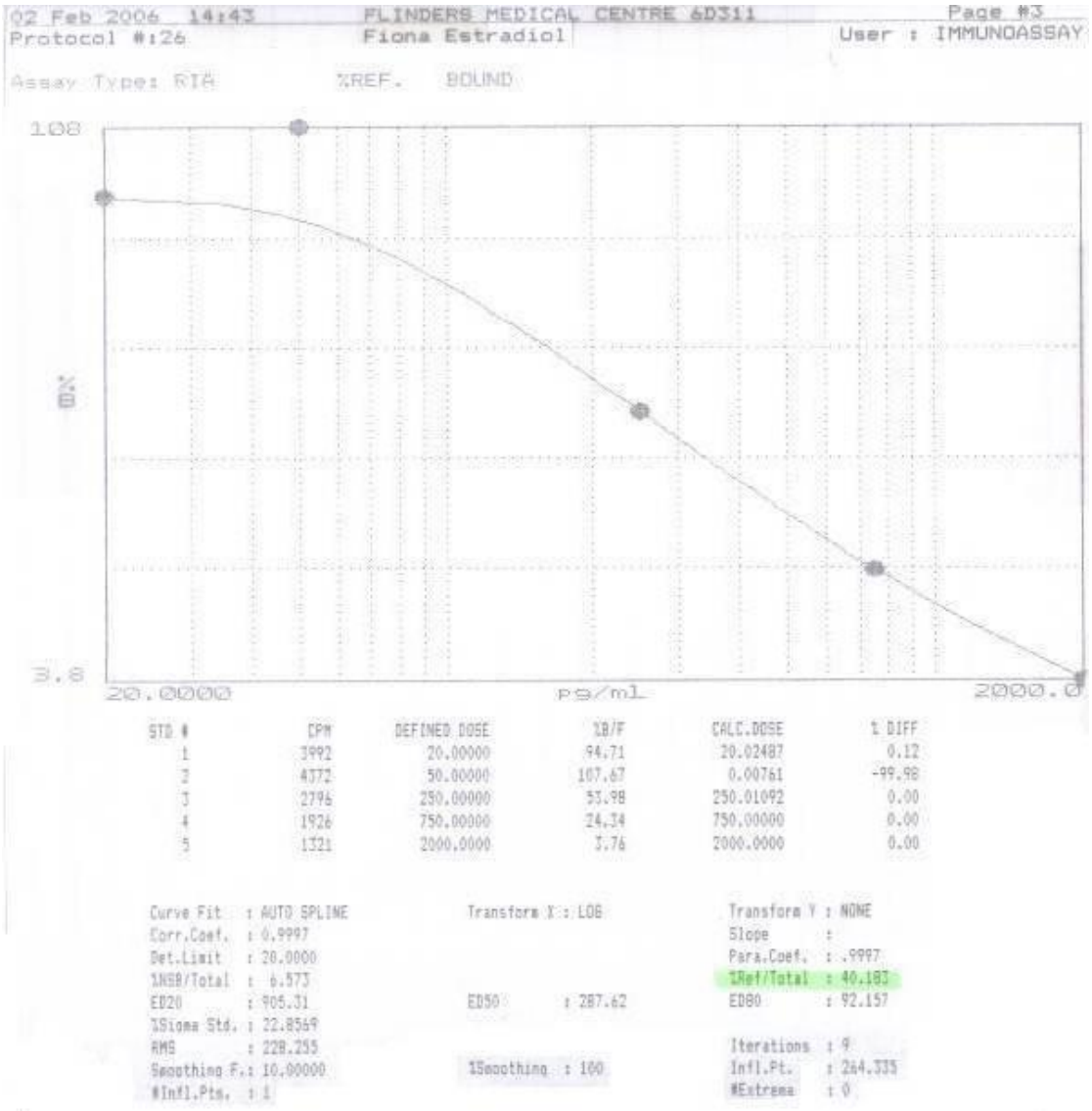


Figure 8-5: Estradiol RIA Standard Curve

X-axis represents concentration of estradiol standards (pg.mL^{-1}) with the y-axis representing % of bound estradiol tracer, hence as estradiol concentration in sample or standard increases the amount of estradiol ^{125}I tracer bound in the fraction decreases. The %Ref/Total refers to the binding of estradiol ^{125}I tracer that can bind from the total amount present. Values should not exceed 50.0%. The %NSB/total is non-specific binding of total amount of total estradiol present in the form of ^{125}I tracer; this value should remain below 10%.

.

8.7 KGN MTT Standard Curve

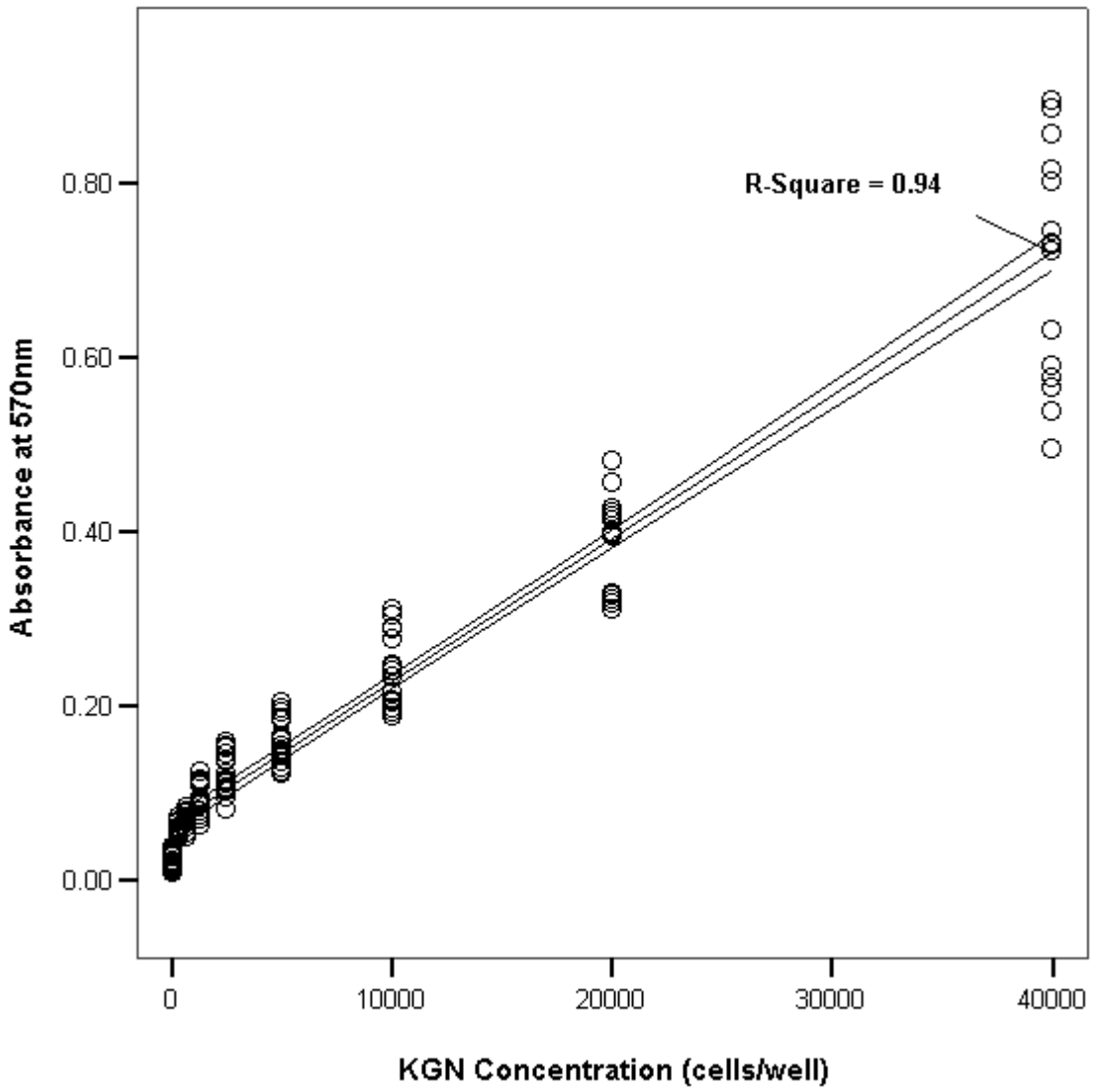


Figure 8-6: MTT Standard Curve for KGN Cells

KGN (n=3) seeded at a densities range of 0 to 4× 10^4 cells/well were allowed to adhere for 24 h in DMEM/F12 + 10% FCS. KGN processed for MTT assay salt (0.5 mg/mL, n=16) for 24. Scatterplot shows linear regression of mean absorbance (570nm) ± 1 SEM to KGN concentration (cells/well). This was carried out by Vicki Edwards (2008), Department of Medical Biotechnology, Health Sciences, Flinders University Australia. This figure refers to section 3.3.5.3 KGN Cells.

Chapter 9. References

Acosta, AA & Kruger, TF 1996, 'The Human Sperm Acrosome Reaction', in *Human Spermatozoa in Assissted Reproduction*, Informa Health Care, pp. 228-33.

Adler, V, Yin, Z, Tew, KD & Ronai, Z 1999, 'Role of redox potential and reactive oxygen species in stress signaling', *Oncogene*, vol. 18, no. 45, pp. 6104-11.

Aharoni, D, Dantes, A, Oren, M & Amsterdam, A 1995, 'cAMP-mediated signals as determinats for Apoptosis in primary granulosa cells', *Experimental Cell Research*, vol. 218, pp. 271-82.

Ahmad, S, Ahmad, A, Schneider, BK & White, CW 2006, 'Cholesterol Interferes with the MTT Assay in Human Epithelial-Like (A549) and Endothelial (HLMVE and HCAE) Cells', *International Journal of Toxicology*, vol. 25, no. 1, pp. 17-23.

Aitken, RJ & Baker, MA 2006, 'Oxidative stress, sperm survival and fertility control', *Molecular and Cellular Endocrinology*, vol. 250, no. 1-2, pp. 66-9.

Aitken, RJ, Gordon, E, Harkiss, D, Twigg, JP, Milne, P, Jennings, Z & Irvine, DS 1998, 'Relative Impact of Oxidative Stress on the Functional Competence and Genomic Integrity of Human Spermatozoa', *Biology and Reproduction*, vol. 59, no. 5, pp. 1037-46.

Aitken, RJ, Harkness, D & Buckingham, DW 1993, 'Analysis of lipid peroxidation mechanisms in human spermatozoa', *Molecular Reproduction and Development*, vol. 35, pp. 302 - 15.

Aitken, RJ, Koopman, P & Lewis, SEM 2004, 'Seeds of concern', *Nature*, vol. 432, no. 7013, pp. 48-52.

Aitken, RJ, Wang, YF, Liu, J, Best, F & Richardson, DW 1983, 'The influence of medium composition, osmolarity and albumin content on the acrosome reaction and fertilizing capacity of human spermatozoa: development of an improved zona-free hamster egg penetration test', *International Journal of Andrology*, vol. 6, no. 2, pp. 180-93.

Alexopoulos, E, Shahid, J, Ongley, HZ & Richardson, MC 2000, 'Luteinized human granulosa cells are associated with endogenous basement membrane-like components in culture', *Molecular Human Reproduction*, vol. 6, no. 4, pp. 324-30.

Alrefai, W & Gill, R 2007, 'Bile Acid Transporters: Structure, Function, Regulation and Pathophysiological Implications', *Pharmaceutical Research*, vol. 24, no. 10, pp. 1803-23.

Alviggi, C, Humaidan, P, Howles, CM, Tredway, D & Hillier, SG 2009, 'Biological versus chronological ovarian age: implications for assisted reproductive technology', *Reproductive Biology and Endocrinology*, vol. 7, pp. 101-13.

Amann, RP 1981, 'A Critical Review of Methods for Evaluation of Spermatogenesis from Seminal Characteristics', *Journal of Andrology*, vol. 2, pp. 37-58.

Amsterdam, A, Gold, RS, Hosokawa, K, Yoshida, Y, Sasson, R, Jung, Y & Kotsuji, F 1999, 'Crosstalk Among Multiple Signaling Pathways Controlling Ovarian Cell Death', *Trends in Endocrinology and Metabolism*, vol. 10, no. 7, pp. 255-62.

Amsterdam, A & Sasson, R 2004, 'Generation and Application of Ovarian Steroidogenic Cell Lines', in E Adashi & A Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California, pp. 389-409.

Antczak, M & Van Blerkom, J 2000, 'The vascular character of ovarian follicular granulosa cells: phenotypic and functional evidence for an endothelial-like cell population', *Human Reproduction*, vol. 15, no. 11, pp. 2306-18.

Arnould, R, Dubois, J, Abikhalil, F, Libert, A, Ghanem, G, Atassi, G, Hanocq, M & Lejeune, FJ 1990, 'Comparison of Two Cytotoxicity Assays-Tetrazolium Derivative Reduction (MTT) and Tritiated Thymidine Uptake-on Three Malignant Mouse Cell Lines Using Chemotherapeutic Agents and Investigational Drugs', *Anticancer Research*, vol. 10, pp. 145-54.

Arthur, JR & Boyd, GS 1974, 'The Effect of Inhibitors of Protein Synthesis on Cholesterol Side-Chain Cleavage in the Mitochondria of Luteinized Rat Ovaries', *European Journal of Biochemistry*, vol. 49, no. 1, pp. 117-27.

Asboth, G, Price, S, Bellinger, J, Ledger, W, Barlow, D & Bernal, A 2001, 'Characterization of adenylyl cyclases in cultured human granulosa cells', *Reproduction*, vol. 121, no. 2, pp. 217-28.

Aziz, DM 2006, 'Assessment of bovine sperm viability by MTT reduction assay', *Animal Reproduction Science*, vol. 92, no. 1-2, pp. 1-8.

Aziz, DM, Ahlswede, L & Enbergs, H 2005, 'Application of MTT reduction assay to evaluate equine sperm viability', *Theriogenology*, vol. 64, no. 6, pp. 1350-6.

Bain, P 2007, 'Gene Expression Profiling of Cylindrospermopsin Toxicity', Doctor of Philosophy thesis, Griffith University.

Bain, P, Shaw, G & Patel, B 2007, 'Induction of p53-Regulated Gene Expression in Human Cell Lines Exposed to the Cyanobacterial Toxin Cylindrospermopsin', *Journal of Toxicology and Environmental Health, Part A*, vol. 70, no. 19, pp. 1687 - 93.

Baker, P & Humpage, A 1994, 'Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray-Darling Basin, Australia', *Marine and Freshwater Research*, vol. 45, no. 5, pp. 773-86.

Baker, PD & Humpage, AR 1994, 'Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray-Darling Basin, Australia', *Marine and Freshwater Research*, vol. 45, no. 5, pp. 773-86.

Bar-Ami, S 1994, 'Increasing progesterone secretion and 3[beta]-hydroxysteroid dehydrogenase activity of human cumulus cells and granulosa-lutein cells concurrent with successful fertilization of the corresponding oocyte', *The Journal of steroid biochemistry and molecular biology*, vol. 51, no. 5-6, pp. 299-305.

Bar-Ami, S, Gitay-Goren, H & Brandes, JM 1989, 'Different morphological and steroidogenic patterns in oocyte/cumulus-corona cell complexes aspirated at in vitro fertilization', *Biology of Reproduction*, vol. 41, no. 4, pp. 761-70.

Bar-Ami, S, Khoury, C, Zlotkin, E & Brandes, JM 1993, 'Increasing progesterone secretion in human granulosa-luteal cells induced by human follicular fluid', *Human Reproduction*, vol. 8, no. 1, pp. 46-52.

Baranao, RI, Dain, L, de Fried, EP & Rumi, L 1995, 'Human Granulosa Cells Express HLA-DR Antigen and are Capable of Synthesizing Interlukin-1', *Hormone and Metabolic Research*, vol. 27, pp. 495-8.

Baud, V & Karin, M 2001, 'Signal transduction by tumor necrosis factor and its relatives', *Trends in Cell Biology*, vol. 11, no. 9, pp. 372-7.

Baumgartner, A, Cemeli, E & Anderson, D 2009, 'The comet assay in male reproductive toxicology', *Cell Biology and Toxicology*, vol. 25, no. 1, pp. 81-98.

Beckman, MW, Polacek, D, Seung, L & Schreiber, JR 1991, 'Human ovarian granulosa cell culture:determination of blood cell contamination and evaluation of possible culture purification steps', *Fertility and Sterility*, vol. 56, no. 5, pp. 881-7.

Ben-Ze've, A & Amsterdam, A 1987, 'In vitro regulation of granulosa cell differentiation. Involvement of cytoskeletal protein expression', *The Journal of Biological Chemistry.*, vol. 262, no. 11, pp. 5366-76.

Benowitz, NL, Jacob, r, P., Jones, RT & Rosenberg , J 1982, 'Interindividual variability in the metabolism and cardiovascular effects of nicotine in man', *Journal Pharmacology and Experimental Therapeutics*, vol. 221, pp. 368-72.

Bernas, T & Dobrucki, JW 2000, 'The Role of Plasma Membrane in Bioreduction of Two Tetrazolium Salts, MTT, and CTC', *Archives of Biochemistry and Biophysics*, vol. 380, no. 1, pp. 108-16.

Best, CL, Griffin, PM & Hill, JA 1995, 'Interferon-Gamma Inhibits Luteinized Human Granulosa-Cell Steroid-Production in-Vitro', *American journal of obstetrics and gynecology*, vol. 172, no. 5, pp. 1505-10.

Best, CL, Pudney, J, Deborah, JA & Hill, JA 1994, 'Modulation of Human Granulosa Cell Steroid Production In Vitro by Tumor Necrosis Factor Alpha: Implications of White Blood Cells in Culture', *Obstetrics and Gynecology*, vol. 84, no. 1, pp. 121-7.

Björndahl, L, Söderlund, SJ, Mohammadieh, M, Pourian, MR & Kvist, U 2004, 'Why the WHO Recommendations for Eoin-Nigrosin Staining Techniques for Human Sperm Vitality Assessment Must Change', *Journal of Andrology*, vol. 25, no. 5.

Bradford, MM 1976, 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248-54.

Breckwoldt, M, Selvaraj, N, Aharoni, D, Barash, A, Segal, I, Insler, V & Amsterdam, A 1996, 'Cell proliferation and apoptosis: Expression of Ad4-BP/cytochrome P450 side chain cleavage enzyme and induction of cell death in long-term cultures of human granulosa cells', *Molecular Human Reproduction*, vol. 2, no. 6, pp. 391-400.

Bruggisser, R, Daeniken, Kv, Jundt, G, Schaffner, W & Tullberg-Reinert, H 2002, 'Interference of Plant Extracts, Phytoestrogens and Antioxidants with the MTT Tetrazolium Assay', *Planta Medica*, vol. 68, no. 5, pp. 445-8.

Bryant, BJ & Knights, KM 2010, *Pharmacology for health professionals*, Mosby, Elsevier, Sydney.

Burns, J, Chapman, AD, Williams, C, Flewelling, L, Carmichael, WW & Pawlowicz, M 2000, 'Cyanotoxic blooms in Florida's (USA) lakes, rivers and tidal estuaries: The recent invasion of toxigenic Cylindrospermopsis raciborskii and consequences for Florida's drinking water supplies.', in *Ninth International Conference on Harmful Algal Blooms*, Hobart, Tasmania, Australia.

Byth, S 1980, 'Palm Island Mystery Disease', *The Medical Journal of Australia*, vol. 2, pp. 40-2.

Carmichael, J, DeGraff, WG, Gazdar, AF, Minna, JD & Mitchell, JB 1987, 'Evaluation of a Tetrazolium-based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity Testing', *Cancer Research*, vol. 47, no. 4, pp. 936-42.

Carmichael, WW, Azevedo, SMFO, An, JS, Molica, RJR, Jochimsen, EM, Lau, S, Rinehart, KL, Shaw, GR & Eaglesham, GK 2001, 'Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins', *Environmental Health Perspectives*, vol. 109, no. 7, pp. 663-71.

Casselman, WGB 1959, 'Lipids', in *Histochemical Technique*, John Wiley & Sons Inc, pp. 54-91.

Chaffin, CL, Dissen, GA & Stouffer, RL 2000, 'Hormonal regulation of steroidogenic enzyme expression in granulosa cells during the peri-ovulatory interval in monkeys', *Molecular Human Reproduction*, vol. 6, no. 1, pp. 11-8.

Chaffkin, ML, Luciano, AA & Peluso, JJ 1993, 'The Role of Progesterone in Regulating Human Granulosa Cell Proliferation and Differentiation *in Vitro*', *Journal of Clinical Endocrinology and Metabolism*, vol. 76, no. 3, pp. 696-700.

Chan, WK & Tan, CH 1987, 'Induction of Aromatase Activity in Porcine Granulosa Cells by FSH and Cyclic Amp', *Endocrine Research*, vol. 13, no. 3, pp. 285 - 99.

Chiswell, RK, Shaw, GR, Eaglesham, G, Smith, MJ, Norris, RL, Seawright, AA & Moore, MR 1999, 'Stability of Cylindrospermopsin, the Toxin from Cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, Temperature, and Sunlight on Decomposition', *Environmental Toxicology*, vol. 14, pp. 155-61.

Chong, MWK, Wong, BSF, Lam, PKS, Shaw, GR & Seawright, AA 2002, 'Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes', *Toxicon*, vol. 40, no. 2, pp. 205-11.

Chu, S, Nishi, Y, Yanase, T, Nawata, H & Fuller, PJ 2004, 'Transrepression of Estrogen Receptor {beta} Signaling by Nuclear Factor-{kappa}B in Ovarian Granulosa Cells', *Molecular Endocrinology*, vol. 18, no. 8, pp. 1919-28.

Claassens, OE, Wehr, JB & Harrison, KL 2000, 'Optimizing sensitivity of the human sperm motility assay for embryo toxicity testing', *Hum. Reprod.*, vol. 15, no. 7, pp. 1586-91.

Clark, BJ, Wells, J, King, SR & Stocco, DM 1994, 'The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR)', *The Journal of Biological Chemistry.*, vol. 269, no. 45, pp. 28314-22.

National Climate Centre 2006, *Annual Climate Summary 2005*, by Climate Analysis Section, Bureau of Meteorology.

Codd, GA, Bell, SG, Kaya, K, Ward, CJ, Beattie, KA & Metcalf, JS 1999, 'Cyanobacterial Toxins, exposure routes and human health', *European Journal of Phycology*, vol. 34, pp. 405-15.

Codd, GA, Lindsay, J, Young, FM, Morrison, LF & Metcalf, JS 2005, 'Harmful Cyanobacteria: From mass mortalities to management measures', in J Huisman, HCP Matthijs & PM Visser (eds), *Harmful Cyanobacteria*, Springer, Netherlands, vol. 3.

Conley, A & Hinshelwood, M 2001, 'Mammalian aromatases', *Reproduction*, vol. 121, no. 5, pp. 685-95.

Conti, M 2002, 'Specificity of the Cyclic Adenosine 3′,5′-Monophosphate Signal in Granulosa Cell Function ', *Biology of Reproduction*, vol. 67, no. 6, pp. 1653-61.

Cree, IA & Andreotti, PE 1997, 'Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines', *Toxicology in Vitro*, vol. 11, no. 5, pp. 553-6.

Davidoff, F 1968, 'Effects of guanidine derivatives on mitochondrial function :I. Phenethylbiguanide inhibition of respiration in mitochondria from guinea pig and rat tissues', *The Journal of Clinical Investigation*, vol. 47, no. 10, pp. 2331-43.

Davis, WL & McKay, CP 1996, 'Origins of life: A comparison of theories and application to Mars', *Origins of Life and Evolution of the Biosphere*, vol. 26, no. 1, pp. 61-73.

De Jonge, CJ, Centola, GM, Reed, ML, Shabanowitz, RB, Simon, SD & Quinn, P 2003, 'Human Sperm Survival Assay as a Bioassay for the Assisted Reproductive Technologies Laboratory', *Journal of Andrology*, vol. 24, no. 1, pp. 16-8.

De Kretser, DM, Loveland, KL, Meinhardt, A, Simorankir, D & Wreford, N 1998, 'Spermatogenesis', *Human Reproduction*, vol. 13 no. Supplement 1, pp. 1-8.

Debnam, PM & Shearer, G 1997, 'Colorimetric Assay for Substrates of NADP+-Dependent Dehydrogenases Based on Reduction of a Tetrazolium Dye to Its Soluble Formazan', *Analytical Biochemistry*, vol. 250, no. 2, pp. 253-5.

DeLeve, LD & Kaplowitz, N 1991, 'Glutathione metabolism and its role in hepatotoxicity', *Pharmacology & Therapeutics*, vol. 52, no. 3, pp. 287-305.

Delforge, JP, Thomas, K, Roux, F, Seiqueira, JC & Ferin, J 1972, 'Time Relationships Between Granulosa Cells Growh and Luteinization, and Plasma Luteinizing Hormone Discharge in Human. I. A Morphometric Analysis', *Fertility and Sterility*, vol. 23, no. 1, pp. 1-11.

Deneke, SM & Fanburg, BL 1989, 'Regulation of cellular glutathione', *American Journal of Physiology - Lung Cellular and Molecular Physiology* , vol. 257, no. 4, pp. L163-73.

Devoto, L, Kohen, P, Vega, M, Castro, O, Gonzalez, RR, Retamales, I, Carvallo, P, Christenson, LK & Strauss, IIIJF 2002, 'Control of human luteal steroidogenesis', *Molecular and Cellular Endocrinology*, vol. 186, no. 2, pp. 137-41.

Dignum, M, Matthijs, HCP, Pel, R, Laanbroek, HJ & Mur, LR 2005, 'Nutrient Limitation of Freshwater Cyanobacteria', in J Huisman, HCP Matthijs & PM Visser (eds), *Harmful Cyanobacteria*, Springer, Netherlands, vol. 3.

Disbrey, BD & Rack, JH 1970, 'Lipids', in *Histological Laboratory Methods*, E&S Livingstone, Edinburg and London, pp. 158-70.

Doody, KJ, Lorence, MC, Mason, JI & Simpson, ER 1990, 'Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle', *J Clin Endocrinol Metab*, vol. 70, no. 4, pp. 1041-5.

Drummond, AE & Findlay, JK 1999, 'The role of estrogen in folliculogenesis', *Molecular and Cellular Endocrinology*, vol. 151, no. 1-2, pp. 57-64.

Duley, J, Stewart, RJ & Allardyce, RA 1979, 'Semiautomated radioassay of in vitro protein synthesis by fibroblasts', *Analytical Biochemistry*, vol. 96, no. 1, pp. 139-43.

Duncan, WC, Cowen, GM & Illingworth, PJ 1999, 'Steroidogenic enzyme expression in human corpora lutea in the presence and absence of exogenous human chorionic gonadotrophin (HCG)', *Molecular Human Reproduction*, vol. 5, no. 4, pp. 291-8.

Dunkel, L, Hirvonen, V & ErkkilaÈ, K 1997, 'Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis', *Cell Death and Differentiation*, vol. 7, no. 4, pp. 171-9.

Duy, TN, Lam, PKS, Shaw, G & Connell, DW 2000, 'Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water', *Reviews of Environmental Contamination and Toxicology*, vol. 163, pp. 113-86.

Edgar, DH, Whalley, KM, Gemmell, JA, James, GB & Mills, JA 1991, 'Effects of in-vitro exposure to HCG on subsequent HCC-responsiveness of human granulosa cells obtained following treatment with GnRH analogue and gonadotrophins: an in-vitro model for luteal phase support', *Human Reproduction*, vol. 6, no. 2, pp. 198-202.

Edwards, RG 1974, 'Follicular Fluid', *Journal Reproduction Fertility*, vol. 37, no. 1, pp. 189-219.

Eliasson, R & Treichl, L 1971, 'Supravital Staining of Human Spermatozoa', *Fertility and Sterility*, vol. 22, no. 2, pp. 134-7.

Endo, T, Aten, RF, Leykin, L & Behrman, HR 1993, 'Hydrogen Peroxide Evokes Antisteroidogenic adn Antigonadotropic Actions in Human Granulosa Luteal Cells', *Journal of Clinical Endocrinology and Metabolism*, vol. 76, no. 2, pp. 337-42.

Enmark, E & Gustafsson, JA 1999, 'Oestrogen receptors -an overview', *Journal of Internal Medicine*, vol. 246, no. 2, pp. 133-8.

Eppig, J 2001, 'Oocyte control of ovarian follicular development and function in mammals', *Reproduction*, vol. 122, no. 6, pp. 829-38.

Espey, LL, Bellinger, AS & Healy, JA 2004, 'Ovulation: An Inflammatory Cascade of Gene Expression', in E Adashi & A Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California, pp. 145-65.

Esterhuizen, AD, Franken, DR, Lourens, JGH & van Rooyen, LH 2001, 'Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF', *Human Reproduction*, vol. 16, no. 1, pp. 138-44.

Fabbro, LD & Duivenvoorden, LJ 1996, 'Profile of a Bloom of the Cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju in the Fitzroy River in Tropical Central Queensland', *Marine Freshwater Research*, vol. 47, pp. 685-94.

Falconer, I 1998, 'Algal Toxins and Human Health', in J Hrubec (ed.), *The handbook of environmental chemistry*, Springer-Verlag, Berlin Heidelberg, vol. 5, Part C Quality and Treatment of Drinking Water II.

Falconer, IR 2001, 'Toxic cyanobacterial bloom problems in Australian waters: risks and impacts on human health', *Phycologia*, vol. 40, no. 3, pp. 228-33.

Falconer, IR, Hardy, SJ, Humpage, AR, Froscio, SM & Tozer, GJ 1999, 'Hepatic and Renal Toxicity of the Blue-Green Alga (Cyanobacterium) *Cylindrospermopsis raciborskii* in Male Swiss Albino Mice', *Environmental Toxicology*, vol. 14, pp. 143-50.

Falconer, IR & Humpage, AR 2001, 'Preliminary evidence for in vivo tumour initiation by oral administration of extracts of the blue-green alga Cylindrospermopsis raciborskii containing the toxin cylindrospermopsin', *Environmental Toxicology*, vol. 16, no. 2, pp. 192-5.

Falconer, IR & Humpage, AR 2006, 'Cyanobacterial (Blue-Green algal) toxins in water supplies: Cylindrospermopsins', *Environmental Toxicology*, vol. 21, no. 4, pp. 299-304.

Fan, W, Yanase, T, Morinaga, H, Gondo, S, Okabe, T, Nomura, M, Komatsu, T, Morohashi, KI, Hayes, TB, Takayanagi, R & Nawata, H 2007, 'Atrazine-Induced Aromatase Expression Is SF-1 Dependent: Implications for Endocrine Disruption in Wildlife and Reproductive Cancers in Humans', *Environmental Health Perspectives*, vol. 115, no. 5, p. 720.

Fastner, J, Heinze, R, Humpage, AR, Mischke, U, Eaglesham, GK & Chorus, I 2003, 'Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of Cylindrospermopsis raciborskii (Cyanobacteria) isolates', *Toxicon*, vol. 42, no. 3, pp. 313-21.

Fatum, M, Natalie, YO, David, S, Joseph, O, Simon, A & Laufer, N 2009, 'Levels of steroidogenic acute regulatory protein and mitochondrial membrane potential in granulosa cells of older poor-responder women', *Fertility and Sterility*, vol. 91, no. 1, pp. 220-5.

Ferran, C, Stroka, DM, Badrichani, AZ, Cooper, JT, Wrighton, CJ, Soares, M, Grey, ST & Bach, FH 1998, 'A20 Inhibits NF-kB Activation in Endothelial Cells Without Sensitizing to Tumor Necrosis Factor–Mediated Apoptosis', *Blood*, vol. 91, no. 7, pp. 2249-58.

Fessard, V & Bernard, C 2003, 'Cell alterations but no DNA strand breaks induced in vitro by cylindrospermopsin in CHOK1 cells', *Environmental Toxicology*, vol. 18, no. 5, pp. 353-9.

Figenschau, Y, Sundsfjord, JA, Yousef, MI, Fuskevag, OM, Sveinbjornsson, B & Bertheussen, K 1997, 'A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells', *Human Reproduction*, vol. 12, no. 3, pp. 523-31.

Fischer, TV & Kahn, RH 1972, 'Histochemical Studies of Rat Ovarian Follicular Cells In Vitro', *In Vitro*, vol. 7, no. 4, pp. 201-5.

Folgero, T, Bertheussen, K, Lindal, S, Torbergsen, T & Oian, P 1993, 'Andrology: Mitochondrial disease and reduced sperm motility', *Human Reproduction*, vol. 8, no. 11, pp. 1863-8.

Fotakis, G & Timbrell, JA 2006, 'In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride', *Toxicology Letters*, vol. 160, no. 2, pp. 171-7.

Franklin, MR & Hathaway, LB 2008, '2-Diethylaminoethyl-2,2-diphenylvalerate-HCl (SKF525A) Revisited: Comparative Cytochrome P450 Inhibition in Human Liver Microsomes by SKF525A, Its Metabolites, and SKF-Acid and SKF-Alcohol ', *Drug Metabolism and Disposition*, vol. 36.

Fraser, LR, Beyret, E, Milligan, SR & Adeoya-Osiguwa, SA 2006, 'Effects of estrogenic xenobiotics on human and mouse spermatozoa', *Human Reproduction*, vol. 21, no. 5, pp. 1184-93.

Freshney, RI 2005, 'Quantitation', in *Culutre of Animal Cells: A Manual of Basic Technique*, Fifth edn, Wiley-Liss, New Jersey, pp. 343-5.

Fritz, AM & Speroff, L 1982, 'The endocrinology of the menstrual cycle: the interaction of folliculogenesis and neuroendocrine mechanisms', *Fertility and Sterility*, vol. 38, no. 5, pp. 509-25.

Froscio, SM, Cannon, E, Lau, HM & Humpage, AR 2009, 'Limited uptake of the cyanobacterial toxin cylindrospermopsin by Vero cells', *Toxicon*, vol. 54, no. 6, pp. 862-8.

Froscio, SM, Fanok, S & Humpage, AR 2009, 'Cytotoxicity screening for the cyanobacterial toxin cylindrospermopsin', *Journal of Toxicology and Environmental Health, Part A: Current Issues*, vol. 72, no. 5, pp. 345-9.

Froscio, SM, Fanok, S & Humpage, AR 2009, 'Cytotoxicity Screening for the Cyanobacterial Toxin Cylindrospermopsin', *Journal of Toxicology and Environmental Health, Part A: Current Issues*, vol. 72, no. 5, pp. 345 - 9.

Froscio, SM, Humpage, AR, Burcham, PC & Falconer, IR 2001, 'Cell-Free Protein Synthesis Inhibition Assay for the Cyanobacterial Toxin Cylindrospermopsin', *Environmental Toxicology*, vol. 16, pp. 408-12.

Froscio, SM, Humpage, AR, Burcham, PC & Falconer, IR 2003, 'Cylindrospermopsin-Induced Protein Synthesis Inhibition and Its Dissociation from Acute Toxicity in Mouse Hepatocytes', *Environmental Toxicology*, vol. 18, pp. 243-51.

Gerlier, D & Thomasset, N 1986, 'Use of MTT colorimetric assay to measure cell activation', *Journal of Immunological Methods*, vol. 94, no. 1-2, pp. 57-63.

Gillies, RJ, Didier, N & Denton, M 1986, 'Determination of cell number in monolayer cultures', *Analytical Biochemistry*, vol. 159, no. 1, pp. 109-13.

Goodwin, CJ, Holt, SJ, Downes, S & Marshall, NJ 1995, 'Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS', *Journal of Immunological Methods*, vol. 179, no. 1, pp. 95-103.

Goodwin, CJ, Holt, SJ, Downes, S & Marshall, NJ 1996, 'The use of intermediate electron acceptors to enhance MTT bioreduction in a microculture tetrazolium assay for human growth hormone', *Life Sciences*, vol. 59, no. 20, pp. 1745-53.

Gore-Langton, RE 1988, 'Cyclosporine differentially affects estrogen and progestin synthesis by rat granulosa cells in vitro', *Molecular and Cellular Endocrinology*, vol. 57, no. 3, pp. 187-98.

Griffiths, DJ & Saker, ML 2003, 'The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin', *Environmental Toxicology*, vol. 18, no. 2, pp. 78-93.

Griswold, MD 1998, 'The central role of Sertoli cells in spermatogenesis', *Seminars in Cell & Developmental Biology*, vol. 9, no. 4, pp. 411-6.

Griveau, JF & Lannou, DL 1997, 'Reactive oxygen species and human spermatozoa: physiology and pathology', *International Journal of Andrology*, vol. 20, no. 2, pp. 61-9.

Grootegoed, JA, Siep, M & Baarends, WM 2000, 'Molecular and cellular mechanisms in spermatogenesis', *Best Practice & Research Clinical Endocrinology & Metabolism*, vol. 14, no. 3, pp. 331-43.

Gross, BA, Mindea, SA, Pick, AJ, Chandler, JP & Batjer, HH 2007, 'Medical management of Cushing disease', *Neurosurgical FOCUS*, vol. 23, no. 3, pp. 1-6.

Guet, P, Royere, D, Paris, A, Lansac, J & Driancourt, MA 1999, 'Aromatase activity of human granulosa cells in vitro: effects of gonadotrophins and follicular fluid', *Human Reproduction*, vol. 14, no. 5, pp. 1182-9.

Hansen, MB, Nielsen, SE & Berg, K 1989, 'Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill', *Journal of Immunological Methods*, vol. 119, no. 2, pp. 203-10.

Hanukoglu, I, Suh, BS, Himmelhoch, S & Amsterdam, A 1990, 'Induction and mitochondrial localization of cytochrome P450scc system enzymes in normal and transformed ovarian granulosa cells', *The Journal of Cell Biology* , vol. 111, no. 4, pp. 1373-81.

Hartshorne, GM 1990, 'Subpopulations of granulosa cells within the human ovarian follicle', *J Reprod Fertil*, vol. 89, no. 2, pp. 773-82.

Harvey, PW & Everett, DJ 2003, 'The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: critical omissions from regulatory endocrine disrupter screening strategies for human health?', *Journal of Applied Toxicology*, vol. 23, no. 2, pp. 81-7.

Havelock, JC, Rainey, WE & Carr, BR 2004, 'Ovarian granulosa cell lines', *Molecular and Cellular Endocrinology*, vol. 228, no. 1-2, pp. 67-78.

Hawkins, PR, Chandrasena, NR, Jones, GJ, Humpage, AR & Falconer, IR 1997, 'Isolation and Toxicity of *Cylindrospermopsis Raciborskii* from an Ornamental Lake', *Toxicon*, vol. 35, no. 3, pp. 341-6.

Hawkins, PR, Runnegar, MTC, Jackson, AB & Falconer, IR 1985, 'Severe Hepatotoxicity Caused by the Tropical Cyanobacterium (Blue-Green Alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Rju Isolated from a Domestic Water Supply Reservoir', *Applied and Environmental Microbiology*, vol. 50, no. 5, pp. 1292-5.

Hecht, NB 1998, 'Molecular mechanisms of male germ cell differentiation', *BioEssays*, vol. 20, no. 7, pp. 555-61.

Hediger, M, Romero, M, Peng, J-B, Rolfs, A, Takanaga, H & Bruford, E 2004, 'The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins', *Pflügers Archiv European Journal of Physiology*, vol. 447, no. 5, pp. 465-8.

Heimler, I, Rawlins, RG, Owen, H & Hutz, RJ 1998, 'Dioxin Perturbs, in a Dose- and Time-Dependent Fashion, Steroid Secretion, and Induces Apoptosis of Human Luteinized Granulosa Cells', *Endocrinology*, vol. 139, no. 10, pp. 4373-9.

Heller, DT, Cahill, DM & Schultz, RM 1981, 'Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes', *Developmental Biology*, vol. 84, no. 2, pp. 455-64.

Hess, J, Angel, P & Schorpp-Kistner, M 2004, 'AP-1 subunits: quarrel and harmony among siblings', *Journal of Cell Science*, vol. 117, no. 25, pp. 5965-73.

Hess, KA, Chen, L & Larsen, WJ 1998, 'The Ovarian Blood Follicle Barrier Is Both Charge- and Size-Selective in Mice', *Biology of Reproduction*, vol. 58, pp. 705-11.

Hillensjo, T, Sjogren, A, Strander, B, Nilsson, L, Wikland, M, Hamberger, L & Roos, P 1985, 'Effect of gonadotrophins on progesterone secretion by cultured granulosa cells obtained from human preovulatory follicles', *Acta Endocrinology*, vol. 110, no. 3, pp. 401-7.

Ho, CKM, Christenson, LK & Strauss, IIIJF 2004, 'Intracellular Cholesterol Dynamics in Steroidogenic Cells', in E Adashi & A Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California, pp. 93-112.

Hoeger, SJ, Hitzfeld, BC & Dietrich, DR 2005, 'Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants', *Toxicology and Applied Pharmacology*, vol. 203, no. 3, pp. 231-42.

Hoeger, SJ, Shaw, G, Hitzfeld, BC & Dietrich, DR 2004, 'Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants', *Toxicon*, vol. 43, no. 6, pp. 639-49.

Hong, X, Qu, J, Chen, J, Cheng, S, Wang, Y, Song, L, Wang, S, Liu, J & Wang, X 2007, 'Effects of trichlorfon on progesterone production in cultured human granulosa-lutein cells', *Toxicology in Vitro*, vol. 21, no. 5, pp. 912-8.

Hosokawa, K, Dantes, A, Schere-Levy, C, Barash, A, Yoshida, Y, Kotsuji, F, Vlodavsky, I & Amsterdam, A 1998, 'Induction of Ad4BP/SF-1, Steroidogenic Acute Regulatory Protein and Cytochrome P450scc Enzyme System Expression in Newly Established Human Granulosa Cell Lines', *Endocrinology*, vol. 139, pp. 4679-87.

Hossain, AM, Osuamkpe, CO & Nagamani, M 2008, 'Extended culture of human spermatozoa in the laboratory may have practical value in the assisted reproductive procedures', *Fertility and Sterility*, vol. 89, no. 1, pp. 237-9.

Hoyer, PB 2001, 'Reproductive toxicology: current and future directions', *Biochemical Pharmacology*, vol. 62, pp. 1557-64.

Hrudey, S, Burch, M, Drikas, M & Gregory, R 1999, 'Remedial Measures', in I Chorus & J Bartman (eds), *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management*, E & FN Spon London.

Hsia, S-M, Yeh, C-L, Kuo, Y-H, Wang, PS & Chiang, W 2007, 'Effects of Adlay (Coix lachryma-jobi L. var. ma-yuen Stapf.) Hull Extracts on the Secretion of Progesterone and Estradiol In Vivo and In Vitro', *Experimental Biology and Medicine*, vol. 232, no. 9, pp. 1181-94.

Hsieh, RH, Au, HK, Wang, R-S, Yang, PS & Tzeng, CR 2004, 'The Role of Mitochondria in the Aging Ovary', in E Adashi & C Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California.

Huang, B-M, Hsiao, K-Y, Chuang, P-C, Wu, M-H, Pan, H-A & Tsai, S-J 2004, 'Upregulation of Steroidogenic Enzymes and Ovarian 17{beta}-Estradiol in Human Granulosa-Lutein Cells by Cordyceps sinensis Mycelium', *Biol of Reproduction*, vol. 70, no. 5, pp. 1358-64.

Huang, KT, Chen, YH & Walker, AM 2004, 'Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids', *Biotechniques*, vol. 37, no. 3, pp. 406-+.

Hughes, CM, Lewis, SE, McKelvey-Martin, VJ & Thompson, W 1998, 'The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity', *Human Reproduction*, vol. 13, no. 5, pp. 1240-7.

Hughes, SF, Haney, AF & Hughes Jr, CL 1990, 'Use of Human Cumulus Granulosa Cells For In Vitro Screening of Reproductive Toxicants', *Reproductive Toxicology*, vol. 4, pp. 11-5.

Huhtaniemi, IT 2004, 'Functional Consequences of Mutations and Polymorphisms in Gonadotropin and Gonadotropin Receptor Genes', in E Adashi & C Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California, pp. 55-78.

Humpage, AR & Falconer, IR 2003, 'Oral Toxicity of the Cyanobacterial Toxin Cylindrospermopsin in Male Swiss Albino Mice: Determination of No Observed Adverse Effect Level for Deriving a Drinking Water Guideline Value', *Environmental Toxicology*, vol. 18, pp. 94-103.

Humpage, AR, Fenech, M, Thomas, P & Falconer, IR 2000, 'Micronucleus induction and chromosome loss in transformed human white cells indicated clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin', ***Mutation Research*****, vol. 472, pp. 155-61.**

Humpage, AR, Fontaine, F, Froscio, SM, Burcham, PC & Falconer, IR 2005, 'Cylindrospermopsin Genotoxicity and Cytotoxicity: Role of Cytochrome P-450 and Oxidative Stress', ***Journal of Toxicology and Environmental Health Part A*****, vol. 68, pp. 739-53.**

Ibrahim, ME, Moussa, MAA & Pedersen, H 1989, 'Efficacy of zona-free hamster egg sperm penetration assay as a predicter of ***in vitro*** **fertilisation',** ***Archives of Androloge*****, vol. 23, no. 3, pp. 267-74.**

Ishiyama, M, Tominaga, H, Shiga, M, Sasamoto, K, Ohkura, Y & Ueno, K 1996, 'A Combined Assay of Cell Viability and ***in Vitro*** **Cytotoxicity with a Highly Water-Soluble Tetrazolim Salt, Neutral Red and Crystal Violet',** ***Biological and Pharmaceutical Bulletin*****, vol. 19, no. 11, pp. 1518-20.**

Jiang, JY, Xiong, H, Cao, M, Xia, X, Sirard, MA & Tsang, BK 2010, 'Mural granulosa cell gene expression associated with oocyte developmental competence', ***Journal of Ovarian Research*****, vol. 3, no. 1, p. 6.**

Kagabu, S, Kodama, H, Fukuda, J, Karube, A, Murata, M & Tanaka, T 1999, 'Inhibitory effects of nitric oxide on the expression and activity of aromatase in human granulosa cells', ***Molecular Human Reproduction*****, vol. 5, no. 5, pp. 396-401.**

Kasper, DL, Braunwald, E, Fauci, AS, Hauser, SL, Longo, DL, Jameson, JL & Isselbacher, KJ 2006, 'Reproductive Endocrinology: Disorders of the Ovary and Female Reproductive Tract', in JL Jameson (ed.), ***Harrison's Endocrinology*****, 16th Ed edn, McGraw-Hill, USA.**

Kasson, BG, Bai, S, Liu, J, Tobin, C & Kessel, B 1993, 'Characterization of a Rapid and Sensitive Enzyme Immunoassay (EIA) for Progesterone Applied to Conditioned Cell Culture Media', ***Journal of Immunoassay and Immunochemistry*****, vol. 14, no. 1, pp. 33 - 49.**

Keren-Tal, I, Suh, B-S, Dantes, A, Lindner, S, Oren, M & Amsterdam, A 1995, 'Involvement of p53 Expression in cAMP-Mediated Apoptosis in Immortalized Granulosa Cells', ***Experimental Cell Research*****, vol. 218, pp. 283-95.**

Kerin, JFP, Moor, RM & Seamark, RF 1976, 'Human Graffian Follicles in Tissue Culture: Correlations Between Oestrogen and Progesterone Production and the site of Δ5-3 β- Hydroxy-Steroid Dehydrogenase Activity', ***Theriogenology*****, vol. 6, no. 6, pp. 605-6.**

Khamsi, F & Roberge, S 2001, 'Granulosa cells of the cumulus oophorus are different from mural granulosa cells in their response to gonadotrophins and IGF-I', ***Journal of Endocrinology*****, vol. 170, no. 3, p. 565.**

Kinkel, AD, Fernyhough, ME, Helterline, DL, Vierck, JL, Oberg, KS, Vance, TJ, Hausman, GJ, Hill, RA & Dodson, MV 2004, 'Oil red-O stains non-adipogenic cells: a precautionary note', ***Cytotechnology*****, vol. 46, no. 1, pp. 49-56.**

Kirchman, D, K'Nees, E & Hodson, R 1985, 'Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems', ***Applied and Environmental Microbiology*****, vol. 49, no. 3, pp. 599-607.**

Knaggs, P, Lambert, A, Proudfoot, F, Nickson, I, Hooper, MAK, Lenton, E & Robertson, WR 1998, 'A rapid method for the measurement of the oxoreductase activity of 11b-hydroxysteroid dehydrogenase in granulosa–lutein cells from patients undergoing in-vitro fertilization', ***Molecular Human Reproduction*****, vol. 4, no. 2, pp. 147-51.**

Kohen, P, Castro, O, Palomino, A, Munoz, A, Christenson, LK, Sierralta, W, Carvallo, P, Strauss, JF, III & Devoto, L 2003, 'The Steroidogenic Response and Corpus Luteum Expression of the Steroidogenic Acute Regulatory Protein after Human Chorionic Gonadotropin Administration at Different Times in the Human Luteal Phase', ***The Journal of Clinical Endocrinology and Metabolism*****, vol. 88, no. 7, pp. 3421-30.**

Kunz, L, Roggors, C & Mayerhofer, A 2007, 'Ovarian acetylcholine and ovarian KCNQ channels: Insights into cellular regulatory systems of steroidogenic granulosa cells', ***Life Sciences*****, vol. 80, no. 24-25, pp. 2195-8.**

Kwintkiewicz, J, Nishi, Y, Yanase, T & Giudice, LC 2009, 'Peroxisome ProliferatorActivated Receptor-γ Mediates Bisphenol A Inhibition of FSH-Stimulated IGF-1, Aromatase, and Estradiol in Human Granulosa Cells', ***Environmental Health Perspectives*****, vol. 118, no. 3.**

Lambert, A, Harris, SD, Knaggs, P & Robertson, WR 2000, 'Improved FSH Sensitisation and aromatase assay in human granulosa-lutein cells', ***Molecular Human Reproduction*****, vol. 6, no. 8, pp. 677-80.**

Landefeld, TD, Campbell, KL & Midgley, JAR 1997, 'Rapid changes in the synthesis of specific ovarian granulosa cell proteins induced by human choriogonadotropin', ***Proceedings of the National Academy of Sciences*****, vol. 76, p. 5153.**

Ledger, W 2009, 'Demographics of infertility ', ***Reproductive BioMedicine Online*****, vol. 18, no. Supplement 2, pp. 11-4.**

Leung, PCK & Armstrong, DT 1980, 'Interactions of Steroids and Gonadotropins in the Control of Steroidogenesis in the Ovarian Follicle', ***Annual Review of Physiology*****, vol. 42, no. 1, pp. 71-82.**

Leung, PCK & Steele, GL 1992, 'Intracellular Signaling in the Gonads', ***Endocrine Reviews*****, vol. 13, no. 3, pp. 476-98.**

Lin, Q, Poon, S, Chen, J, Cheng, L, HoYuen, B & Leung, P 2009, 'Leptin interferes with 3',5'-Cyclic Adenosine Monophosphate (cAMP) signaling to inhibit steroidogenesis in human granulosa cells', *Reproductive Biology and Endocrinology*, vol. 7, no. 1, p. 115.

Liu, DY & Baker, GHW 1992, 'Tests of human sperm function and fertilization in vitro', *Fertility and Sterility*, vol. 58, no. 3, pp. 465-81.

Lobb, D & Younglai, E 2006, 'A simplified method for preparing IVF granulosa cells for culture', *Journal of Assisted Reproduction and Genetics*, vol. 23, no. 2, pp. 93-5.

Lobb, DK, Soliman, SR, Daya, S & Younglai, EV 1998, 'Steroidogenesis in luteinized granulosa cell cultures varies with follicular priming regimen', *Human Reproduction*, vol. 13, no. 8, pp. 2064-7.

Lobb, DK, Soliman, SR, Daya, S & Younglai, EV 1998, 'Steroidogenesis in luteinized granulosa cell cultures varies with follicular priming regimen', *Human Reproduction*, vol. 13, no. 8, pp. 2064-7.

Lobb, DK & Younglai, EV 2001, 'Granulosa–luteal cell function in vitro and ovarian stimulation protocols', *Human Reproduction*, vol. 16, no. 1, pp. 195-6.

Loukides, JA, Loy, RA, Edwards, R, Honig, J, Visintin, I & Polan, ML 1990, 'Human follicular fluids contain tissue macrophages', *The Journal of Clinical Endocrinology and Metabolism*, vol. 71, no. 5, pp. 1363-7.

Lovell, T, Gladwell, R, Groome, N & Knight, P 2002, 'Modulatory effects of gonadotrophins and insulin-like growth factor on the secretion of inhibin A and progesterone by granulosa cells from chicken preovulatory (F1-F3) follicles', *Reproduction*, vol. 123, no. 2, pp. 291-300.

Macklon, NS & Fauser, BCJM 1998, 'Follicle development during the normal menstrual cycle', *Maturitas*, vol. 30, no. 2, pp. 181-8.

Mangelsdorf, DJ, Thummel, C, Beato, M, Herrlich, P, Schütz, G, Umesono, K, Blumberg, B, Kastner, P, Mark, M, Chambon, P & Evans, RM 1995, 'The nuclear receptor superfamily: The second decade ', *Cell*, vol. 83, no. 6, pp. 835-9.

Manna, PR, Huhtaniemi, IT & Stocco, DM 2004, 'Detection of hCG Responsive Expression of the Steroidogenic Acute Regulatory Protein in Mouse Leydig Cells', *Biological Procedures Online*, vol. 6, no. 1, pp. 83-93.

Manna, PR, Kero, J, Tena-Sempere, M, Pakarinen, P, Stocco, DM & Huhtaniemi, IT 2001, 'Assessment of Mechanisms of Thyroid Hormone Action in Mouse Leydig Cells: Regulation of the Steroidogenic Acute Regulatory Protein, Steroidogenesis, and Luteinizing Hormone Receptor Function', *Endocrinology*, vol. 142, no. 1, pp. 319-31.

Martinat, N, Crépieux, P, Reiter, E & Guillou, F 2005, 'Extracellular signal-regulated kinases (ERK) 1, 2 are required for luteinizing hormone (LH)-induced steroidogenesis in primary Leydig cells and control steroidogenic acute regulatory (StAR) expression', *Reproduction Nutrition Development*, vol. 45, no. 1, pp. 101-8.

Mattison, DR 1983, 'The mechanisms of action of reproductive toxins', *American journal of industrial medicine*, vol. 4, no. 1-2, p. 65.

McAllister, JM, Mason, JI, Byrd, W, Trant, JM, Waterman, MR & Simpson, ER 1990, 'Proliferating human granulosa-lutein cells in long term monolayer culture: expression of aromatase, cholesterol side-chain cleavage, and 3 beta-hydroxysteroid dehydrogenase', *The Journal of Clinical Endocrinology and Metabolism*, vol. 71, no. 1, pp. 26-33.

McKay, J & Moeller, A 2001, 'Is risk associated with drinking water in Australia of significant concern to justify mandatory regulation?', *Environmental Management*, vol. 28, no. 4, pp. 469-81.

MediLexicon 2011, *Toxin*, MediLexicon International Ltd, Bexhill-on-Sea, UK, <http://www.medilexicon.com/medicaldictionary.php?t=92741>.

Mendiola, J, Torres-Cantero, AM, Moreno-Grau, JM, Ten, J, Roca, M, Moreno-Grau, S & Bernabeu, R 2008, 'Exposure to environmental toxins in males seeking infertility treatment: a case-controlled study ', *Reproductive BioMedicine Online*, vol. 16, no. 6, pp. 842-50.

Miller, WL 1988, 'Molecular biology of steroid hormone synthesis', *Endocrine Reviews*, vol. 9, no. 3, pp. 295-318.

Minehata, FM, Goto, Y, Inoue, N, Sakamaki, K, Chedrese, PJ & Manabe, N 2007, 'Anti-apoptotic activity of porcine cFLIP in ovarian granulosa cell lines', *Molecular Reproduction and Development*, vol. 74, no. 9, pp. 1165-70.

Moore, MR, Seawright, RR, Chiswell, R, Norris, RL & Shaw, GR 1998, 'Proceedings of the British Toxicology Society Annual Congress University of Surrey, Guilford 19-22 April 1998', *Human and Experimental Toxicology*, vol. 17, no. 9, p. 503.

Morgan, CD, Mills, KC, Lefkowitz, DL & Lefkowitz, SS 1991, 'An improved colorimetric assay for tumor necrosis factor using WEHI 164 cells cultured on novel microtiter plates ', *Journal of Immunological Methods*, vol. 145, pp. 259-62.

Morris, DS, Dunn, RL, Schuster, TG, Ohl, DA & Smith, GD 2007, 'Ideal Culture Time for Improvement in Sperm Motility From Testicular Sperm Aspirates of Men With Azoospermia', *The Journal of Urology*, vol. 178, no. 5, pp. 2087-91.

Mosmann, T 1983, 'Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays', *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55-63.

Mueller, H, Kassack, MU & Wiese, M 2004, 'Comparison of the Usefulness of the MTT, ATP, and Calcein Assays to Predict the Potency of Cytotoxic Agents in Various Human Cancer Cell Lines', *Journal of Biomolecular Screening*, vol. 9, no. 6, pp. 506-15.

Mur, LR, Skulberg, OM & Utkilen, H 1999, 'Cyanobacteria in the Environment', in I Chorus & J Bartman (eds), *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management*, E & FN Spon on behalf of the WHO, Londond.

Murdoch, RN & White, IG 1967, 'Studies of the Metabolsim of Human Spermatozoa', *Journal Reproduction and Fertilty*, vol. 16, pp. 351-61.

Murphey, BD 2004, 'Luteinization', in E Adashi & A Leung (eds), *The Ovary*, Second Edition edn, Elsevier Academic Press, California, pp. 185-200.

Murphey, SJ, Rolden, ERS & Yanagimachi, R 1986, 'Effect of extracellular cations and energy substrates on acrosome reaction or precapacitated guinea pig spermatozoa', *Gamete Research*, vol. 14, pp. 1-10.

Nakajima, M, Iwata, K, Yamamoto, T, Funae, Y, Yoshida, T & Kuroiwa, Y 1998, 'Nicotine Metabolism in Liver Microsomes from Rats with Acute Hepatitis or Cirrhosis', *Drug Metabolism and Disposition*, vol. 26, pp. 36-41.

Nasr-Esfahani, MH, Aboutorabi, R, Esfandiari, E & Mardani, M 2002, 'Sperm MTT viability assay: A new method for evaluation of human sperm viability', *Journal of Assisted Reproduction and Genetics*, vol. 19, no. 10, p. 477.

Nebert, DW & Russell, DW 2002, 'Clinical importance of the cytochromes P450', *The Lancet*, vol. 360, no. 9340, pp. 1155-62.

Negri, AP, Jones, GJ, Blackburn, SI, Oshima, Y & Onodera, H 1997, 'Effect Of Culture and Bloom Development and of Sample Storage on Paralytic Shellfish Poisons in the Cyanobacterium Anabaena Circinalis', *Journal of Phycology*, vol. 33, no. 1, pp. 26-35.

Nelson, DM, Enders, AC & King, BF 1978, 'Cytological events involved in protein synthesis in cellular and syncytial trophoblast of human placenta. An electron microscope autoradiographic study of [3H]leucine incorporation', *The Journal of Cell Biology* , vol. 76, no. 2, pp. 400-17.

Ng, EHY, Tang, OS & Ho, PC 2000, 'The significance of the number of antral follicles prior to stimulation in predicting ovarian responses in an IVF programme', *Human Reproduction*, vol. 15, no. 9, pp. 1937-42.

Nishi, Y, Yanase, T, Mu, Y-M, Oba, K, Ichino, I, Saito, M, Nomura, M, Mukasa, C, Okabe, T, Goto, K, Takayanagi, R, Kashimura, Y, Haji, M & Nawata, H 2001, 'Establishment and Characterization of a Steroidogenic Human Granulosa-Like Tumor Cell Line, KGN, That Expresses Functional Follicle-Stimulating Hormone Receptor', *Endocrinology*, vol. 142, no. 1, pp. 437-45.

Norris, RLG, Seawright, AA, Shaw, GR, Senogles, P, Eaglesham, GK, Smith, MJ, Chiswell, RK & Moore, MR 2002, 'Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse', *Toxicon*, vol. 40, no. 4, pp. 471-6.

Norris, RLG, Seawright, AA, Shaw, GR, Smith, MJ, Chiswell, RK & Moore, MR 2001, 'Distribution of ^{14}C Cylindrospermopsin *in vivo* in the Mouse', *Environmental Toxicology*, vol. 16, p. 498.

Nuojua-Huttunen, S, Tomas, C, Bloigu, R, Tuomivaara, L & Martikainen, H 1999, 'Intrauterine insemination treatment in subfertility: an analysis of factors affecting outcome', *Human Reproduction*, vol. 14, no. 3, pp. 698-703.

O'Toole, SA, Sheppard, BL, Phil, D, McGuinness, EPJ, Gleeson, NC, Yoneda, M & Bonnar, J 2003, 'The MTS assay as an indicator of chemosensitivity/resistance in malignant gyneacological tumours', *Cancer Detection and Prevention*, vol. 27, pp. 47-54.

Ohno, K, Araki, N, Yanase, T, Nawata, H & Iida, M 2004, 'A Novel Nonradioactive Method for Measuring Aromatase Activity Using a Human Ovarian Granulosa-Like Tumor Cell Line and an Estrone ELISA', *Toxicol. Sci.*, vol. 82, no. 2, pp. 443-50.

Ohtani, I, Moore, RE & Runnegar, MTC 1992, 'Cylindrospermopsin: a potent hepatotoxin form the blue-green alga *Cylindrospermopsis raciborskii*', *Journal of American Chemical Society*, vol. 114, no. 20, pp. 7941-2.

Ombelet, W, Cooke, I, Dyer, S, Serour, G & Devroey, P 2008, 'Infertility and the provision of infertility medical services in developing countries', *Human Reproduction Update*, vol. 14, no. 6, pp. 605-21.

Orlando, C, Caldini, AL, Fiorelli, G, Cuomo, S & Serio, M 1982, 'ATP and ADP content of human ejaculated spermatozoa', *International Journal of Andrology*, vol. 5, no. 5, pp. 497-502.

Pacifici, R, Altieri, I, Gandini, L, Lenzi, A, Passa, AR, Pichini, S, Rosa, M, Zuccaro, P & Dondero, F 1995, 'Environmental Tobacco-Smoke - Nicotine and Cotinine Concentration in Semen', *Environmental Research*, vol. 68, no. 1, pp. 69-72.

Padisak, J 1997, '*Cylindrospermopsis raciborskii* (Wolszynsk) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and a review of its ecology', *Archiv fuer Hydrobiologie. Supplementband*, vol. 107, no. 4, pp. 563-93.

Panesar, NS, Chan, KW & Ho, CS 2003, 'Mouse Leydig tumor cells produce C-19 steroids, including testosterone', *Steroids*, vol. 68, no. 3, pp. 245-51.

Patrizio, P, Fragouli, E, Bianchi, V, Borini, A & Wells, D 2007, 'Molecular methods for selection of the ideal oocyte ', *Reproductive BioMedicine Online*, vol. 15, no. 3, pp. 346-53.

Patrizio, P, Tucker, MJ & Guelman, V 2003, *A Color Atlas for Human Assisted Reproduction: Laboratory and Clinical Insights*, Lippincott Williams & Wilkins.

Payne, AH & Hales, DB 2004, 'Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones', *Endocrine Reviews*, vol. 25, no. 6, pp. 947-70.

Peluso, JJ 2003, 'Progesterone as a regulator of granulosa cell viability', *The Journal of steroid biochemistry and molecular biology*, vol. 85, no. 2-5, pp. 167-73.

PerkinElmer 2002, *ATPlite Luminescence ATP Detection Assay System*, PerkinElmer Life Sciences, Boston, < http://www.perkinelmer.com/lifesciences>.

Peterson, RN & Freund, M 1970, 'ATP Synthesis and Oxidative Metabolism in Human Spermatozoa', *Biology of Reproduction*, vol. 3, no. 1, pp. 47-54.

Petty, RD, Sutherland, LA, Hunter, EM & Cree, IA 1995, 'Comparison of MTT and ATP-based assays for the measurement of viable cell number', *Journal of Bioluminescence and Chemiluminescence*, vol. 10, no. 1, pp. 29-34.

Phy, J, Stocco, DM & Prien, SD 2002, 'Differences in steroidogenic acute regulatory protein expression from the luteinizing-granulosa cells of patients undergoing in vitro fertilization with embryo transfer: Implications for cycle outcomes* 1', *American journal of obstetrics and gynecology*, vol. 186, no. 5, pp. 872-5.

Pichini, S, Zuccaro, P & Pacifici, R 1994, 'Drugs in Semen', *Clinical Pharmokinetics*, vol. 26, no. 5, pp. 356-73.

Plumb, JA, Milroy, R & Kaye, SB 1989, 'Effects of the pH Dependence of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide-Formazan Absorption on Chemosensitivity Determined by a Novel Tetrazolium-based Assay', *Cancer Research*, vol. 49, pp. 4435-40.

Pozzolini, M, Scarfì, S, Benatti, U & Giovine, M 2003, 'Interference in MTT cell viability assay in activated macrophage cell line', *Analytical Biochemistry*, vol. 313, no. 2, pp. 338-41.

Prochaska, HJ & Santamaria, AB 1988, 'Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: A screening assay for anticarcinogenic enzyme inducers', *Analytical Biochemistry*, vol. 169, no. 2, pp. 328-36.

Promega 2003, 'CellTiter96® AQueous One Solution Cell Proliferation Assay', no. Technical Bulletin#TB112,<http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf_649>.

Quinn, MC, McGregor, SB, Stanton, JL, Hessian, PA, Gillet, WR & Green, DP 2006, 'Purification of granulosa cells from human ovarian follicular fluid using granulosa cell aggregates', *Reproduction, Fertility and Development*, vol. 18, pp. 501-8.

Ressom, R, San Soong, F, Fitzgerald, J, Turczynowicz, L, El Saadi, O, Roder, D, Maynard, T & Falconer, I 1994, *Health Effects of Toxic Cyanobacteria (Blue-Green Algae)*, Australian Government Publishing Service.

Revelli, A, Modotti, M, Piffaretti-Yanez, A, Massobrio, M & Balerna, M 1994, 'Steroid receptors in human spermatozoa', *Human Reproduction*, vol. 9, no. 5, pp. 760-6.

Reynolds, CS & Walsby, AE 1975, 'Water-Blooms', *Biological Reviews*, vol. 50, pp. 437-81.

Richards, JS 1994, 'Hormonal Control of Gene Expression in the Ovary', *Endocrine Reviews*, vol. 15, no. 6, pp. 725-51.

Richards, JS, Liu, Z & Shimada, M 2008, 'Immune-like mechanisms in ovulation', *Trends in Endocrinology & Metabolism*, vol. 19, no. 6, pp. 191-6.

Rizzino, A, Rizzino, H & Sato, G 1979, 'Defined Media and the Determination of Nutritional and Hormonal Requirements of Mammalian Cells in Culture', *Nutrition Reviews*, vol. 37, no. 12, pp. 369-76.

Rodgers, RJ, Vella, CA, Rodgers, HF, Scott, K & Lavranos, TC 1996, 'Production of Extracellular Matrix, Fibronectin and Steroidogenic Enzymes, and Growth of Bovine Granulosa Cells in Anchorage-independent Culture', *Reproduction, Fertility and Development*, vol. 8, pp. 249-57.

Rodríguez, E, Onstad, GD, Kull, TPJ, Metcalf, JS, Acero, JL & von Gunten, U 2007, 'Oxidative elimination of cyanotoxins: Comparison of ozone, chlorine, chlorine dioxide and permanganate', *Water Research*, vol. 41, no. 15, pp. 3381-93.

Rogers, EH, Zehr, RD, Gage, MI, Humpage, AR, Falconer, IR, Marr, M & Chernoff, N 2007, 'The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation', *Toxicon*, vol. 49, no. 6, pp. 855-64.

Ronen-Fuhrmann, T, Timberg, R, King, SR, Hales, KH, Hales, DB, Stocco, DM & Orly, J 1998, 'Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary', *Endocrinology*, vol. 139, no. 1, pp. 303-15.

Runnegar, MT, Kong, SM, Zhong, YZ, Ge, JL & Lu, SC 1994, 'The Role of Glutathione in the Toxicity of a Novel Cyanobacterial Alkaloid Cylindrospermopsin in Cultured Rat Hepatocytes', *Biochemical and Biophysical Research Communications*, vol. 201, no. 1, pp. 235-41.

Runnegar, MTC, Kong, SM, Zhong, YZ & Lu, SC 1995, 'Inhibition of Reduced Glutathione Synthesis By Cyanobacterial Alkaloid Cylindrospermopsin in Cultured Rat Hepatocytes', *Biochemical Pharmacology*, vol. 49, no. 2, pp. 219-25.

Russell, DL & Robker, RL 2007, 'Molecular mechanisms of ovulation: co-ordination through the cumulus complex', *Human Reproduction Update*, vol. 13, no. 3, pp. 289-312.

Said, TM, Agarwal, A, Sharma, RK, Thomas, JAJ & Sikka, SC 2005, 'Impact of sperm morphology on DNA damage caused by oxidative stress induced by [beta]-nicotinamide adenine dinucleotide phosphate', *Fertility and Sterility*, vol. 83, no. 1, pp. 95-103.

Said, TM, Aziz, N, Sharma, RK, Lewis-Jones, I, Thomas, AJ & Agarwal, A 2005, 'Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients', *Asian Journal of Andrology*, vol. 7, no. 2, pp. 121-6.

Salamonsen, LA, Nie, G, Dimitriadis, E, Robb, L & Findlay, JK 2001, 'Genes involved in implantation', *Reproduction, Fertility and Development* vol. 13, no. 1, pp. 41 - 9

Saliba, E & Henrot, A 2001, 'Inflammatory mediators and neonatal brain damage', *Biol Neonate*, vol. 79, pp. 224-7.

Salustri, A, Ulisse, S, Yanagishita, M & Hascall, VC 1990, 'Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta', *Journal of Biological Chemistry*, vol. 265, no. 32, pp. 19517-23.

Sanderson, JT 2006, 'The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals', *Toxicological Sciences*, vol. 94, no. 1, pp. 3-21.

Sanocka, D, Miesel, R, Jedrzejczak, P, Chelmonska-Soyta, A & Kurpisz, M 1997, 'Effect of reactive oxygen species and the activity of antioxidant systems on human semen; association with male infertility', *International Journal of Andrology*, vol. 20, no. 5, pp. 255-64.

Saotome, K, Morita, H & Umeda, M 1989, 'Cytotoxicity test with simplified crystal violet staining method using microtitre plates and its application to injection drugs', *Toxicology in Vitro*, vol. 3, no. 4, pp. 317-21.

Sasson, R & Amsterdam, A 2002, 'Stimulation of Apoptosis in Human Granulosa Cells from in Vitro Fertilization Patients and Its Prevention by Dexamethasone: Involvement of Cell Contact and Bcl-2 Expression', *The Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 7, pp. 3441-51.

Saxena, D, Safi, R, Little-Ihrig, L & Zeleznik, JA 2004, 'Liver Receptor Homolog-1 Stimulates the Progesterone Biosynthetic Pathway during Follicle-Stimulating Hormone- Induced Granulosa Cell Differentiation', *Endocrinology*, vol. 145, no. 8, pp. 3821-9.

Schipper, I, Fauser, BCJM, Gaver, EBO, Zarutskie, PW & Dahl, KD 1993, 'Development of a human granulosa cell culutre model with follicle stimulating hormone responsiveness', *Human Reproduction*, vol. 8, no. 9, pp. 1380-6.

Schmidt, CL, Kendall, JZ, Dandekar, PV, Quigley, MM & Schmidt, KL 1984, 'Characterization of long-term monolayer cultures of human granulosa cells from follicles of different size and exposed in vivo to clomiphene citrate and hCG', *Journal of Reproduction and Fertility*, vol. 71, no. 1, pp. 279-87.

Seawright, AA, Nolan, CC, Shaw, GR, Chiswell, RK, Norris, RL, Moore, MR & Smith, MJ 1999, 'The oral toxicity for mice of the tropical cyanobacterium Cylindrospermopsis raciborskii (Woloszynska)', *Environmental Toxicology*, vol. 14, no. 1, pp. 135-42.

BoM National Climate Centre 2006, *Annual Climate Summary 2005*, by Section, CA.

Seifer, D, MacLaughlin, D, Penzias, A, Behrman, H, Asmundson, L, Donahoe, P, Haning, R, Jr & Flynn, S 1993, 'Gonadotropin-releasing hormone agonist-induced differences in granulosa cell cycle kinetics are associated with alterations in follicular fluid mullerian-inhibiting substance and androgen content', *The Journal of Clinical Endocrinology and Metabolism*, vol. 76, no. 3, pp. 711-4.

Seifert, M, McGregor, G, Eaglesham, G, Wickramasinghe, W & Shaw, G 2007, 'First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, Lyngbya wollei (Farlow ex Gomont) Speziale and Dyck', *Harmful Algae*, vol. 6, no. 1, pp. 73-80.

Senogles, PJ, Shaw, G, Smith, M, Norris, R, Chiswell, R, Mueller, J, Sadler, R & Eaglesham, G 2000, 'Degradation of the cyanobacterial toxin cylindrospermopsin from *Cylindrospermopsis raciborskii*, by chlorination', *Toxicon*, vol. 38, no. 9, pp. 1203-13.

Sevin, BU, Peng, ZL, Perras, JP, Ganjei, P, Penalver, M & Averette, HE 1988, 'Application of an ATP-bioluminescence assay in human tumor chemosensitivity testing', *Gynecologic Oncology*, vol. 31, no. 1, pp. 191-204.

Shalgi, R, Kraicer, P, Rimon, A, Pinto, M & Sferman, N 1973, 'Proteins of Human Follicular Fluid: The Blood-Follicle Barrier', *Fertility and Sterility*, vol. 24, no. 6, pp. 429-33.

Shaw, GR, Seawright, AA, Moore, MR & Lam, PKS 2000, 'Cylindrospermopsin, A Cyanobacterial Alkaloid: Evaluation of Its Toxicologic Activity. ', *Therapeutic Drug Monitoring*, vol. 22, no. 1, pp. 89-92.

Shen, X, Lam, PKS, Shaw, GR & Wickramasinghe, W 2002, 'Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin', *Toxicon*, vol. 40, pp. 1499-501.

Siddiqui, EJ, Shabbir, MA, Mikhailidis, DP, Mumtaz, FH & Thompson, CS 2006, 'The effect of serotonin and serotonin antagonists on bladder cancer cell proliferation', *BJU International*, vol. 97, no. 3, pp. 634-9.

Simard, J, Ricketts, M-L, Gingras, S, Soucy, P, Feltus, FA & Melner, MH 2005, 'Molecular Biology of the 3β- Hydroxysteroid Dehydrogenase/Δ^5-Δ^4 Isomerase Gene Family', *Endocrine Reviews*, vol. 26, no. 4, pp. 525-85.

Sivonen, K & Jones, G 1999, 'Cyanobacterial Toxins', in I Chorus & J Bartman (eds), *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management*, E & FN Spon on behalf of the WHO, Londond.

Smith, LP, Nierstenhoefer, M, Yoo, SW, Penzias, AS, Tobiasch, E & Usheva, A 2009, 'The Bile Acid Synthesis Pathway Is Present and Functional in the Human Ovary', *PLoS ONE*, vol. 4, no. 10, p. e7333.

Soboloff, J, Sasaki, H & Tsang, BK 2001, 'Follicular Stage-Dependent Tumor Necrosis Factor a-Induced Hen Granulosa Cell Integrin Production and Survival in the Presence of Transforming Growth Factor a In Vitro', *Biology of Reproduction*, vol. 65, pp. 477-87.

Steinberg, CEW & Hartmann, HM 1988, 'Planktonic bloom-forming Cyanobacteria and the eutrophication of lakes and rivers', *Freshwater Biology*, vol. 20, pp. 279-87.

Stenekes, N, Colebatch, HK, Waite, TD & Ashbolt, NJ 2006, 'Risk and governance in water recycling public acceptance revisited', *Science Technology & Human Values*, vol. 31, no. 2, pp. 107-34.

Stephansson, O, Kieler, H, Granath, F & Falconer, H 2009, 'Endometriosis, assisted reproduction technology, and risk of adverse pregnancy outcome', *Human Reproduction*, vol. 24, no. 9, pp. 2341-7.

Steuerwald, NM 2007, 'Maternal age-related differential global expression profiles observed in human oocytes', *Reproductive BioMedicine Online*, vol. 14, no. 6, pp. 700-8.

Stewart, I, Webb, P, Schluter, P, Fleming, L, Burns, J, Gantar, M, Backer, L & Shaw, G 2006, 'Epidemiology of recreational exposure to freshwater cyanobacteria - an international prospective cohort study', *BMC Public Health*, vol. 6, p. 93.

Stocco, DM & Clark, BJ 1996, 'Regulation of the acute production of steroids in steroidogenic cells', *Endocrine Reviews*, vol. 17, no. 3, pp. 221-44.

Stocco, DM & Clark, BJ 1996, 'Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis', *Biochemical Pharmacology*, vol. 51, no. 3, pp. 197-205.

Stocco, DM, Clark, BJ, Reinhart, AJ, Williams, SC, Dyson, M, Dassi, B, Walsh, LP, Manna, PR, Wang, X, Zeleznik, AJ & Orly, J 2001, 'Elements involved in the regulation of the StAR gene', *Molecular and Cellular Endocrinology*, vol. 177, no. 1-2, pp. 55-9.

Stocco, DM & Strauss, IIIJF 1998, 'Intramitochondrial cholesterol transfer in steroidogenic cells', in TY Chang & DA Freeman (eds), *Intracellular Cholesterol Trafficking*, Kluwer Academic, Boston, pp. 169-82.

Stocco, DM, Wang, X, Jo, Y & Manna, PR 2005, 'Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought', *Molecular Endocrinology*, vol. 19, no. 11, pp. 2647-59.

Stoklosowa, S, Bahr, J & Gregoraszczuk, E 1978, 'Some Morphological and Functional Characteristics of Cells of the Porcine Theca Interna in Tissue Culture', *Biology of Reproduction*, vol. 19, no. 4, pp. 712-9.

Stylianou, E & Saklatvala, J 1998, 'Interleukin-1 ', *The International Journal of Biochemistry & Cell Biology*, vol. 30, no. 10, pp. 1075-9.

Sukenik, A, Reisner, M, Carmeli, S & Werman, M 2006, 'Oral toxicity of the cyanobacterial toxin cylindrospermopsin in mice: Long-term exposure to low doses', *Environmental Toxicology*, vol. 21, no. 6, pp. 575-82.

Tapanainen, J, Leinonen, PJ, Tapanainen, P, Yamamoto, M & Jaffe, RB 1987, 'Regulation of human granulosa-luteal cell progesterone production and proliferation by gonadotropins and growth factors', *Fertility and Sterility*, vol. 48, no. 1, pp. 576-80.

Teare, JM, Islam, R, Flanagan, R, Gallagher, S, Davies, MG & Grabau, C 1997, 'Measurement of Nucleic Acid Concentrations Using the DyNA Quant™ and the GeneQuant™', *Biotechniques*, vol. 22, no. 6, pp. 1170-4.

Teerds, KJ & Dorrington, JH 1993, 'Immunohistochemical Localization of 3-Beta-Hydroxysteroid Dehydrogenase in the Rat Ovary During Follicular Development and Atresia', *Biology of Reproduction*, vol. 49, no. 5, pp. 989-96.

Templeton, A, Morris, JK & Parslow, W 1996, 'Factors that affect outcome of in-vitro fertilisation treatment', *Lancet*, vol. 348, no. 9039, pp. 1402-6.

Terao, K, Ohmori, S, Igarshi, K, Ohtani, I, Watanabe, MF, Harada, KI, Ito, E & Watanabe, M 1994, 'Electron Microscope Studies on Experimental Poisoning in Mice Induced by Cylindrospermopsin Isolated from Blue-Green Alga *Umezakia Natans*', *Toxicon*, vol. 32, no. 7, pp. 833-43.

Thomas, JL, Duax, WL, Addlagatta, A, Kacsoh, B, Brandt, SE & Norris, WB 2004, 'Structure/function aspects of human 3 beta-hydroxysteroid dehydrogenase', *Molecular and Cellular Endocrinology*, vol. 215, no. 1-2, pp. 73-82.

Thompson, JL, Manore, M, M & Vaughan, LA 2011, *The Science of Nutrition*, 2nd edn, Pearsons Education Inc, San Francisco, USA.

Tomsu, M, Sharma, V & Miller, D 2002, 'Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters', *Human Reproduction*, vol. 17, no. 7, pp. 1856-62.

Tsutsumi, R, Hiroi, H, Momoeda, M, Hosokawa, Y, Nakazawa, F, Koizumi, M, Yano, T, Tsutsumi, O & Taketani, Y 2008, 'Inhibitory Effects of Cholesterol Sulfate on Progesterone Production in Human Granulosa-like Tumor Cell Line, KGN', *Endocrine Journal*, vol. 55, no. 3, pp. 575-81.

Tureck, RW & Strauss, JFd 1982, 'Progesterone synthesis by luteinized human granulosa cells in culture: the role of de novo sterol synthesis and lipoprotein-carried sterol', *The Journal of Clinical Endocrinology and Metabolism*, vol. 54, no. 2, pp. 367-73.

Ubaldi, F 2005, 'Review: Management of Poor Responders in IVF', *Reproductive BioMedicine Online*, vol. 10, no. 2, pp. 235-46.

Vaughan, D 2002, *Histology Learning System*, Oxford University Press, 9th May 2011, <http://www.bu.edu/histology/m/t_female.htm>.

Vigue, C, Vigue, L & Huszar, G 1992, 'Adenosine Triphosphate (ATP) Concentrations and ATP/Adenosine Diphosphate Ratios in Human Sperm of Normospermic, Oligospermic, and Asthenospermic Specimens and in their Swim-up Fractions:Lack of Correlation Between ATP Parameters and Sperm Creatine Kinase Concentrations', *Journal of Andrology*, vol. 13, no. 4, pp. 305-11.

Vistica, DT, Skehan, P, Scudiero, D, Monks, A, Pittman, A & Boyd, MR 1991, 'Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production', *Cancer Research*, vol. 51, no. 10, pp. 2515-20.

Voutilainen, R, Tapanainen, J, Chung, BC, Matteson, KJ & Miller, WL 1986, 'Hormonal regulation of P450scc (20,22-desmolase) and P450c17 (17 alpha-hydroxylase/17,20-lyase) in cultured human granulosa cells', *The Journal of Clinical Endocrinology and Metabolism*, vol. 63, no. 1, pp. 202-7.

Wade, MG, Foster, WG, Younglai, EV, McMahon, A, Leingartner, K, Yagminas, A, Blakey, D, Fournier, M, Desaulniers, D & Hughes, CL 2002, 'Effects of subchronic exposure to a complex mixture of persistent contaminants in male rats: systemic, immune, and reproductive effects', *Toxicological Sciences*, vol. 67, no. 1, p. 131.

Wagner, U, Schlebusch, H, Vanderven, H, Vanderven, K, Diedrich, K & Krebs, D 1990, 'Accumulation of Pollutants in the Genital-Tract of Sterility Patients', *Journal of Clinical Chemistry and Clinical Biochemistry*, vol. 28, no. 10, pp. 683-8.

Walsh, LP, McCormick, C, Martin, C & Stocco, DM 2000, 'Roundup Inhibits Steroidogenesis by Disrupting Steroidogenic Acute Regulatory (StAR) Protein Expression', *Environmental Health Perspectives*, vol. 108, no. 8, p. 769.

Wang, L, Brannstrom, M, Pascoe, V & Norman, R 1995, 'Cellular composition of primary cultures of human granulosa-lutein cells and the effect of cytokines on cell proliferation', *Reproduction, Fertility and Development*, vol. 7, no. 1, pp. 21-6.

Wattenberg, LW 1958, 'Microscopic Histochemical Demonstartion of Steroid - 3beta-OL Dehydrogenase in Tissue Sections', The *Journal Histochemistry and Cytochemistry*, vol. 6, no. 4, pp. 225-32.

WHO 1999, 'World Health Organisation laboratory manual for the examination of human semen and sperm-cervical mucus interaction', in Fourth edn, Cambridge University Press, pp. 1-128.

Yie, SM, Brown, GM, Liu, GY, Collins, JA, Daya, S, Hughes, EG, Foster, WG & Younglai, EV 1995, 'Melatonin and steroids in human pre-ovulatory follicular fluid: seasonal variations and granulosa cell steroid production', *Human Reproduction*, vol. 10, no. 1, pp. 50-5.

Yong, EL, Baird, DT, Reichert, LE & Hillier, SG 1992, 'Hormonal Regulation of the Growth and Steroidogenic Function of Human Granulosa Cells', *Journal of Clinical Endocrinology and Metabolism*, vol. 74, no. 4, pp. 842-9.

Young, FM, Menadue, MF & Lavranos, TC 2005, 'Effects of the insecticide amitraz, an α2-adrenergic receptor agonist, on human luteinized granulosa cells', *Human Reproduction*, vol. 20, pp. 3018-25.

Young, FM, Micklem, J & Humpage, AR 2008, 'Effects of blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells in vitro', *Reproductive Toxicology*, vol. 25, no. 3, pp. 374-80.

Young, FM, Phungtamdet, W & Sanderson, BJS 2005, 'Modification of MTT assay conditions to examine the cytotoxic effects of amitraz on the human lymphoblastoid cell line, WIL2NS', *Toxicology in Vitro*, vol. 19, no. 8, pp. 1051-9.

Young, FM, Phungtamdet, W & Sanderson, BJS 2005, 'Optimisation of an MTT Assay for the Determination of Amitraz Toxicity Using a Human Lymphoblastoid Cell Line', *Toxicology in Vitro*, vol. 19, no. 8, pp. 1051-9.

Yousef, MI, Kamel, KI, El-Guendi, MI & El-Demerdash, FM 2007, 'An in vitro study on reproductive toxicity of aluminium chloride on rabbit sperm: The protective role of some antioxidants', *Toxicology*, vol. 239, no. 3, pp. 213-23.

Yu, F-Q, Han, C-S, Yang, W, Jin, X, Hu, Z-Y & Liu, Y-X 2005, 'Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially', *J Endocrinology*, vol. 186, no. 1, pp. 85-96.

Zlotkin, T, Farkash, Y & Orly, J 1986, 'Cell-Specific Expression of Immunoreactive Cholesterol Side-Chain Cleavage Cytochrome P-450 During Follicular Development in Rat Ovary', *Endocrinology*, vol. 119, no. 6, pp. 2809-20.

Zonneveld, PV, Te Velde, ER & Koppeschaar, HPF 1994, 'Low luteal phase serum progesterone levels in regularly cycling women are predictive of subtle ovulation disorders', *Gynecological Endocrinology*, vol. 8, no. 3, pp. 169-74.

Zosmer, A, Elder, M & Sullivan, M 2002, 'The production of progesterone and 5, 6-epoxyeicosatrienoic acid by human granulosa cells', *The Journal of steroid biochemistry and molecular biology*, vol. 81, no. 4-5, pp. 369-76.

Zreik, TG, Kodaman, PH, Jones, EE, Olive, DL & Behrman, H 1999, 'Identification and characterization of an ascorbic acid transporter in human granulosa–lutein cells', *Molecular Human Reproduction*, vol. 5, no. 4, pp. 299-302.