Role of Chemotherapeutics in Ovarian Failure

by

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SUMMARY

Breast cancer not only causes morbidity but is also responsible for reducing the quality of life among younger premenopausal survivors due to chemotherapy-induced premature ovarian failure and infertility. This doctoral thesis examines the toxic effects of commonly used breast cancer chemotherapeutics (doxorubicin and cyclophosphamide) on ovarian carcinoma (KGN) cells and murine ovarian follicles *in vitro*.

The breast cancer chemotherapy regimen has adverse side-effects caused by the generation of reactive oxygen species (ROS), as well as the specific mechanisms of action targeting proliferating cells. Tocopherols (alpha, beta, gamma and delta) have proven antioxidant activity. Moreover, previous studies demonstrated cytotoxic effects of gamma tocopherol (gToc) against breast cancer cells *in vitro* and *in vivo.* Hence, it was hypothesised that the addition of gToc to doxorubicin and cyclophosphamide would neutralize ROS generated during chemotherapy and increase ovarian follicle cell viability and hormone synthesis.

Clinically relevant doses of the activated form of cyclophosphamide, 4-hydroxycyclophosphamide (4OHCYC), were not toxic to ovarian carcinoma cells *in vitro*. Therefore, the dose of doxorubicin (5µM) at which 50% of KGN cells were killed (EC50) was combined with 4OHCYC at a dose (0.5µM) that maintains the 10:1 ratio found in patient serum. This combination significantly reduced KGN cell viability after 72h continuous exposure but the addition of gToc neither inhibited nor stimulated KGN cell viability or estrogen production. This study showed that doxorubicin but not 4OHCYC was cytotoxic to ovarian carcinoma cells at clinically relevant doses *in vitro*. Therefore, the inhibitory concentrations (IC values) of doxorubicin, 4OHCYC and gamma tocopherol against the MCF-7 breast cancer cell line were determined.

To study the direct effects of chemotherapeutics and antioxidants on follicles, it was necessary to isolate them from ovarian tissue in a way that avoided structural damage and maintained the physiological functions and viability of the follicles. Therefore, an easy, economical and robust method for isolating follicles from ovarian tissue and assessing the viability of the follicular cells were developed and characterised. The latter involved disaggregating follicles to generate a single cell suspension that could be assessed by trypan blue assay. There was a linear relationship between granulosa cells (GC) per follicle and follicle diameter (R^2: 0.9). From this study it was determined that morphologically intact, freshly isolated murine secondary follicles (125±6µm to 185±6µm) have 287

to 488 GCs of which only 61% to 72% are viable. This study also showed that follicle cell viability was not affected by the follicle isolation technique. However, the numbers of GCs derived from image analysis of intact, whole fluorescent-stained follicles were approximately 50% lower than from disaggregated follicles combined with trypan blue assessment. This difference was attributed to the 3-dimensional structure of the intact follicles because this may have obscured the fluorescent signal. The granulosa cell numbers derived from this study were higher than values estimated using fixed ovarian sections. It was concluded that the direct quantification of viable GC isolated from disaggregated follicles is the most accurate and reproducible method for assessing GC number and viability.

Follicles cultured in a 2D *in vitro* system did not grow during a 72h culture period. Their spherical structure was disrupted but the cell viability was similar to that found in freshly isolated follicles. Follicles produced 0.84±0.07pg/mL estrogen and 5.66±6.82pg/mL progesterone after 24h which was lower than that produced in a 3D culture system. Since the 2D culture of follicles led to the disruption of follicle morphology and dispersion of GCs across the surface of the culture plate, it was decided to examine and develop 3D follicle culture systems. Two 3D matrices were examined in a 48h study. Matrigel was performed because it supported the maintenance of spherical follicle morphology (47% with high M1 or M2 grade), granulosa cell viability (64% to 69%) and synthesis of both estrogen and progesterone. None of the 2D or 3D culture conditions examined supported AMH synthesis. Therefore, Matrigel was selected for further studies in which murine secondary follicles were exposed to commonly used breast cancer chemotherapeutics with or without antioxidants.

Doxorubicin and 4OHCYC alone and in combination were more toxic to primary derived follicular GCs within an intact follicle *in vitro*, than to the breast cancer cell line MCF-7, because the IC25 values of doxorubicin and 4OHCYC obtained from 24h MCF-7 exposures killed higher proportions (74% and 63% respectively) of cells inside intact murine secondary follicles *in vitro*. The combination of doxorubicin and 4OHCYC was additive; instead of killing 50% of the cells as exposure to the combined IC25 values of the two chemotherapeutics would predict, the combination killed 84% of the follicular cells. Exposure to the IC25 value of gamma tocopherol (gToc) obtained from the MCF-7 cells however was not at all cytotoxic to follicle cells and the addition of gToc to the doxorubicin and 4OHCYC combination also had no additional cytotoxicity. However, in this study the vehicle control for gToc, 0.3% DMSO, also maintained the morphological integrity and viability of follicles. Leading to the conclusion that the study should be repeated with a higher concentration of gToc to be able to give an unequivocal description of its separate effects on follicles.

Studies conducted during this PhD project together support the hypothesis that commonly used chemotherapeutics for treating breast cancer have direct damaging effects on the 3D structure of follicles. Exposure to chemotherapeutics disrupted the basal membrane of follicles which led to the loss of theca cells and further damage to follicle integrity. Together the loss of follicle integrity and the death of GCs led to reduced hormone synthesis. If this occurs *in vivo*, disruption to follicles would contribute to premature ovarian failure. Although the co-administration of gamma tocopherol did not entirely protect follicles from the damaging effect of chemotherapeutics, it demonstrated promise in that it maintained follicle integrity, follicle cell viability and hormone production when administered alone. Hence it is recommended that further studies should be conducted in which higher doses of gamma tocopherol are combined with the doxorubicin and cyclophosphamide.

This PhD project therefore evaluated the effects of commonly used chemotherapeutics on ovarian follicles. Findings increase understanding of the role of these chemotherapeutics in ovarian failure among women of reproductive age and support further research to develop chemotherapeutics with minimum or no effect on women's fertility. Overall, this project will contribute to improving the quality of life of cancer survivors by identifying some of the mechanisms of action of chemotherapeutic induced premature ovarian failure. The finding that a concentration of gToc that kills 25% of breast cancer cells but has no cytotoxicity to primary-derived follicles *in vitro* suggest that if gToc was found to be safe to add to patient's chemotherapeutic (doxorubicin and cyclophosphamide) regimens *in vivo*, it has the potential to increase anticancer efficacy, possibly reduce the number of chemotherapy cycles required, and so reduce adverse side-effects to the ovary.

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.

Mohammad Asaduzzaman

Date: 24 May 2018

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‘Have patience. All things are difficult before they become easy.’

Sheikh Saa’di (1210 – 1291)

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PUBLISHED WORK

The following is a list of peer-reviewed articles and conference presentations during candidature -

Peer-reviewed Articles-

1. Young, F, Drummond, J, Akers, E, Bartle, L, Kennedy, D & Asaduzzaman, M 2017, 'Effects of ovarian disaggregation on adult murine follicle yield and viability', *Reproduction, Fertility and Development*, vol. 29, no. 12, pp. 2400-2410.

2. Asaduzzaman, M, Gonzalez, DF & Young, F 2018, 'Ovarian follicle disaggregation to assess granulosa cell viability', *International Journal of Clinical Medicine,* vol. 9, pp. 377-399.

3. Figueroa, D, Asaduzzaman, M & Young, F 2018, 'Real time monitoring and quantification of reactive oxygen species in breast cancer cell line MCF-7 by 2', 7'–dichlorofluorescin diacetate (DCFDA) assay', *Journal of pharmacological and toxicological methods*, vol. 94, pp. 26-33.

4. Asaduzzman, M, Cui, X, Zhang, H and Young, F 2018, 'Three-Dimensional In Vitro Culture of Murine Secondary Follicles in a Defined Synthetic Matrix', *Journal of Biomaterials and Nanobiotechnology*, vol. 9, no. 3, p.244.

Accepted for publication

1. Young, F, Austin, S, Ho, D & Asaduzzaman, M 2018, 'Effect of Glyphosate and Roundup on steroidogenic cell viability and estrogen synthesis *in vitro*', *International Journal of Toxicology*.

Article Submitted for publication

1. Asaduzzaman, M, Rodgers, RJ & Young, FM 2018, 'Quantification of viable granulosa cells in murine ovarian follicles', *Biotechnic & Histochemistry*. **(Chapter 3, Section 3.3 in this thesis)**

2. Figueroa, D, Asaduzzaman, M & Young, F 2018, Effect of chemotherapeutics and γ-Tocopherol on MCF-7 breast adenocarcinoma and KGN ovarian carcinoma cell lines *in vitro*', *Biomed Research International.* **(Chapter 2, Section 2.6 in this thesis)**

Conference Presentations

Mohammad Asaduzzaman, David Kennedy, Xiaolin Cui, Hu Zhang, Fiona Young 2016 Culturing Mouse Ovarian Follicles in 3D Culture Matrix, Fertility society of Australia, Perth, Western Australia, 4-7 September 2016.

Mohammad Asaduzzaman, Roopayamini Fai Letchumann, Fiona Young 2017 A Comparison of Open and Closed Vitrification Systems using murine follicles, Mid-Year meeting of Scientists in Reproductive Technology (SIRT), Auckland, New Zealand, 6-7 May 2017.

Mohammad Asaduzzaman, Jordan Lee Durrans and Fiona Young 2018, The effect of cryopreservation on murine follicle growth and hormone synthesis *in vitro*, accepted for oral presentation in SIRT Mid-Year Meeting 2018, Hunter Valley, Newcastle, 5-6 May 2018.

Abstracts

Mohammad Asaduzzaman, John Drummond, David Kennedy, Fiona Young 2015 Quantification of granulosa cells in mouse ovarian follicles, Endocrine Society of Australia – Society of Reproductive Biology, Adelaide, South Australia 23 – 26 Aug, Abstr 296.

John Drummond, Fiona Young, Mohammad Asaduzzaman, David Kennedy 2015 Effect of ovarian disaggregation on murine follicle yield and quality, Endocrine Society of Australia – Society of Reproductive Biology, Adelaide, South Australia 23 – 26 Aug, Abstr 298.

Fiona Young, John Drummond, Mohammad Asaduzzaman, 2015 Ovarian disaggregation affects murine follicle yield & quality, 4th World Congress of International Society for Fertility Preservation, Shanghai, China, November 13-15 Nov, Abstr 086.

David Kennedy, John Drummond, Mohammad Asaduzzaman, Herman Fernandes, Fiona Young 2016 Assessment of Mouse Follicle Viability after Cryopreservation, Fertility society of Australia, Perth, Western Australia, 4-7 September 2016, Abstr 98.

GLOSSARY OF ABBREVIATIONS

2D:	Two Dimensional
3D:	Three Dimensional
4HPCYP:	4-hydroperoxycyclophosphamide
4OHCYC:	4-hydroxycyclophosphamide
AA:	Arachidonic Acid
AC:	Doxorubicin (Adriamycin) + Cyclophosphamide
AFC:	Antral Follicle Count
AMH:	Anti-Mullerian Hormone
AMHRII:	AMH type II receptors
ANOVA:	Analysis of Variance
AR:	Androgen Receptor
ATCC:	America Type Culture Collection
aToc:	Alpha Tocopherol
AYA:	Adolescent and Young Adult
BMP:	Bone Morphogenetic Proteins
BSA:	Bovine Serum Albumin
cAMP:	Cyclic Adenosine Monophosphate
COC:	Cumulus Oocyte Complex
COH:	Controlled Hyper Stimulation
CoV:	Coefficients of Variation
CRA:	Chemotherapy Related Amenorrhea
CV:	Crystal Violet
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	Deoxyribonucleic Acid
Dox:	Doxorubicin

dToc: Delta Tocopherol

E2: Estradiol

ECM: Extracellular Matrix

EDTA: Ethylene Diamine Tetra-Acetic Acid

EGF: Epidermal Growth Factor

EIA: Enzyme-Linked Immunoassay

ELISA: Enzyme-Linked Immunosorbent Assay

EthD 1: Ethidium Homodimer 1

EtOH: Ethanol

FA: Fibrin-Alginate

FA-IPN: Fibrin-Alginate Interpenetrating Network

FCS: Foetal Calf Serum

FSH: Follicle Stimulating Hormone

FSHR: FSH Receptor

GC: Granulosa Cell

GDF: Growth and Differentiating Factor

gToc: Gamma Tocopherol

GVB: Germinal Vesicle Break-down

HBSS: Hanks Balanced Salt Solution

IC: Inhibitory Concentration

InB: Inhibin B

IP: Intraperitoneal

ITS: Insulin, Transferrin and Selenium

IVF: *In Vitro* Fertilization

IVM: *In vitro* Maturation

LD: Live-Dead

LH: Luteinizing Hormone

MA:	Matrigel-alginate
MEM:	Minimal Essential Medium
MIS:	Mullerian Inhibiting Substance
mRNA:	Messenger Ribonucleic acid
NO:	Nitric Oxide
P4:	Progesterone
PARP:	Poly-ADP-Ribose Polymerase
PBS:	Phosphate Buffered Saline
PCOS:	Polycystic Ovary Syndrome
ROS:	Reactive Oxygen Species
SD:	Standard Deviation
TB:	Trypan Blue
TGF:	Transforming Growth Factor

1. CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

The treatment of cancers in adolescent and young adult (AYA) women can cause premature ovarian failure and infertility (Ben-Aharon et al. 2010a; Knight & Glister 2006; Meirow et al. 1999; Morgan et al. 2012; Oktem & Oktay 2007a; Petrillo et al. 2011; Roti et al. 2012; Yucebilgin et al. 2004; Yuksel et al. 2015). Ovarian granulosa cells (GC) synthesise and secrete anti mullerian hormone (AMH), and serum AMH concentrations in female oncology patients decrease as chemotherapy proceeds (Anders et al. 2008; Anderson et al. 2006; Anderson & Cameron 2011; Aslam et al. 2011; Decanter et al. 2010; Fong et al. 2008; Lawrenz et al. 2012; Lie Fong et al. 2008; Lutchman Singh et al. 2007; Oktay et al. 2006; Partridge et al. 2010; Peigne & Decanter 2014; Singh et al. 2007). However, the direct effects of chemotherapeutic agents on ovarian follicle cell viability and AMH production have not been examined.

AMH is produced by the proliferating GCS of small growing follicles (Baarends et al. 1995; Broekmans et al. 2008; Hanna et al. 2006; Pask et al. 2004; Peigne & Decanter 2014; Stubbs et al. 2005; Weenen et al. 2004b; Zec et al. 2011), and it has been suggested that cytotoxic chemotherapy might decrease the production of AMH by decreasing the numbers of viable GCs (Figure 1. 5). The recruitment of small inactive primordial follicles into the growing pool of follicles is suppressed by AMH (Durlinger et al. 2002; Durlinger et al. 1999; Themmen 2005), therefore the indirect removal of AMH by chemotherapy would result in the recruitment of more primordial follicles (which would otherwise remain quiescent) into the growing pool of follicles. These in turn would be killed by subsequent cycles of chemotherapy, and lead to premature ovarian failure. In Asian countries, 25% of breast cancer patients are premenopausal (Tiong et al. 2014). This cohort of young women is subject to the adverse impact of infertility on their post-cancer quality of life. Improved understanding of the effects of cancer and chemotherapy on aspects of ovarian and follicular physiology may lead to the development of anti-cancer treatments with additional benefits for preserving fertility in cancer survivors.

A combined regimen of doxorubicin and cyclophosphamide is commonly used to treat breast cancer patients but the effect of this chemotherapeutic regimen on ovarian follicles has not been examined. This PhD thesis presents an examination of the effects of clinically relevant doses of doxorubicin and cyclophosphamide on murine follicle cell viability and hormone production *in vitro*.

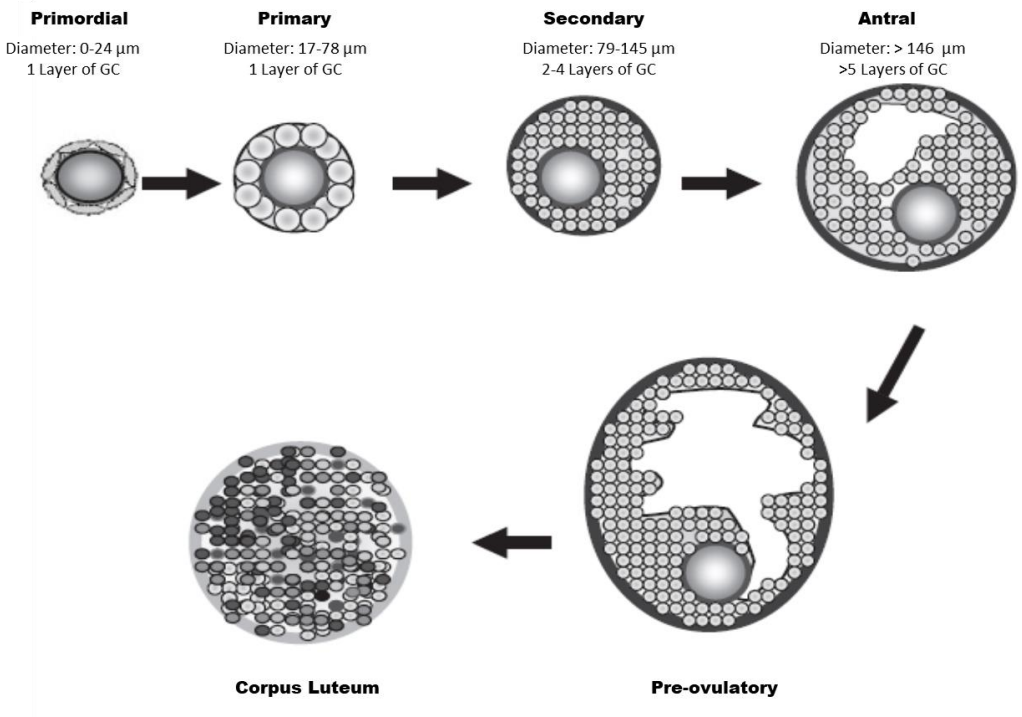
1.2 Folliculogenesis

Folliculogenesis is the process of ovarian follicle development. In the foetus, the primitive germ cells divide and differentiate to form oogonia. These oogonia are surrounded by somatic cells which in later stages differentiate into the cells that form the follicle (Eppig 2001; Gougeon 1996; Wallace & Kelsey 2010). A small fraction of the dormant primordial follicles are recruited to grow in a process known as follicle activation (Fortune et al. 2000; Gougeon 1996). An ovarian follicle passes through a series of developmental stages: primordial (quiescent), primary, secondary (pre-antral), tertiary (antral) and finally the pre-ovulatory large antral follicle stage (*Figure 1. 1*).

In the process of folliculogenesis, the oocyte of the primary follicle increases in size and secretes zona pellucida proteins as well as paracrine factors. These proteins and growth factors subsequently stimulate granulosa cell proliferation (Chen et al. 2015; Hernandez-Coronado et al. 2016; Hutt, McLaughlin & Holland 2006; Kawamura, Kawamura & Hsueh 2016; Plancha et al. 2005; Weenen et al. 2004a). Young and McNeilly (2010) identified one layer of flattened GCs in primordial follicles and showed that the GCs become cuboidal in primary follicles. Secondary follicles consist of 2 to 4 layers of GCs whereas antral follicles have more than 5 layers (Hutt, McLaughlin & Holland 2006; Young & McNeilly 2010). The diameters of follicles reflect the number of granulosa cell layers, and the numbers of GC in mouse follicles range from 0 to 24 (primordial), 17-78 (primary), 79-145 (early-Mid Secondary) and 146-215 (late secondary-incipient antral) and >216 μm (antral) (Bagger et al. 1993; Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Gougeon 1986; Gougeon & Chainy 1987; Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Liu et al. 2000; Pedersen & Peters 1968; Salmon, Handyside & Joyce 2004; Segino et al. 2005; Smitz & Cortvrindt 2002; Tsuji, Sowa & Nakano 1985). Secondary follicles are characterised by the differentiation of theca cells from the stroma which become mature theca cell layers in antral follicles (Gougeon 1996; Young & McNeilly 2010). Further follicular development results in the formation of a fluid filled antrum (Gougeon 1996; Weenen et al. 2004a) (Figure 1. 1).

Follicular development is tightly regulated by hormones, gonadotrophins and growth factors, and occurs through two recruitment processes; the entry of primordial follicles into the growing pool and the selection of a dominant follicle from the pool of growing secondary follicles (Fortune et al. 2000; Kawamura, Kawamura & Hsueh 2016; Knight & Glister 2006; Regan et al. 2017; Zec et al. 2011). During birth human ovaries contain 266 to 472 thousand resting follicles, a number which is reduced to 180,000 by puberty and less than 1000 at menopause (Gougeon 1996; Kelsey et al. 2011;

Liew et al. 2017; McLaughlin et al. 2015; Wallace & Kelsey 2010). After puberty, a small group of resting follicles are recruited in the growing pool and in each menstrual cycle, one dominant follicle undergoes ovulation. This dominant follicle is developed from a primordial follicle that was recruited to the growing pool approximately 6 to 12 months previously (Erickson & Danforth 1995; Erickson & Shimasaki 2001; Fortune et al. 2000; Zec et al. 2011).



	Flattened Granulosa cells (GC)		Oocyte
	Cuboidal Granulosa cells (GC)		Antrum

Figure 1. 1: Stages of follicle maturation (adapted from Barnett et al. (2006)).

After being recruited primordial follicles pass through primary, secondary, antral and pre-ovulatory stages before ovulation. Diameters are for mouse follicle and were derived from Griffin, Jeanine et al. (2006) and numbers of granulosa cell (GC) layers were described by Young and McNeilly (2010)

A primordial follicle consists of a small immature prophase stage oocyte surrounded by one layer of flattened (squamous) GCs (Telfer, E. E. & Zelinski, M. B. 2013). Human primordial follicles of 30 to 50 µm grow into pre-antral follicles of 100 to 200 µm (Gosden et al. 1993; McLaughlin, M. et al. 2014) whereas in mice, the primordial follicles are 0 to 24 µm and grow into secondary follicles of 79-145 µm (Griffin, Jeanine et al. 2006). Throughout reproductive life, from puberty to menopause, a combination of signals stimulates a small percentage of the primordial follicles to grow, which

means that the GCs start to proliferate and the oocytes increase in size (Eppig & O'Brien 1996; Erickson & Danforth 1995; Kidder & Mhawi 2002; Liew et al. 2017; Telfer et al. 2000; Weenen et al. 2004a).

1.2.1 Role of FSH, AMH, Androgen and Growth Factors in Follicle Development

Follicle development until the pre-antral stage is independent of the gonadotrophins; follicle stimulating hormone (FSH) and luteinising hormone (LH) released from pituitary gland and modulated by inhibin A and B that are produced during menstrual cycle from the female ovaries (De Kretser et al. 2004; Findlay et al. 2000; Groome et al. 1996; Makanji et al. 2009), while growth of follicles beyond the antral stage is known as gonadotrophin dependent (Dewailly et al. 2016). Androgen receptors (AR) are expressed maximally by preantral follicles but are thereafter reduced in large antral and pre-ovulatory follicles. It is therefore suggested that the role of androgen in folliculogenesis is in the development of follicles until the preantral stage (Catteau-Jonard et al. 2008; Hillier, Tetsuka & Fraser 1997; Lenie & Smitz 2009). Androgen produced by larger follicles stimulates the expression of anti-Mullerian hormone (AMH) gene and acts on the AR present in growing follicles (Lebbe & Woodruff 2013; Pigny et al. 2003). FSH receptors (FSHR) are expressed only by large preantral follicles which supports the effect of FSH on later stages of folliculogenesis (Gougeon 1996; Yamoto, Shima & Nakano 1992). Also, the early development of follicles to preantral stage in FSHR knockout mice (Durlinger et al. 2001) supported the FSH independent growth of follicles during early stages. However, Campbell et al. (2004) reported FSH induced growth of preantral follicles but only large preantral follicle expresses FSHR which is not in agreement of FSH stimulated follicle growth. However, it has been reported that FSH stimulates FSHR expression indirectly by androgen (Lebbe & Woodruff 2013) to enhance the growth of follicles in early stage. In addition, the selection of a dominant follicle is controlled by FSH (Zec et al. 2011) and FSH stimulates aromatase activity in the GCs in larger follicles which increases the conversion of androgen to estrogen (Sullivan et al. 1999).

In FSH null mice, primordial follicles were recruited into the growing pool and continued to grow to pre-antral stages (Eppig & O'Brien 1996) indicating that FSH was not required for the recruitment of primordial follicles. Dong et al. (1996) reported that primary follicle development did not occur in mice lacking Growth and Differentiating Factor 9 (GDF-9). In GDF-9 inactivated mice, follicle development did not proceed beyond primary follicle stage (Hanrahan et al. 2004). It was concluded that the activation of primordial follicles was not regulated by FSH but was controlled by GDF-9. Furthermore, BMP-15 (Bone Morphogenetic Proteins) is closely related to GDF-9, and BMP-15 null

mice were sub-fertile (Yan et al. 2001), and BMP-15 inactivated ewes were completely infertile (Juengel et al. 2002). These two factors (GDF-9 and BMP-15) are synthesized by the oocytes of growing primary or larger follicles and stimulate the proliferation of GCs (Knight & Glister 2006). Granulosa cell proliferation was also increased by Activin, BMP-2, 5, and 6, which are produced by GCs, BMP-2, -4 and -7 by theca cells and BMP-6 by oocytes (*Figure 1. 2*) (Knight & Glister 2006). Therefore, a combination of autocrine (Activin, BMP-2, 5, 6) and paracrine (GDF-9, BMP-6, BMP-15) signals regulate the growth of follicles. The two-way communication (autocrine and paracrine) takes place through the junctions between GCs and oocytes (Weenen et al. 2004a).

Picton (2001) suggested that the store of germ cells produces an inhibitory substance that maintains the balance between the growing and resting follicle numbers. Her prediction was partially substantiated by the finding that the genes that regulated the formation of primary follicles from primordial follicles were NOBOX and Foxl2 (Schmidt et al. 2004). Foxl2 was responsible for differentiating the squamous GCs of primordial follicles into the cuboidal GCs of primary follicles (Schmidt et al. 2004) whereas NOBOX was produced by oocytes and accelerated oocyte development. It was reported that NOBOX null mice had fewer numbers of oocytes than wild type (Rajkovic et al. 2004). These studies therefore indicated that squamous GCs were differentiated by Foxl2, the oocyte was developed by NOBOX, and that NOBOX and Foxl2 played a role in the transition from primordial to primary follicle.

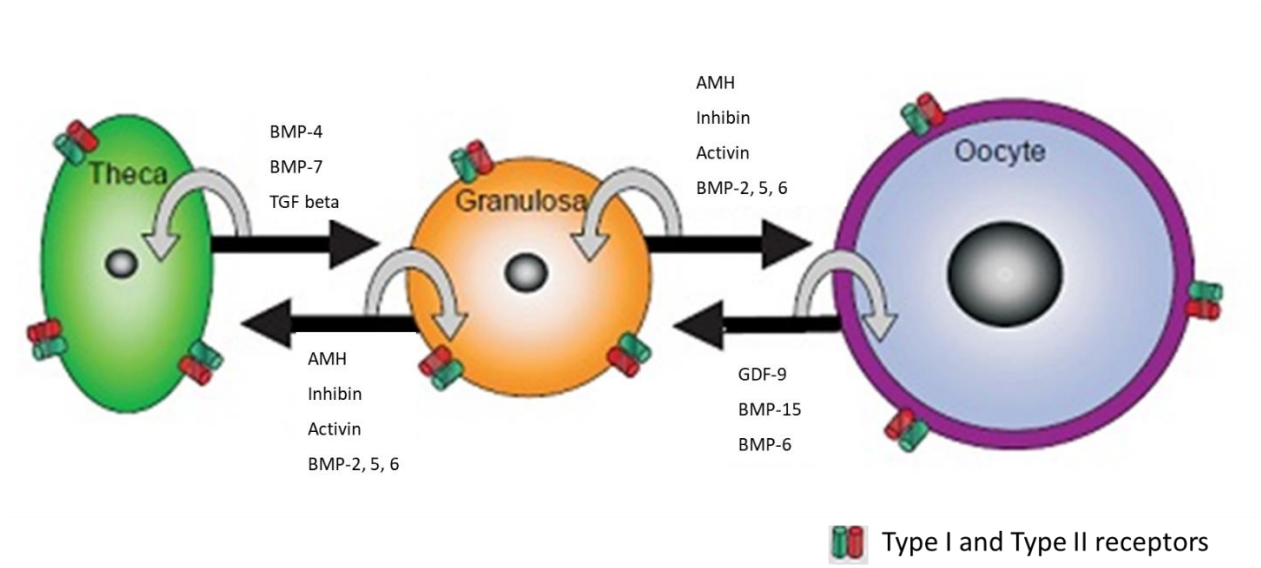


Figure 1. 2: Regulation during folliculogenesis, adopted from (Knight & Glister 2006).

All the growth factors belong to the Transforming Growth Factor (TGF) beta superfamily and work bi-directionally between GCs and theca, and GCs and oocyte. These growth factors exert autocrine (grey arrow) and paracrine (black arrow) activities through binding with type I or II receptors present on theca, GCs and oocyte. (AMH: Anti Mullerian Hormone, BMP: Bone Morphogenetic Proteins, GDF: Growth and Differentiation Factor)

1.3 Steroidogenesis in the Follicle

Steroidogenesis is the process where biologically active steroid hormones are produced from cholesterol. Although different glands produce different hormones, the basic steroidogenic pathway is similar with variations due to cell types (Miller & Auchus 2011). Follicles are one of the sites for steroidogenesis and can produce steroid hormones *in vitro* (Bergh et al. 1993; Fortune & Eppig 1979; Johnson et al. 2017; Lenie & Smitz 2009; Murray et al. 1998; Nestler et al. 1987; Young & McNeilly 2012). Production of sex-hormones by follicles is essential for the functioning of ovary and pregnancy maintenance (Zebian 2010). The basic pathway consists of cytochrome P450 induced production of pregnenolone from cholesterol which can take place either in the mitochondria or endoplasmic reticulum. The rate limiting step is the transportation of free cholesterol from cytoplasm to mitochondria through endocytosis (Miller 2008; Miller & Auchus 2011; Young, FM, Micklem, J & Humpage, AR 2008). However, the steroidogenic pathway involving cytochrome P450 is associated with generation of reactive oxygen species (ROS) which are detoxified by cellular intrinsic antioxidant glutathione peroxidase (Devine, Perreault & Luderer 2012; Hall 1988; Hanukoglu 2006). Although pregnenolone is not a hormone it is the precursor of all other hormones. Follicular GCs get the supply of cholesterol from low density lipoprotein present in plasma mainly derived from diet (Mason & Rainey 1987). Compared to other steroidogenic pathways in different

glands and organs, ovarian steroidogenesis is complex because the process of hormone production takes place in two types of cells (theca and GCs) (Figure 1. 3) present in ovarian follicles (Haggstrom 2014; Miller & Auchus 2011). Steroidogenesis starts in GCs after cAMP induced expression of P450scc enzyme which converts cholesterol to pregnenolone (Miller & Auchus 2011; Voutilainen et al. 1986; Whitelaw et al. 1992; Zebian 2010). Thereafter pregnenolone is converted to progesterone by 3βHSD2. Progesterone and pregnenolone diffuse out from the GC to the theca cells which produce dehydroepiandrosterone (DHEA) through the activity of P450c17 (Smyth et al. 1993). This is acted on by 3β-Hydroxysteroid Dehydrogenase 2 (3βHSD2) to produce androstenedione. A small fraction of androstenedione is then converted to testosterone by the action of aldo-keto reductase 1C3 (AKR1C3). Also, testosterone is produce via androstenediol from DHEA by 17βHSD1. The rest of the androstenedione then returns to the GC which converts it to estrone through CYP450aromatase activity thereafter to estradiol by 17βHSD1 under FSH stimulation. Testosterone produced in theca cells also acts as substrate for P450aromatase to produce estradiol (Boon & Simpson 2012; Miller & Auchus 2011; Pruitt 2016; Smyth et al. 1993; Strauss 2014; Strauss, Modi & McAllister 2014; Young, FM et al. 2012) (Figure 1. 3).

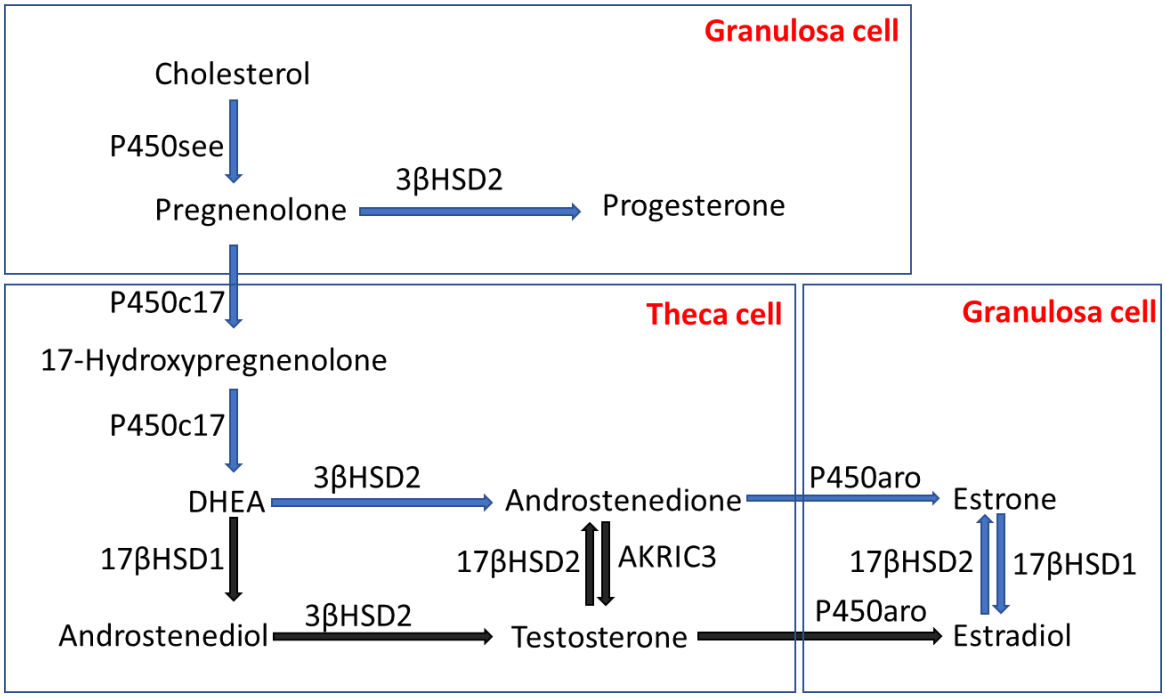


Figure 1. 3: Synthesis of steroid hormone in ovarian follicle

Androgens are synthesised from cholesterol in ovarian GCs. Hormone synthesis starts in GCs by the induction of P450see to produce pregnenolone which transfers to theca cells to produce other androgens; androstenedione and testosterone which are returned to the GCs from converting to estrogen and estradiol.

1.3.1 Role of AMH in Folliculogenesis

Until puberty, mature antral follicles undergo atresia, but after puberty, a small number of mature antral follicles are selected for ongoing development. The gonadotrophins (FSH and LH) stimulate the antral follicles to develop further into pre-ovulatory follicles (Dewailly et al. 2016; McGee & Hsueh 2000). In addition to the genes and growth factors described above, anti-Mullerian hormone (AMH) also plays an important role in two transitional stages; primordial follicle activation and dominant follicle selection (Figure 1. 4). AMH firstly inhibits the recruitment of primordial follicles in the growing pool (Durlinger et al. 2002) and secondly inhibits the FSH induced cyclic recruitment of dominant follicles by reducing the sensitivity of larger follicles to FSH (Figure 1. 4) (Choi & Rajkovic 2006; Dewailly et al. 2016; Diclemente et al. 1994; Durlinger et al. 2001). In simplest model, small growing follicles produce androgen to express AR and FSHR to support follicle growth in response to FSH indirectly by increased production of AMH. Zec et al. (2011) summarized the possible actions of AMH in the ovary as being inhibition of follicular activation and growth and as inhibition of FSH-stimulated growth by inhibiting granulosa cell proliferation (Figure 1. 4).

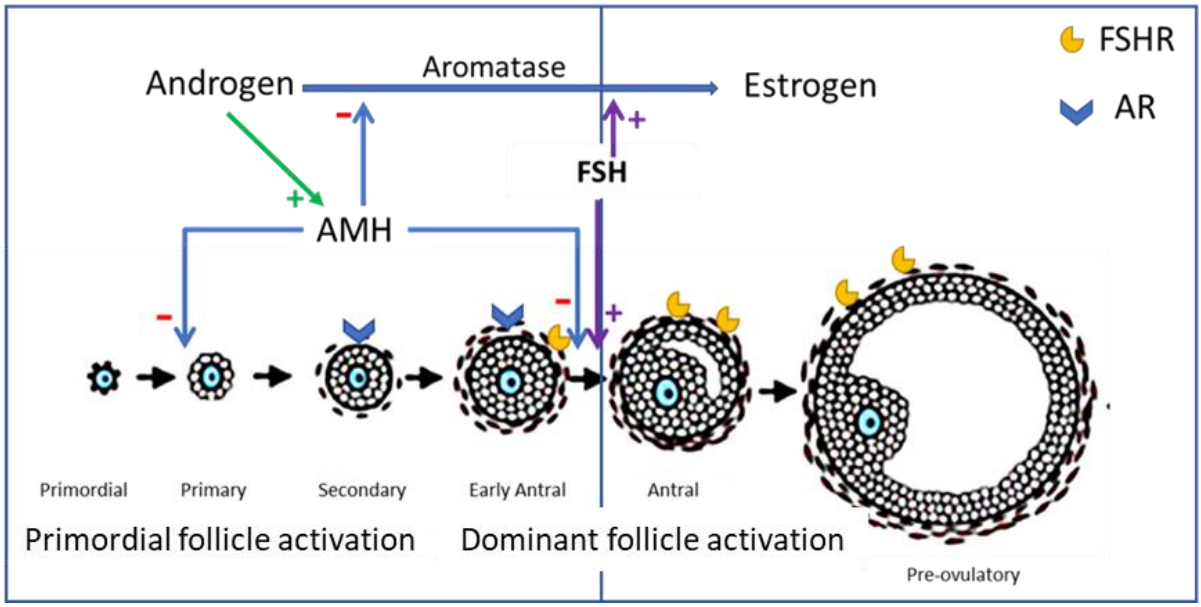


Figure 1. 4: Interaction of androgen, FSH and AMH during follicular growth.

Early development of follicle to early antral follicle is not dependent on FSH while growth of follicle from antral to ovulation is dependent on FSH. Androgen receptors (AR) present in GCs of secondary and early antral follicles while FSH receptors (FSHR) are expressed in GCs of larger antral follicles. FSH contribute to cyclic selection and promotes aromatase activity to increase estrogen production. AMH play role in primary selection and indirectly in dominant selection by reducing FSH sensitivity to larger follicles. Androgen stimulates AMH production by the small growing follicles. Image adopted from Orisaka et al. (2009) and Zec et al. (2011).

1.3.1.1 Activation of Primordial Follicles

Durlinger et al. (1999) examined the role of AMH in the mouse ovary by comparing AMH null mice with wild type mice of different ages. Four-month-old AMH null mice had lower numbers of primordial follicles and thirteen-month-old mice had almost no primordial follicles in comparison to wild type mice. An *in vivo* study in which AMH null mice and wild type mice were exposed to low and high levels of FSH found that there were higher numbers of growing follicles in the AMH null mice (Durlinger et al. 2001). These showed that AMH inhibited the recruitment of primordial follicles into the growing pool so that AMH deficient mice had more growing follicles and lower numbers of primordial follicles. In a later study, Durlinger et al. (2002) found that mouse ovaries cultured with AMH *in vitro* had 40 percent fewer growing follicles in comparison to control ovaries. It was indicated that primordial follicle pool depleted gradually in AMH knockout mice resulting in early cessation of oestrus cycle. This suggested the inhibitory effect of AMH on primordial follicle activation (Visser & Themmen 2005). In another study, ovaries from postnatal day 2 mice were treated with AMH *in vitro* and that treatment reduced the number of activated growing follicles (Choi & Rajkovic 2006). These studies confirm the concept that AMH has an inhibitory effect on the activation of mouse primordial follicles.

In a simple model, after primordial follicle activation, oocytes from primary and secondary follicles produce BMP-15 and GDF-9. These two transforming growth factors stimulate GCs to secrete AMH. The secreted AMH then inhibits and prevents the further recruitment of more primordial follicles into the growing pool (Knight & Glister 2006) (Figure 1. 2).

1.3.1.2 Cyclic Recruitment

AMH also has a role in inhibiting antral follicle growth in the cyclic recruitment phase. AMH reduced aromatase activity and LH receptor expression in porcine and postnatal rat GCs (Diclemente et al. 1994). During the follicular phase, FSH stimulates granulosa cell aromatase activity and induces LH receptors on the GCs (Sullivan et al. 1999). In later *in vivo* study, low and high level of FSH in AMH null mice produced higher number of growing follicles (Durlinger et al. 2001). These studies confirm the role of AMH in decreasing sensitivity of larger follicles to FSH (Choi & Rajkovic 2006; Diclemente et al. 1994; Durlinger et al. 2001).

Thus, AMH controls the primordial follicle pool by inhibiting initial recruitment (Durlinger et al. 2002; Durlinger et al. 1999; Visser & Themmen 2005). In the later stages of folliculogenesis FSH induces the recruitment of antral follicles (Dee 2004; Durlinger et al. 2001; Weschler 2003) while AMH

secreted by the growing follicles counteracts the stimulatory effects of FSH and prevents the cyclic recruitment of antral follicles (Figure 1. 2).

1.4 AMH in Animal Ovaries

A study examining rat ovaries used immunohistochemistry and mRNA in situ hybridization to show that the expression of AMH mRNA started on postnatal day 3 and was localised to the GCs (Baarends et al. 1995). The same study reported that AMH and AMH receptor type II (AMHRII) mRNA was expressed by the GCs of preantral and antral follicles, but that corpora lutea and large antral follicles expressed little or no AMH or AMHRII mRNA (Baarends et al. 1995). Although AMH mRNA was detected in GCs of primary follicles the AMH protein was only detected in small secondary follicles and AMH protein levels continued to increase to a maximum in large secondary follicles before declining in small antral follicles. The expression of AMH mRNA was up to 3 times lower in large antral follicles than small secondary follicles (Baarends et al. 1995).

Immunohistochemistry was used to characterise the expression of AMH in ovaries from Tammar Wallabys (*Macropus eugenii*). Rabbit polyclonal or C-20 goat polyclonal antibodies raised against human AMH were used as primaries and anti-rabbit or anti-goat IgG conjugated to peroxidase were used as secondary antibodies with hematoxylin counterstaining. Primary or transitional follicles stained weakly whereas secondary follicles stained well for AMH. In early antral follicles, AMH protein was detected in the GCs surrounding the oocyte whereas atretic follicles ceased producing AMH. This study showed that all mural and cumulus GCs of antral follicles synthesized AMH (Pask et al. 2004).

Mouse antihuman AMH antibody (clone 5/6) was used to examine the role of AMH in transition of primordial to primary follicles in polycystic ovaries and normo-ovulatory ovaries (Stubbs et al. 2005). Ovarian sections were incubated with mouse antihuman antibody. Expression of AMH was positive in primary follicles but relatively less intense in primordial and transitioning follicles. Also there was less AMH positive staining in primordial and transitioning follicles of polycystic ovaries in comparison to normo-ovulatory women (Stubbs et al. 2005). The mouse antihuman AMH antibody could stain AMH in follicles at all stages of development and staining was strongest in GCs (Stubbs et al. 2005). The above findings suggest that AMH is produced by the growing follicles (primary to small antral follicle) until the dominant follicle is recruited for further maturation and ovulation.

1.5 AMH in Human Ovaries and Serum

Gonadal tissues from 135 patients aged from 6 weeks post conception to 38 years of age were collected from two hospital archives to examine the expression of AMH by immunohistochemistry using monoclonal mouse anti-human AMH primary antibody. Early ovarian samples (9 to 34 weeks) did not express AMH because there were only primordial and primary follicles. AMH was detected in ovarian tissue at 36 weeks of gestation in secondary follicles where the oocyte was surrounded by cuboid GCs that expressed AMH (Rajpert-De Meyts et al. 1999).

In another study, the expression of AMH in ovarian sections collected from 12 normally cycling women of 19 to 44 years was investigated by immunohistochemistry. There was no AMH expression in primordial follicles, weak or little expression in 74% of primary follicles and the highest expression was found in secondary, preantral and small antral follicles (≤4 mm in diameter). Expression of AMH gradually reduced in larger (4-8 mm in diameter) antral follicles (Weenen et al. 2004a). Hanna et al. (2006) measured the AMH levels in follicular fluid by using the DSL AMH ELISA kit and found that the average concentration of AMH in human antral follicular fluid was 4 ng/mL (range: 0.3-16). The concentration of AMH decreased with increasing follicular size and the concentration was nil in follicles larger than 9 mm (Hanna et al. 2006). In summary, in humans AMH is produced by primary, secondary and small antral follicles.

La Marca et al. (2006) measured serum levels of AMH using the AMH/MIS ELISA kit from Immunotech in 12 women 18 to 24 years of age by taking blood samples every day during the menstrual cycle. It was found that the AMH level was lowest (3.4 ± 1.1 ng/mL) at day 14 and highest at day 12 (3.8 ± 1.2 ng/mL), but the difference between the highest and lowest AMH levels was not significant. In another study Hehenkamp et al. (2006) examined blood AMH levels using the AMH DSL ELISA in 44 menstruating women (25-47 years) across seven menstrual cycles and reported that the AMH levels were not significantly different from cycle to cycle or within a single cycle period. These two studies demonstrated that the menstrual cycle did not have any effect on serum AMH levels in healthy fertile women.

Kelsey et al. (2011) reviewed 3,260 published data points (including their own data) to develop a validated model of serum AMH concentrations from conception until menopause. Analysis of all the published data showed that AMH levels decreased after birth and started to increase after two years of age to reach the peak level at 24.5 years of age, after this serum AMH concentrations decreased until menopause. In another study, AMH levels were measured in blood samples from 17,120

women of 24 to 50 years of age in the United States of America. It was found that AMH levels declined with increasing age at a rate of 0.2 ng/mL/year until 35, 0.1 ng/mL/year from 36 to 40, and 0.1 ng/mL/year after 40 years of age (Seifer, Baker & Leader 2011). In general, AMH levels become measurable shortly after birth and continue to increase until they reach a peak when women are in their mid-20's, then decline thereafter until menopause.

In humans, the maximum number of 7 million ovarian follicles is found in the foetus at mid-gestation. This number then declines to 2 million at birth, half a million at puberty, 25 thousands at the age of 37 years, and less than one thousand at menopause (Wallace & Kelsey 2010). This progressive decline in follicle count with age is due to follicular atresia. Van Rooij et al. (2002) examined serum AMH levels and numbers of antral follicles in 130 patients undergoing IVF, on day 3 of a normal cycle. The numbers of antral follicles were determined by ultrasound. It was found that the serum AMH levels were dependent on the antral follicle numbers. In another study, La Marca et al. (2005) examined the origin of serum AMH in 24 women of late reproductive age of 44 ± 2.8 years, 14 menopausal women of 56 ± 4 years and 9 regularly cycling women of 43 ± 4 years who underwent oophorectomy. Blood samples were collected before and after surgery. AMH was undetectable in 2 out of 24 late reproductive aged women and undetectable in 13 out of 14 postmenopausal and all oophorectomy women. This indicated that AMH could be only from the ovarian origin. Hence serum AMH levels can be used as an indirect marker that reflects the number of follicles transitioning from primordial to antral stage.

1.5.1 Association Between AMH Level and Fertility

Initially serum AMH levels were used as an indicator of granulosa cell tumors (Broekmans et al. 2008), but recently, serum AMH levels have been used as a predictor of ovarian reserve. The relationship between AMH and follicular reserve was examined by comparing AMH null mice with wild type mice at different ages (25 days, 4 and 13 months) (Durlinger et al. 1999). This study reported higher number of preantral and small antral follicles in the 25 days and 4 months old mice but fewer numbers of primordial follicles in the 4 and 13 months old mice that were compared to wild type (Durlinger et al. 1999). This suggested depletion of AMH could be associated with depleted follicular reserve which subsequently can shorten reproductive life.

Gnoth et al. (2008) described a correlation between AMH levels and the response to controlled hyper stimulation (COH) in 316 *in vitro* fertilization (IVF) patients. The average AMH level was 1.7 ± 2.9 ng/mL, but when AMH levels were less than or equal to 1.26 ng/mL, four oocytes were retrieved

98% of the time. This study showed that there is an indirect relationship between AMH, follicular reserve and COH response.

Polycystic ovary syndrome (PCOS) is a common disorder of folliculogenesis that leads to infertility through the production of more small antral but fewer large ovulatory antral follicles (Webber et al. 2003). The ovarian abnormality in 32 PCOS patients (16 with normal regular cycles and 16 with oligomenorrhoea) was compared to 24 women with normal and regular cycles (Webber et al. 2003). PCOS patients in both groups had higher numbers of growing primary follicles but decreased numbers of primordial follicles compared with controls. Also anovulatory PCOS patients had six times higher numbers of small antral follicles than ovulatory PCOS patients (Webber et al. 2003). This results supported Durlinger et al. (1999) in that they suggested an intrinsic factor contributing to the depletion of follicular reserve in PCOS patients.

Stubbs et al. (2005) examined the effect of AMH on follicular progression from primordial to primary follicles in the ovaries of PCOS patients. Tissues from 16 polycystic ovaries were collected and AMH expression in 1403 follicles examined. The AMH positive primordial and transitional (from primordial to primary) follicles in polycystic ovaries and normal ovaries were 32% and 74% respectively. In another study, Pigny et al. (2006) used the Gen II ELISA to compare serum AMH levels in 73 PCOS patients with 96 controls. The AMH levels in PCOS patients and controls were 81.6 and 33.5 pmol/L which was significantly different. Catteau-Jonard et al. (2008) identified the increased expression of AMH protein by GCs from small follicles in POCS patients compared to controls. In that study, follicular fluids were collected from follicles of 8 to 13 mm and 17-22 mm in diameter. The proteins were purified, the concentrations were determined, and the samples examined by western immunoblot using an anti AMH monoclonal antibody. It was found that the AMH levels were higher in the follicular fluid of PCOS patients than in the control group. Together these studies indicate that higher serum AMH levels in PCOS patients may have inhibited the recruitment of primordial follicles to the growing pool.

In PCOS there is a “block” at early to mid-antral stage and the follicles do not undergo atresia (Webber et al. 2003). This means that follicles underwent primary selection, but cyclic recruitment is stopped. Therefore, the GCs present in these groups of small antral follicles produces more AMH (Catteau-Jonard et al. 2008) with subsequent inhibition of dominant follicle selection by counteracting the stimulatory effect of FSH (Choi & Rajkovic 2006; Diclemente et al. 1994; Durlinger et al. 2001; Visser et al. 2006).

1.6 Effect of Chemotherapy on Ovarian Follicles

The most commonly occurring cancers among women worldwide are breast, lung, thyroid, colon, uterine, Hon-Hodgkin lymphoma, pancreatic, ovarian, liver and stomach cancers (Torre et al. 2015; Torre et al. 2016). Breast cancer is the most diagnosed cancer among women worldwide, an estimated to 1,676,600 new cases in 2012 (Torre et al. 2015). Diagnosed cancer patients are treated with different chemotherapeutics (Gnaneswaran, Deans & Cohn 2012; Kim, Klemp & Fabian 2011; Park et al. 2013; Plowchalk & Mattison 1992; Tsai-Turton, M. et al. 2007; Yucebilgin et al. 2004). After administration of chemotherapeutics, these drugs reach the target organ, and other non-target organs through systemic circulation. Hence, it is possible that chemotherapeutics may have unwanted cytotoxic effects on ovary and ovarian follicles to alter the fertility of women of reproductive age (De Vos, Smitz & Woodruff 2014; Devine, Perreault & Luderer 2012; Johnson et al. 2017; Laronda et al. 2014; Luderer 2014; Salama & Woodruff 2017; Shea, Woodruff & Shikanov 2014; Xiao et al. 2015).

Schmidt et al. (2005) cryopreserved cortical ovarian tissues from 22 patients before starting chemotherapy with a range of treatment regimens with different combinations of cyclophosphamide, epirubicin, 5-fluorouracil, methotrexate, adriamycin, bleomycin, vincristine, dacarbazine, hydroxydaunorubicin and etoposide. Ovarian function was characterized by ultrasonography on the remaining ovaries after chemotherapy to measure the ovarian volume, and to count antral follicles. Nine out of 22 patients had no antral follicles, with ovarian volume less than 1.3 cubic centimetre, indicating ovarian failure whereas 59% patients had 6 antral follicles with normal ovarian function. In another study, the effect of chemotherapy on primordial follicle reserve was examined in 26 patients. Of these, 10 had received chemotherapy either with alkylating agents (n=5) or non-alkylating agent (n=5) previously and 16 patients had not been exposed to chemotherapy previously. Primordial follicles in every fifth section of the ovarian tissue were counted. The numbers of primordial follicles were 5.4 ± 1.32 and 9.6 ± 2.2 in ovarian cortical pieces collected from chemotherapy and control patients respectively ($p=0.05$) (Oktay & Oktem 2007). Patients treated with alkylating agents had fewer (2.9±1.1) primordial follicles in comparison to the patient treated with non-alkylating agents (7.9±1.6) ($p<0.05$).

The effect of chemotherapy on fertility is dependent on a number of factors such as age of the patient, dose and number of cycles of chemotherapy (Kim, Klemp & Fabian 2011; Xiao et al. 2017). For example, every cycle of cyclophosphamide can decease the reproductive age of a woman by 1.5

to 3 years. Therefore, a patient treated with cyclophosphamide 2.4 to 3 g/m^2 for 3 to 4 months will lose the equivalent of 10 reproductive years (Kim, Klemp & Fabian 2011). The toxic effects vary according to the chemotherapeutic agents; alkylating agents such as cisplatin procarbazine, cyclophosphamide and the nitrosoureas are toxic to ovaries at high doses (Gnaneswaran, Deans & Cohn 2012).

1.6.1 Breast Cancer and Fertility

In Australia, there were 15,050 breast cancer female patients in 2012 of which one in 46 were below the age of 50 (Ferlay et al. 2015). Similarly, in the United States, 30% of cancers are breast cancer , estimated to 180,000 per year (DeSantis et al. 2014). Unlike developed countries, the breast cancer survivors in Asian countries are about 12 years younger. It has been reported that one in four breast cancer patients are below 35 years of age (Tiong et al. 2014). Reports indicated that, with improved diagnosis and treatment, the five year survival rate for breast cancer has increased from 80 to 90% (Ferlay et al. 2015; Morgan et al. 2012) which means that there are higher number of premenopausal breast cancer survivor female patients (Jeruss & Woodruff 2009; Walter, Xu & Woodruff 2017).

Chemotherapy related amenorrhea (CRA) was examined in a follow up study on 44 early stage breast cancer patients of 21 to 51 years old (mean age 40 years) who were exposed to different chemotherapy regimens as shown in Table 1. 1 (Anders et al. 2008).

Table 1. 1: Chemotherapy regimen breast cancer patients were exposed to in study by Anders et al. (2008)

Chemotherapy regimen	**Number of breast cancer patients**
Adriamycin+Cyclophosphamide (AC)	13
AC followed by Taxol	9
Docetaxel	8
AC followed by Taxotere	5
Dose dense AC	3
AC with neo adjuvant Taxol and Seloda	4
5-Fluorouracil+Epirubicin+Cyclophosphamide	1
Docetaxel+Carboplatin+Trastuzumab with adjuvant AC	1
Trastuzumab	8

Serum AMH levels were measured at no more than three weeks before starting chemotherapy, three to seven weeks after chemotherapy, six months and one year after completion of chemotherapy. There were significant differences in pre-chemotherapy serum AHM levels between women less than 35 years of age and above 35 years of age (2.72 vs 0.47 ng/mL, $p<0.0001$). After three to seven weeks, serum AMH levels sharply declined to 0.03 ng/mL in both groups but the decline was more pronounced in the older women (Anders et al. 2008). After 5 months of chemotherapy, although the AMH levels slightly increased to 0.26 ng/mL for the younger women, one year post chemotherapy the AMH levels were similar in both groups (0.03 ng/mL vs 0.07 ng/mL) (Anders et al. 2008). In addition, it was reported that the risk of CRA increased in women with lower pre-chemotherapy serum AMH levels. This study indicated that besides chemotherapy there is an inverse relation between serum AMH level and age of the women.

In another study, Partridge et al. (2010) compared markers to predict the ovarian reserve of 20 women (mean age 36.8 years) who survived early stage breast cancer with controls (n=20, mean age 36.9 years). Breast cancer patients were treated with one of the following chemotherapy regimens (Table 1. 2).

Table 1. 2: Chemotherapeutic regimens patients were exposed to in study by Partridge et al. (2010)

Chemotherapy regimens	**Number of patients**
Adriamycin + cyclophosphamide (AC)	9
AC followed by dense paclitaxel	8
AC followed by paclitaxel	1
High dose Adriamycin followed by cyclophosphamide+methotrexate+5-fluorouracil	1
High dose Adriamycin followed by cyclophosphamide+adriamycin+5-fluorouracil	1

Ovarian reserve was predicted by antral follicle count (AFC), AMH, FSH, inhibin B (InB), and E2 (Partridge et al. 2010). All of these markers showed different values for control and treatment groups. For example, the controls had higher values for antral follicle count (9.5 vs 6), inhibin B (33.09 vs 11.54), and AMH (1.35 vs 0.43) but were lower for FSH (6.9 vs 9.0) and E2 (38 vs 44.26) (Partridge et al. 2010). However, although all these markers showed different values from controls,

the differences in AMH levels showed the highest statistical significance, and this indicated a better marker to predict ovarian reserve after chemotherapy.

Chemotherapeutic effects on serum AMH levels were followed up for five years in 42 breast cancer patients and compared to 14 women with breast cancer but without chemotherapy (control group). 33 women remained in the study for five years but others were withdrawn due to disease recurrence, oophorectomy or hysterectomy (Anderson & Cameron 2011). Serum AMH levels were measured once a year for the study period of 5 years. Pre-treatment serum AMH levels were 1.29±0.21 ng/mL and 0.74±0.15 ng/mL for the chemotherapy and the non-chemotherapy group respectively, but the serum AMH levels declined to undetectable limits (0.09±0.02 ng/mL) after five years, in contrast to the non-chemo group in which serum AMH levels remained unchanged (0.22±0.11 ng/mL). In both the groups the serum AMH level declined gradually, but the extent of decline was higher in the chemotherapy group (Anderson & Cameron 2011).

Ovarian exposure to chemotherapeutics used for treating breast cancer has been reported to cause premature ovarian failure leading to infertility. The most diagnosed cancer among female is breast cancer which is commonly treated with chemotherapy regimen comprising of doxorubicin (Adriamycin) and cyclophosphamide. Hence, this chemotherapy regimen has been chosen to apply in this project to expose to murine ovarian follicle to examine their direct effect of this chemotherapy regimen on ovarian follicular cell viability and hormone synthesis.

1.6.2 Other Cancers and Fertility

Many haematological cancers affect young patients who will have their post-cancer quality of life adversely affected by infertility. Serum AMH concentrations were used to predict the ovarian reserve after chemotherapy in 25 haematological cancer survivors of 20 to 35 years and in 42 controls of mean age 29.9 years (normo-ovulatory women) (Fong et al. 2008). Patients were allocated to, group A (n=13, mean age 29.4 years) treated with either alkylating agents such as procarbazine and ifosfamide (n=10) or non-alkylating agents such as methotrexate and idarubicin (n=3) and group B (n=12, mean age 25.3 years) who underwent stem cell transplantation followed by total body irradiation in association with high dose (60 mg/kg) chemotherapy with procarbazine (Fong et al. 2008). Pre-chemotherapy serum AMH levels were 1.0, 0.9 and 2.1 for group A, B and control patients but after chemotherapy serum AMH levels were 0.3, 0 and 1.3 μg/L respectively. This study suggested that radiotherapy in conjunction with chemotherapy with alkylating agents reduced serum AMH levels more than chemotherapy with alkylating agents alone.

In another study, the level of AMH was measured before and after chemotherapy in 17 women of 19 to 35 years of age. Patients were diagnosed with different types of cancers including breast cancer, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and Ewing sarcoma and were treated with different chemotherapeutic agents including bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone, methotrexate, teniposide, camustine, prednisone, rituximab, etopophos, epirubicin, docetaxel, 5-flurouracil, dactinomycin and ifosfamide in different combinations. The serum AMH levels decreased from 2.7 ± 1.0 to 1.1 ± 0.6 ng/mL after the first cycle and to 0.4 ± 0.4 ng/mL after the second cycle (Rosendahl et al. 2010). AMH levels were measured before, during and after administration of either adriamycin, bleomycin, vinblastine, dacarbazine (ABVD group) or cyclophosphamide, to 30 young women (average age 24 years) with lymphoma. The AMH levels declined sharply after starting chemotherapy and this decrease continued until the end of chemotherapy. AMH levels started to increase 3 months after competing chemotherapy for all women except those who were treated with cyclophosphamide (Decanter et al. 2010). Findings thus suggest that chemotherapy reduces the level of serum AMH among patients irrespective of cancer types.

1.6.3 Effect of Chemotherapy on Serum AMH Level

Since serum AMH is an indirect indicator of ovarian reserve it has been used as a marker for fertility. Oktay et al. (2006) measured AMH levels in three breast cancer patients before and after chemotherapy and found that AMH levels declined after each course of chemotherapy. In another study, fifty premenopausal women of 28 to 52 years of age with breast cancer were recruited for assessing the effect of chemotherapy on AMH levels (Anderson et al. 2006). The chemotherapeutic treatment regimen differed from patient to patient (Table 1. 3)

Table 1. 3: Chemotherapy regimens breast cancer patients were exposed to in study by Anderson et al. (2006)

Regimen	Chemotherapeutics	Number of patients	Duration (wks)
FEC-T	5-fluorouracil + epirubicin + cyclophosphamide followed by docetaxel	8	24
CMF	Cyclophosphamide + methotrexate + 5-fluorouracil	3	18
E-CMF (Non-trial)	Epirubicin followed by CMF	10	24
E-CMF (Trail of accelerated chemotherapy: TACT)	Epirubicin followed by CMF	8	28

AMH levels were measured before, during and 6 months after completion of chemotherapy. Before initiation of the chemotherapy, the AMH levels were correlated to the age of the patients and demonstrated a negative correlation ($r = –0.45$, $P = 0.004$). During the treatment period of 12 months, AMH levels fell sharply from 1.13 ± 0.63 ng/mL to 0.15±0.04 ng/mL after 12 months. There was also a fall in antral follicle numbers from 5.8±0.7 to 2.1±0.3 after 12 months and a related reduction in ovarian volume from 4.3±1.3 to 3.0±0.2 mL (Anderson et al. 2006). Six months after completion of chemotherapy the AMH levels were found to increase gradually (Anderson et al. 2006). In later study, serum AMH levels of 22 breast cancer patients of 22 to 42 years of age were measured before and after chemotherapy (Singh et al. 2007). Two chemotherapeutic regimens were used; the first consisted of epirubicin, cyclophosphamide, methotrexate and 5-fluorouracil and the second consisted of 5-fluorouracil, epirubicin and cyclophosphamide. The serum AMH levels before and after chemotherapy (regimen 1) were 6.77 ± 1.7 ng/mL and 0.95 ± 0.34 ng/mL respectively (Singh et al. 2007).

AMH is produced by growing follicles before being recruited for dominance. It is thus possible that chemotherapy kills the proliferating GCs in growing follicles and thus AMH production is reduced. As a secondary effect of decreased AMH level, more of the follicles are activated and are killed by subsequent chemotherapy cycles. Hence the ovarian reserve is declined and fewer numbers of follicles grow to the antral stage.

1.6.4 Follicle Burn-out the Ovary

Women have a fixed number of dormant primordial follicle at birth and the number of resting primordial follicle in ovary at any stage of life indicates the reproductive lifespan of a woman (Findlay et al. 2015b, 2015a, 2015c; Liew, Vaithiyanathan & Hutt 2016; Tremellen & Savulescu 2014; Wallace & Kelsey 2010). During puberty, an adolescent girl has 180,000 resting primordial follicles of which a small group of follicles are being recruited to grow to produce mature egg through the reproductive age. Naturally, the number of primordial follicle at menopause reach to 1000 (Gougeon 1996; Kelsey et al. 2011; McLaughlin et al. 2015; Wallace & Kelsey 2010). A follicle surrounded by layers of GCs grow through folliculogenesis under tight regulation by AMH, growth factors, gonadotrophins and hormones (Knight & Glister 2006; Orisaka et al. 2009; Zec et al. 2011). During follicular development, GCs proliferate and support the growth of oocyte. However, when chemotherapy is used to treat cancers, chemotherapies are also exposed to ovarian follicular cells and kill proliferating GCs in growing follicles (Devine, Perreault & Luderer 2012; Mahajan 2015; Raz et al. 2002), but GCs of secondary follicles synthesize AMH which regulates the follicle reserve in

ovary (Kollmann et al. 2015; Visser et al. 2006) by inhibiting primary activation of dormant primordial follicle and inhibiting cyclic recruitment of antral follicles for dominance (Dee 2004; Diclemente et al. 1994; Durlinger et al. 2001; Knight & Glister 2006). Therefore, on exposure to chemotherapy, proliferating GCs are killed and the sources of AMH is removed which thereafter activates more of the resting primordial follicles into the growing pool which are being killed by subsequent chemotherapy cycles (*Figure 1. 5*) (Baarends et al. 1995; Stubbs et al. 2005; Woodard & Bolcun-Filas 2016). Also, the death of GCs leads to the death of oocyte (Hughes J R, M & C 1991; Liew, Vaithiyanathan & Hutt 2016). With the continuation of chemotherapy cycles, this cycle of primary activation of follicles and apoptosis of proliferating GCs continue and lead to follicle burn out of the fixed number of resting follicles in the ovary to cause premature ovarian insufficiency. This suggest that follicle burn out is one possible hypothesis in explaining the reduction of primordial follicle reserve after chemotherapy. Also, oocytes in resting primordial follicles are extremely sensitive to DNA damage on exposure to chemotherapeutics (Hutt et al. 2013; Kerr, Jeffrey B. et al. 2012; Kerr, Jeffrey B et al. 2012; Kim et al. 2013; Meirow et al. 2010). The DNA damage induced loss of primordial follicle is not reversed even after survival from cancer after chemotherapy (Kerr, Jeff B et al. 2012). This means that on exposure to chemotherapeutics ovarian reserve of primordial follicles is reduced because of DNA damage of resting oocytes while growing follicles are killed by chemotherapy cycles to reduce the reproductive age of women.

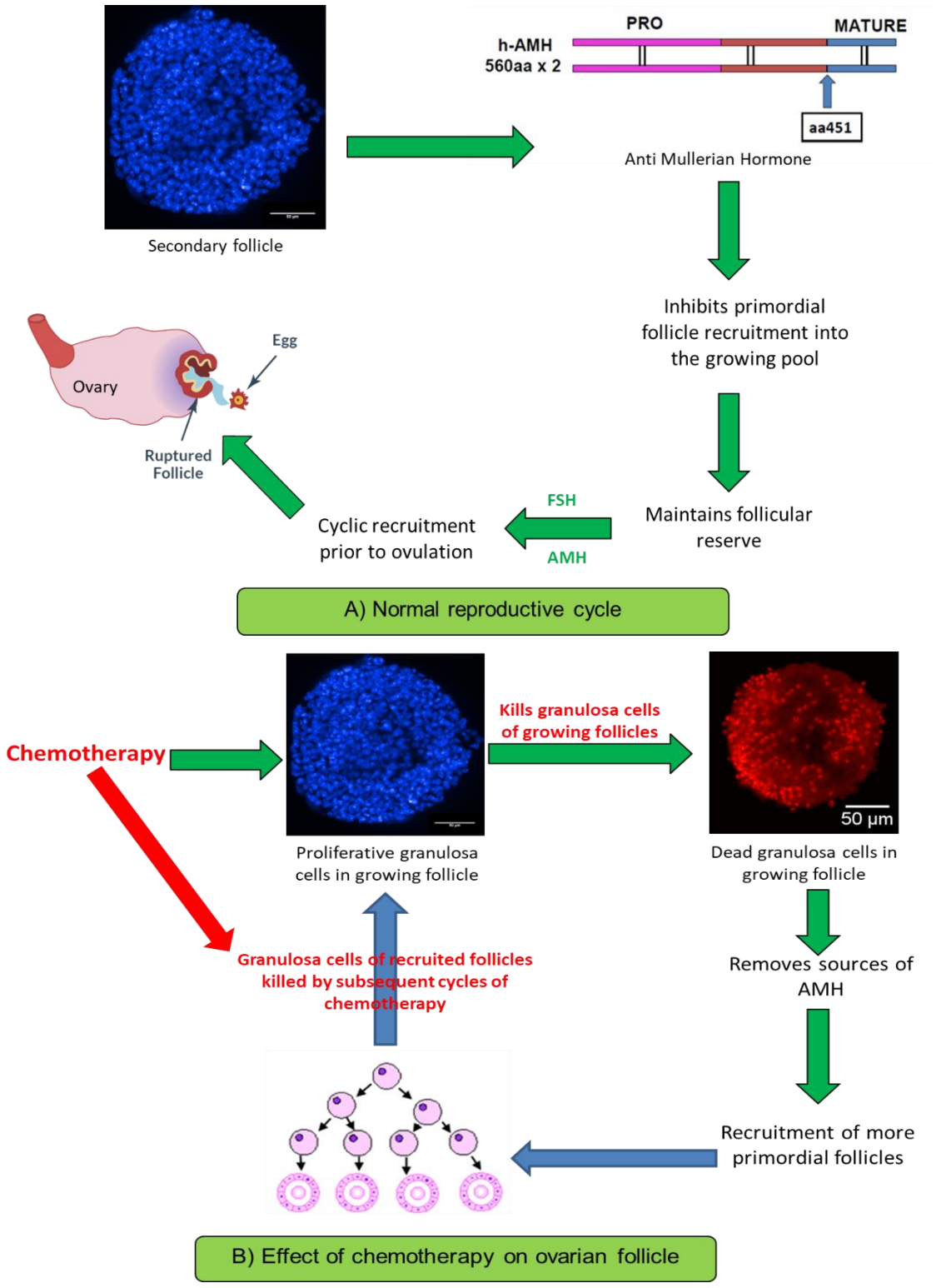


Figure 1. 5: Normal reproductive cycle (A) and effect of chemotherapy on ovarian follicle and AMH production (B)

Small group of dormant primordial follicles are recruited in the growing pool to grow further to secondary follicle stage which produce anti Mullerian hormone (AMH) which inhibits the recruitment of primordial follicle to maintain follicular reserve and fertility before menopause (A). on exposure to chemotherapy, removal of AMH synthesis source due to kill of proliferative GCs in growing follicles results recruitment of more primordial follicles and subsequently killed by following chemotherapy cycles (B).

However, the direct effects of chemotherapeutics on ovarian follicles have not been examined *in vitro*. Therefore, this PhD project aims to examine the direct effects of chemotherapeutics on murine ovarian follicular granulosa cell viability and hormone synthesis to better understand infertility among cancer survivors.

1.7 Animal Studies Examining Chemotherapy and Fertility

Female mice were exposed to cyclophosphamide at intraperitoneal (IP) doses of 0, 75, 200 and 500 mg/kg body weight in normal saline. Differential follicle counts were done on serial sections of ovaries after 1 to 14 days treatment. Cyclophosphamide significantly decreased the numbers of primordial and antral follicles (Plowchalk & Mattison 1992). The response was dose dependent and after 3 days all the primordial follicles were destroyed after administration of the 500mg dose. Growing follicles demonstrated less sensitivity to cyclophosphamide but the total ovarian volume was reduced by 30 to 40% by one day after administration of the 500mg dose. Morphometric examination suggested that follicular volume loss was due to loss of antral follicles, corpora lutea and interstitial tissue (Plowchalk & Mattison 1992).

Cyclophosphamide is detoxified in the body by glutathione peroxidise, an intrinsic antioxidant. Depletion of glutathione after treatment with cyclophosphamide increased granulosa cell apoptosis in rats (Tsai-Turton, M. et al. 2007). A human granulosa cell line (COV434) treated with buthionine sulfoximine to inhibit glutathione synthesis was co-treated with 4-hydroperoxycyclophosphamide (4HPCYP) and this caused significant induction of granulosa cell apoptosis (Tsai-Turton, M. et al. 2007). These studies suggest that chemotherapy with using cyclophosphamide may increase oxidative stress, decrease antioxidants and increase granulosa cell apoptosis.

Paclitaxel is a widely used to treat breast cancer. Paclitaxel 7.5 mg/kg or cisplatin 5mg/kg were administered to rats and after 7 days the ovaries were collected and stained with haematoxylin and eosin to enable enumeration of the primordial follicles. The numbers of primordial follicles in the control, paclitaxel and cisplatin groups were 23.1 ± 16.1, 10.3 ± 13.0 and 13.9 ± 15.2 respectively (Yucebilgin et al. 2004). The effect of paclitaxel on fertility was examined in rats by administering five doses of Paclitaxel at a dose of 5 mg/kg to rats every 3 days. The paclitaxel-treated and control animals were sacrificed and the numbers of follicles in ovarian sections were examined. Paclitaxel reduced the antral follicle count significantly but did not significantly affect the primordial and pre-antral follicle counts. This indicated that paclitaxel was not toxic to small or medium follicles but was only toxic to large follicles (Tarumi et al. 2009).

In 2009, the potential for lactoferrin to prevent ovarian damage caused by cyclophosphamide was evaluated. Lactoferrin is a milk-derived natural compound that has therapeutic actions against microbial infections and is believed to have activity in preventing cancer development. In the study conducted by Horiuchi et al. (2009), 2% bovine lactoferrin was co-administered with cyclophosphamide 200 mg/kg every alternate week for 24 weeks to female mice. Control group was administered 2% bovine serum albumin instead of lactoferrin. After 8 and 24 weeks of treatment, the expression of Adamts 1 mRNA and Soh1h 1 mRNA were significantly reduced in the control group in comparison to lactoferrin group. Adamts 1 is expressed by preovulatory follicular GCs and thus its expression is indicative of viable GCs in the growing follicle pool. On the other hand, Soh1h 1 is expressed by oocytes in primordial follicles. Once primordial follicles are recruited, the expression of Soh1h1 disappears. Hence Soh1h1 and Adamts 1 expressions are markers for the dormant and growing follicular reserve. Cyclophosphamide suppressed the expression of these two genes and lactoferrin exerted a protective effect against cyclophosphamide-induced follicular depletion. Lactoferrin promoted expression of Adamts 1 which is associated with follicular granulosa cell viability.

Ovarian toxic effects after 5 weeks treatment with single dose of busulfan and cyclophosphamide were evaluated by examining histological sections of ovaries from 28 mice. Germ cells were completely depleted in 13 out of 28 mice and the remaining 15 mice exhibited about 90% depletion of primordial and growing follicles in comparison to controls (Park et al. 2013). The effects were irreversible, because 15 weeks after ceasing treatment, 22 mice still did not produce any embryos.

Damage to the ovary and depletion of follicles were examined after 12h and 7day of 5 fluorouracil (5-FU) infusion at a dose of 150 mg/kg to mice (Lambouras et al. 2018). Resting follicle counts was not affected but the number of growing follicles (secondary and antral) immediately but restored at normal level after 7 days suggesting acute ovarian toxicity of 5-FU but is not associated with long term infertility of female cancer survivors.

Above studies clearly suggest the ovo-toxicity of chemotherapies which were measured in terms of ovarian follicle (primordial and antral) count but the effect of chemotherapies on growing follicle, follicular GCs and hormone synthesis are poorly understood. The prevention of cytotoxicity of modified chemotherapy regimens on follicular cell viability may help in conserving fertility among cancer survivors and improving living standards.

1.8 Development of Ovo-protective Chemotherapy

Previous sections (1.6.1 to 1.6.4) described the detrimental effects of chemotherapy on fertility. Among the chemotherapies, mostly used regimen is the combination of doxorubicin (Adriamycin) and cyclophosphamide (AC) (Ganz et al. 2017; Guo et al. 2011; Nahleh et al. 2016; Shulman et al. 2014; Singh et al. 2017; Swenson et al. 2003) which exert their effect by killing cancer cells but at the same time generates reactive oxygen species (ROS) which is responsible for adverse-effects including cardiomyopathy, amenorrhea, and infertility (Ataya et al. 1990; Detti et al. 2013; Goodman & Hochstein 1977; Himelstein-Braw, Peters & Faber 1978; Kalyanaraman et al. 2002; Minotti, Cairo & Monti 1999; Oktay & Oktem 2007; Singal & Panagia 1984) (detail discussed in chapter 6). Some preliminary studies suggest that concomitant administration of antioxidants can have beneficial effects in neutralizing ROS generated by AC and subsequently reduces chemotherapy induced adverse effects (Figure 1. 4). Among various antioxidants, below antioxidants were reported to have antioxidant property, cytotoxicity and higher bioavailability.

Table 1. 4: List of potential antioxidants

Antioxidant	Properties	References
Alpha tocopherol (aToc)	Serum concentration is highest	Lu, G et al. (2010)
	Co-administration with doxorubicin reduced cardiotoxicity	(Myers, McGuire & Young 1976; Myers et al. 1977)
	Co-administration with f-fluorouracil, adriamycin and cyclophosphamide did not have any cardioprotective effect	Legha et al. (1982)
	Reduced the incidences of prostate and bowel cancer	Blot et al. (1993)
Gamma tocopherol (gToc)	Dominant form in human diet Low level of gToc increased cardiovascular problems	Baumeister et al. (2009)
	Inhibited lung carcinoma cell growth	Lu, G et al. (2010)
	Inhibited breast, colon and prostate cancer	Smolarek, AK and Suh, N (2011)
Delta tocopherol (dToc)	Inhibited lung carcinoma cell growth	Lu, G et al. (2010)
	Increased pro-apoptotic proteins	Smolarek, AK and Suh, N (2011)
Q10	Cardio protective	(Baggio et al. 1994; Overvad et al. 1999; Pepping 1999)
	Stimulates immunity	(Folkers et al. 1993; Lockwood et al. 1994)
	Suppress cancer cell growth	(Folkers 1974; Folkers et al. 1978; Lockwood et al. 1995)
	Breast cancer treated with chemotherapy reduced Q10 in blood	(Lockwood et al. 1994; Portakal et al. 2000)
	Supplementation with 390mg Q10 reduced the tumor size	(Lockwood et al. 1994)

These antioxidants were therefore considered to expose to cancer cells (MCF7 and KGN) and to follicles to examine their effects on cell viability and hormone synthesis.

1.9 Ovarian Disaggregation to Harvest Follicles

To study the direct effects of chemotherapeutics on follicles, it is necessary to isolate follicles from murine ovaries before exposure to chemotherapeutics *in vitro*. An ovarian disaggregation method requires the enzymatic or mechanical disaggregation of ovarian extracellular matrix in which follicles remain embedded.

1.9.1 Ovarian Extracellular Matrix

The ovarian extracellular matrix (ECM) provides the microenvironment for the development of follicles (Salani et al. 2007) and is composed of amorphous and fibrous components. *In vivo*, follicles remain embedded in ECM of ovary which consist of collagen, tenascin and proteoglycans (Salani et al. 2007; Salvetti et al. 2003; Woodruff & Shea 2007). The cellular compartments of an ovarian follicle (GCs, cumulus oocyte complex, basal laminae and theca cells) however are embedded in the ECM of the follicle which consists of collagen, hyaluronic acid and fibrinogen (Gomes et al. 1999; Irving-Rodgers et al. 2010; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; van Wezel, Ingrid L & Rodgers, Raymond J 1996). Collagen provides fibrous structures with elasticity, a property that is required for the spatial development of expanding follicles. Though collagen I is abundant in ovarian tissue, collage IV is predominant in the theca cell region (Woodruff & Shea 2007). Dysregulation of the ovarian ECM was reported in breast, ovarian, colorectal and pancreatic cancers and tenascin C, procollagen I and II and collagen IV were found to facilitate the development of tumors in ovarian cancers (Salani et al. 2007).

1.9.2 Ovarian Tissue Disaggregation Methods

Isolation of follicles from ovarian tissue requires the breakdown of ovarian extracellular matrix (ECM). Composition of ECM varies between species and depends on age of the animal. The variation may include difference in proportions of proteins, collagens, glycolipids and lipids. Also, the ovaries of younger mice (pre-pubertal) are less fibrous than older mice due to presence of higher amount of collagen and therefore follicle isolation from younger mice is easier (Dorphin et al. 2012; Kagawa et al. 2007; Kristensen et al. 2010; Picton, H et al. 2008), but follicles from post pubertal mice would be better model for exposing chemotherapy to follicles.

Two methods are commonly used for isolating and collecting follicles from mouse ovarian tissue; enzyme mediated disaggregation and mechanical disaggregation using needles (Table 1. *5*).

Table 1. 5: Summary of ovarian disaggregation methods

Publication	Method	Yield of Follicles	Method
Torrance, Telfer and Gosden (1989)	1.5 mg/mL Collagenase type I + DNase I 40U/mL	Not provided	Half ovaries from 10-day old mice were incubated in a 10 mL tube containing enzymes in medium with gentle rotation at 37°C for 30 minutes. After incubation follicles were pelleted out at 100-200 g and re-suspended in 3 mL media
Hulshof et al. (1994)	Mechanical	2918 pre-antral follicles per bovine ovary	Each ovary was dissected into small sections in a petri dish containing 10 mL PBS and fragments were transferred into a tube containing 10 mL PBS and resuspended for suction aspiration. The upper suspension was decanted to a centrifuge tube and follicles were pelleted out at 70g for 5 min and resuspended in 10 mL PBS.
Cortvrindt, R, Smitz, J and Van Steirteghem, A (1996)	Mechanical (25.5 g needle)	30-40 early preantral follicles per mouse ovary	Ovaries from 1-21 day old mice were placed in 500 µL L15 media and dissected with 25.5 g needle to release follicles.
Eppig and Schroeder (1989)	Mechanical	Not provided	Punching out of antral follicles from ovaries of 16-28 day old mice
Spears et al. (1994)	Mechanical	Not provided	Murine ovaries were dissected in media using fine and sharp needles to isolate preantral follicles of 190±10µm diameter.
Boland and Gosden (1994)	Mechanical	Not provided	Microdissection of ovarian tissue from 24-day old mice to isolate preantral follicles of 180±10 µm
Eppig and O'Brien (1996)	0.1% crude collagenase + 0.02% DNase	Not provided	Oocyte-granulosa cell complexes were isolated from mouse ovaries using 0.1% crude collagenase and 0.02% DNase
Park et al. (2005)	Mechanical	Not provided	Secondary follicles were dissected out of ovaries using 1 mL syringe needles
	Enzymatic	Not provided	Ovaries were exposed to collagenase I (600 IU) and DNase I in PBS for 30 minutes at 37°C before repeated pipetting to isolate follicles.
Dolmans et al. (2006)	Collagenase type 1	263 follicles per human ovary section of 2.5 X 2.5 to 10X8 mm	1 mg/mL collagenase type IA or 0.04 mg/mL Liberase Blendzyme 3 in 10 mL PBS at 37°C for 60 minutes with mechanical disruption using pipette. Tissue disaggregation was stopped by adding 10 mL of PBS with 10% FBS. Follicles were pelleted out by centrifugation at 50 g for 10 min at 4°C.
	Liberase	156 follicles per human	

		ovary section of 2.5 X 2.5 to 10X8 mm	
Choi, Gong and Lim (2008)	Collagenase type 1	28.5 follicles per porcine ovary	Ovarian slices were treated with 10 mL of 285 CDU/mg (Collagenase Degrading Units) collagenase type I in DMEM for 30 minutes in a shaking incubator at 39°C for 30 minutes with 5% CO_2. Disaggregated follicles were transferred to a petri dish and washed with DMEM.
Kristensen et al. (2010)	Collagenase IV + Liberase	Thousands of follicles collected from human ovary	Ovarian tissue (30µm thick) was treated with 0.2mg/mL Collagenase IV or 0.04mg/mL Liberase for 70-80 minutes at 37°C. Disaggregated ovarian suspension was then aspirated up and down to release follicles. Using a stereomicroscope follicle were identified and collected in PBS.
Shikanov et al. (2011)	0.1% collagenase +0.1% DNase + needle	Not provided	Mouse ovaries were treated with 0.1% collagenase and 0.1% DNase in 1-2 mL media in a petri dish and incubated for 15 minutes at 37°C and 5% CO_2. After incubation follicles were disaggregated free of ovarian tissue using needles
Dorphin et al. (2012)	Mechanical	Not provided	Dissected follicles out from ovaries under stereomicroscope using 27g needle
Young et al. (2017)	590 U/mL collagenase IV, 590u/mL animal origin free (AOF) collagenase IV, 1180U/mL AOF collagenase IV	91±12 follicles (mostly secondary) per ovary	Half ovaries from 3-month-old mice were incubated at 37°C for 30 minutes before mechanical disaggregation for 5 minutes using needles. Isolated follicles were collected using glass pipettes.

When mechanical isolation of ovarian follicles was compared to enzyme induced disaggregation of ovarian tissue, mechanical disaggregation yielded fewer number of follicles and is more optimized to larger follicles such as secondary and antral follicles (Table 1. *5*). However, mechanical isolation of follicles is more favourable for the follicular integrity during *in vitro* culture as evident from less disruption in follicle morphology though the whole follicle viability, maturation of oocyte and embryo development were reported similar to enzyme disaggregated follicles (Demeestere et al.

2002; Dolmans et al. 2006; Nagano et al. 2007; Park et al. 2005; Young et al. 2017). In contrast, enzyme disaggregation is responsible for basement membrane disruption and granulosa cell detachment and thereafter separation of oocyte out of follicle and thereafter follicle growth is compromised (Telfer et al. 2000). A later study by Nagano et al. (2007) suggested that follicle survival rate was higher for the mechanically isolate follicles. Shortly, though mechanical isolation is optimized for larger secondary follicles, it yields higher grade of morphologically intact follicles similar to intact follicles embedded in the ovaries *in vivo* and hence are more suitable model for further *in vitro* development of follicle and exposure to chemotherapeutics for evaluating their effects on viability and steroidogenesis.

1.9.3 Follicle Disaggregation with Hyaluronidase

A follicle consists of an oocyte surrounded by GCs. These GCs are embedded in follicular ECM and provide the spherical shape of the follicles. As already stated, follicular extracellular matrix consists of collagen type IV, hyaluronic acid and fibrinogen (Gomes et al. 1999; Irving-Rodgers et al. 2010; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; van Wezel, Ingrid L & Rodgers, Raymond J 1996; Woodruff & Shea 2007). Basal lamina of ovarian follicle separates GC from other vasculature which is composed of collagen IV, laminin, perlecan and nidogen (Irving-Rodgers et al. 2010). Hyaluronic acid is produced by follicular cumulus GCs through expression of hyaluronan synthase, and is secreted onto surface of GCs to form the extracellular matrix (Rodgers, Irving-Rodgers & Russell 2003). Woodruff and Shea (2007) reported that theca cell compartments contain collagenase IV. Therefore, disaggregation of follicles to isolate GCs requires break down of collagen IV in the theca cell layer and thereafter digestion of hyaluronic acid in the granulosa cell layers.

Yang and Honaramooz (2007) treated minced testes with 0.02% hyaluronidase in DMEM for 20 minutes to isolate testes cells. Mohammed (2014) collected the cumulus-oocyte-complexes (COCs) from mouse antral follicles by applying 0.05% (200 IU/mL) hyaluronidase in PBS for 30 minutes to the follicles, then the GCs were removed by pipetting. Although many researchers have used hyaluronidase to disaggregate COCs, the enzyme may have deleterious effects on the cumulus GCs. (Orimoto et al. 2008) transfected mouse primary GCs with EGFP-fused hyaluronidase plasmids and found that three expressed hyaluronidases (Hyal-1, Hyal-3 and Hyal-2) significantly increased granulosa cell apoptosis. The level of apoptosis induction was higher for Hyal-1 and Hyal-3 than for Hyal-2. In non-transfected cells the mRNA levels of Hyal-1, Hyal-2, and Hyal-3 were significantly increased in apoptotic GCs as well as in atretic follicles (Orimoto et al. 2008). The induction of apoptosis by hyaluronidases was independent of the enzymes catalytic activity, because the inactive

Hyal-1 mutant (D157A/E159A) was as efficient as the wild-type enzyme in causing apoptosis. The activation of the extrinsic apoptotic signalling pathway was involved in induction, because increased levels of cleaved caspase-8, caspase-3, and poly-ADP-ribose polymerase (PARP) were observed upon hyaluronidase ectopic expression (Orimoto et al. 2008). Hence there is a concern that applying hyaluronidase to disaggregate follicles may compromise the viability of harvested GCs. Although COCs have been disaggregated there are no reports describing the disaggregation of whole follicles to produce a single cell suspension or generate a preparation of proliferating FSH-responsive GCs.

1.10 *In Vitro* Culture of Ovarian Follicle

The *in vitro* culture of ovarian follicles is a useful method for *in vitro* maturation of follicles and to investigate the effects of chemotherapeutics on different sized ovarian follicles. Researchers have used both 2D and 3D culture systems. In this section, these two culture systems are evaluated to find the best culture system and conditions for murine follicle growth *in vitro*.

1.10.1 2D Culture Systems

Ovarian follicles have been cultured in 2D system where follicles allowed to grow on a flat surface of culture dish. Researchers use 2D systems for culturing follicles because it is inexpensive, established model with lot of published literatures and easy to examine the change over time. Published reports on 2D culture of ovarian follicles are listed in Table 1. 6. Minimal essential medium (MEM) was evaluated by Eppig and Schroeder (1989) for its suitability to support follicle growth and development *in vitro*. Mouse oocyte granulosa cell complexes (COC) from antral follicles were isolated from 12-day old mice and cultured for 10 days at 37°C under 5% oxygen (O_2), 5% carbon dioxide (CO_2) and 90% nitrogen (N_2) in MEM supplemented with 5% FBS, 0.23 mM pyruvic acid, 0.01% phenol red, 50 mg/L streptomycin sulfate, 75 mg/L penicillin G potassium salt and 0.05 mM 3-isobutyl-1-methylxanthin. 3-isobutyl-1-methylxanthin (IBMX) (believed to maintain oocyte in meiotic arrest). The oocyte diameter increased from 56±0.29 µm to 68±0.23 µm during the 10-day culture period but these were significantly smaller than diameters of freshly isolated oocytes (74±0.35µm) from equivalent age (22 days) follicles *in vivo*. Oocyte maturation was determined in terms of germinal vesicle break-down (GVB) and the production of a polar body. It was reported that when IBMX was replaced by IBMX free media and 1 µg/mL FSH was added after 10 days of culture, 87% of oocytes underwent GVB in comparison to 57% of oocytes cultured in media without FSH after a 16-hour maturation period. These results indicated the importance of FSH for oocyte maturation (Eppig & Schroeder 1989) and were supported by evidence that FSH stimulated GC

proliferation and follicle growth after preantral stage, that follicle maturation beyond preantral stage is FSH dependent and induced by AMH through increased FSHR expression and sensitivity of larger follicles to FSH (Choi & Rajkovic 2006; Dewailly et al. 2016; Diclemente et al. 1994; Durlinger et al. 2001; Lebbe & Woodruff 2013). Although 2D systems supported oocyte growth, it was not at the same level as *in vivo* growth, and 2D culture systems were not thought to be favourable for germinal vesicle break-down.

Spears et al. (1994) introduced a droplet culture system where isolated murine primary follicles were cultured in a 20µL droplet of MEMα media under 50µL oil in humidified condition with 5% carbon dioxide for 6 days, and the medium was replenished every day. Follicles doubled in size in comparison to the initial diameter of 190±10µm. This type of culture system was later used to evaluate follicle co-culture by culturing two pre-antral follicles in wells of a 96 well flat bottom plate containing 100µL MEMα under oil. The follicles were either allowed to grow side by side touching each other or not touching each other for 6 days. In another group, follicles were initially cultured in contact for 2 days and then separated for subsequent 4 days (Spears, De Bruin & Gosden 1996). Follicular growth in terms of diameter was indistinguishable between follicles cultured without touching each other and follicles cultured individually but the conjoined follicles developed quick adhesion and the growth rate was not equal for each conjoined follicle. It was reported that only one in the conjoined and co-cultured follicles reached antral stage and the overall development was significantly less in comparison to follicles cultured alone (Spears, De Bruin & Gosden 1996). Although initially this seems to be a negative result, after separating the conjoined follicles on day 2, the smaller follicles grew faster and by day 6 developed into Graafian follicles and were comparable to follicles cultured alone. This study suggested that the dominant follicle may have suppressive effects on neighbouring follicles *in vivo* in the ovary and *in vitro* cultured in media, but later study suggested that cocultured follicles supported follicle integrity, growth and survival before dominant selection (primary to preantral stage) by paracrine signals between follicles (Hornick et al. 2013).

Eppig and O'Brien (1996) examined the *in vitro* development of oocytes in COCs isolated from primordial follicles from 6, 8 and 12-day old mice. COCs with one or more layers of GCs were isolated using collagenase type I and DNase, then cultured on Coster Transwell-COL membrane inserts in Coaster six-well cluster dishes containing 4 mL Waymouth medium with penicillin G, Pyruvic acid, BSA, ITS and Fetuin (Eppig & O'Brien 1996). COCs were cultured for 16, 14 and 10 days at 37°C under 5% O_2, 5% CO_2, and 90% N_2 and every two days half the culture medium was replaced with fresh

medium. Oocytes grew from 2.1 to 5.3 folds and reached the size of an oocyte comparable to those found in antral follicle of 18-day old mice. Similar results were observed when COCs were cultured in MEM medium maturation (Eppig & Schroeder 1989). Both MEM and Waymouth culture media were optimized for oocyte maturation and so likely to be suitable for follicle *in vitro* culture as well, but the follicle growth was not as successful as the *in vivo* maturation process. This was attributed to the lack of metabolic cooperation between oocyte and cumulus cells in the 2D culture system and was based on the premise that granulosa cell proliferation and function play an important role in *in vivo* oocyte development (Buccione et al. 1987; Eppig 1977, 1979). Therefore *in vitro* maturation and culture of follicles should focus on maintaining the integrity of the follicular structure.

Cortvrindt, R, Smitz, J and Van Steirteghem, A (1996) used a mouse follicle *in vitro* culture system similar to Spears et al. (1994) in which 20 µL droplets of MEM medium were supplemented with FSH under oil. In their protocol ovaries were collected from 14-day old mice and only the intact and regular shaped follicles with one or two layers of GCs and visible oocytes were selected for culture. The selected follicles were cultured separately at 37°C with 5% CO_2, and the media were refreshed on alternate days. The follicles attached to the bottom of the culture vessel, and the spherical shape of the follicles was lost. However the GCs continued to proliferate until the 16th day, there was no atresia and the oocytes also continued to grow (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996). This 2D *in vitro* system was considered sub-optimal however because the 3D structure of the follicle is essential for oocytes to transport amino acids and undergo glycolysis and cholesterol biosynthesis (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989) and hence this 2D culture system would not support *in vitro* maturation of a fertilisable oocyte.

O'Brien, Pendola and Eppig (2003) collected whole ovaries from new born mice. They followed a two-step culture protocol in which the first medium for ovary culture was Waymouth MB752 supplemented with 0.23 mM pyruvic acid, 10 mg/mL streptomycin sulfate, 75 mg/mL penicillin G and 10% FBS. The ovaries were cultured for 8 days at 37°C with 5% CO_2. After that the cumulus-oocyte complexes were collected using collagenase (type not defined) and DNase. The COCs were cultured on Costar Transwell-Col membranes for 14 days (O'Brien, Pendola & Eppig 2003). Later the COCs were cultured for 8 days in modified essential medium-α (MEMα) without ribonucleosides and deoxyribonucleosides and supplemented with 3 mg/mL bovine serum albumin (BSA), antibiotics, insulin, transferrin, and selenium (ITS) and fetuin. Epidermal growth factor (EGF) or FSH were added

after 6 days of culture. This modified culture protocol matured 44% oocytes to metaphase II in comparison to 17% in the Waymouth media. Additionally, 62% of oocytes developed in MEMα initially underwent GVB in response to FSH and EGF during a 17-hour maturation period. This was consistent with the findings of Eppig and Schroeder (1989) and supported the proposal that addition of FSH after initial development improved oocyte competency for further maturation in terms of GVB and production of polar bodies. The original Waymouth medium supported the growth and development of cumulus–oocyte complexes (COCs) during the early stages of culture in comparison to MEMα whereas MEMα was optimal for the later stages. It was also found that high concentrations of glucose were beneficial for *in vitro* oocyte development (O'Brien, Pendola & Eppig 2003). The Waymouth medium used for the first stage of culture contained 27.8 mM of glucose and MEMα contained 5.5 mM of glucose. In addition, MEMα medium also contained a relatively high concentration of ascorbic acid (0.25 mM) which was thought to promote the growth of the COC (O'Brien, Pendola & Eppig 2003) possibly because of its antioxidant function. But it was later thought that the use of treated membranes disrupted the spatial arrangements of the GCs and that the enzymatic isolation of the follicle and perturbation of the basal lamina that surrounds the follicle might have also caused detachment of the cumulus GCs from the oocytes (Desai et al. 2010).

Telfer et al. (2008) applied a two-step culture system where human ovarian sections were first cultured in strips in 300 µL McCoy's 5a media for 6 days in humidified conditions at 37°C with 5% CO_2. After 6 days of culture antral follicles were mechanically dissected out of strips and cultured individually in wells of a V-bottom 96 well plate in 150 µL of media with or without activin for 4 days at 37°C with 5% CO_2. The follicles did not grow during the 4day culture period and also estradiol production was decreased from 132±12 to 105±13 pg/mL (Telfer et al. 2008). Previous study suggested that follicle growth in the later stage beyond preantral stage requires low glucose for oocyte maturation (O'Brien, Pendola & Eppig 2003) but McCoy's 5a media was high in glucose and did not contain any ascorbic acid to neutralize the ROS produced in high oxygen culture condition. Therefore, the follicle growth as well as the hormone production may have been compromised.

Wang et al. (2011) used the droplet culture system as developed by Cortvrindt, R, Smitz, J and Van Steirteghem, A (1996) to culture preantral mouse follicles that were isolated from vitrified ovarian tissues. The follicles were cultured individually for 12 days at 37$^{\circ C}$ with 5% CO_2. Follicles degenerated and some extruded the oocyte, although 75% of cultured follicles survived and 62.2% of oocytes matured to metaphase II stage. However the survival of the follicles was only based on visual inspection of follicle morphology (oocyte surrounded by GCs and theca cells) which did not reflected

the survival of follicular cells, nor growth of follicles or steroidogenic function. Morevoer, oocytes were extruded which might be because of loss of spherical structure of the follicles.

In summary, 2D culture systems for growing follicles are associated with reduced growth of oocytes and maturation (Eppig & O'Brien 1996; Eppig & Schroeder 1989), inhibition of dominant follicle selection (Spears, De Bruin & Gosden 1996), loss of 3D structure of the follicle (Wang et al. 2011) and decreased hormone production (Telfer et al. 2008). Although Spears et al. (1994) reported follicular growth, this increase in diameter might be attributed to the flattening of follicle and to loss of spherical structure rather than to an increase in size due to granulosa cell proliferation in follicle. In addition, denuded oocyte when cultured in 2D culture systems may compromise the gap junctions between GCs and granulosa cell to oocyte and lead to poor maturation (Eppig 1977, 1979; Telfer 1998). Though 2D culutre of ovarina follicle is easy and inexpensive, these 2D *in vitro* culture systems do not mimic the real *in vivo* cell environment and are associated with poor predictivity. Therefore, the 3D structure of follicles is important for follicle and oocyte maturation and hence the concept of 3D culture systems evolved for *in vitro* growth of follicles. Moreover, loss of spherical structure of follicle causes stress to gap junctions. Both loss of spherical structure and stress to gap junction may contribute to extrusion of oocytes and imparied steroidogenesis (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989; Telfer et al. 2008).

1.10.2 3D Culture Systems

3D culture systems evolved from the inspiration to provide cells environment that represents tissues outside the human body. In follicle culture, the concept of 3D culture system was introduced to maintain the 3D structure of follicles and to allow the growth of GCs in all directions, maintain gap junctions between GCs and oocyte in order to support paracrine functions (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989; Woodruff & Shea 2007). Therefore, the use of 3D culture system for *in vitro* culture of follicle has been increased in 21st century. Published reports on follicle culture in 3D culture system are presented in Table 1. 6.

To our knowledge, 3D culture system for ovarian follicle culture was first used to culture murine small preantral follicles in collage gel by Telfer, Torrance and Gosden (1990). Isolated preantral follicles were suspended in 10 µL collagen gel solution and allowed to set at 37°C for 2-3 minutes. Set gel containing follicle was then suspended in 20 µL collagen gel and allowed to set before adding

2 mL M199 medium with 5% calf serum and cultured for 5 days at 37°C with 5% CO_2. Follicles only grew from small preantral follicles to large preantral stage. Most importantly, follicles did not develop theca cell layers. Collagen gel 3D culture system did not support follicle growth beyond antral stage.

The role of FSH in follicle maturation was examined by culturing secondary follicles from 100 to 180 µm with increasing concentrations of FSH (0, 5, 10, 25 and 50 mIU/mL) in a 3D alginate based culture system (Kreeger et al. 2005). Secondary follicles with 2 layers of GCs (100 to 130 µm) were cultured in a 1.5% alginate with 0.2 mg/mL collagen 3D culture matrix, while the larger secondary follicles with multiple layers of GCs were cultured in 1.5% alginate without collagen. Individual follicles with a minimum amount of medium were pipetted into droplets (2 to 3 µL) of culture matrix and suspended on a polypropylene mesh with a 0.1 mm mesh size before immersion into sterile $CaCl_2$ (50 mM) for alginate cross-linking (Kreeger et al. 2005). The resulting beads were then cultured in separate wells (one follicle/bead/well) of a 96 well plate containing 100 µL MEMα at 37°C in 5% CO_2. Finally, FSH was added in increasing concentrations and the follicles were cultured for 8 days. Follicular growth was measured by determining diameters under an inverted microscope. The alginate based culture matrix promoted follicular growth and retained the three dimensional structure (Kreeger et al. 2006; Kreeger et al. 2005). Both two-layered and multi-layered secondary follicles exhibited dose-dependent size increases in diameter but although the survival of the smaller follicles was not significantly affected by the FSH dose, the survival of the larger follicles was dose dependent and the highest survival was reported at 5 mIU/mL (72%) and 10 mIU/mL (69.2%) FSH (Kreeger et al. 2005). This indicated that the growth of smaller follicles was independent of FSH, and this was further supported by a study in which primordial follicles in FSHβ null mice (Kumar et al. 1997) and FSH receptor null mutant mice (Abel et al. 2000) grew to pre-antral stage but without antrum formation. However this study also reported that in the absence of FSH, there was limited proliferation of GCs and no detectable progesterone or estradiol production (Kreeger et al. 2005). In comparison to smaller follicles, oocytes from the larger multilayered follicles were more competent to produce mature oocyte through meiosis in a dose response manner with increasing concentration of FSH, whereas in the absence of FSH oocytes were not healthy and only 14.3% and 15.4% of the oocytes were meiotically competent (Kreeger et al. 2005). This suggested that in early stages, FSH promotes granulosa cell proliferation and in the later stages it helps in oocyte maturation.

Later Kreeger et al. (2006) used a modified alginate matrix by incorporating ECM components including laminin and synthetic peptides such as Arg–Gly–Asp (known as RGD peptide) developed by Kreeger, Woodruff and Shea (2003) to culture secondary murine follicles at 37°C in 5% CO_2. In general, this modified culture matrix created an *in vivo* like follicular morphology. The ECM components promoted follicular growth, differentiation and oocyte meiotic competence (Kreeger et al. 2006). It was reported that incorporation of RGD helped to increase granulosa cell adhesion and the production of progesterone and estradiol (Kreeger, Woodruff & Shea 2003). Incorporation of laminin into the alginate base culture matrix was less important for follicular *in vitro* culture in terms of survival and growth in comparison to alginate-RGD matrix (Kreeger et al. 2006). Another study suggested that an alginate hydrogel-based 3D culture system supported murine follicular growth with production of steroids, formation of antrum, differentiation of theca cells and oocyte maturation. One mature egg was subsequently fertilized *in vitro* and transferred into a host mother, and gave rise to a live, healthy pup (Xu et al. 2006). It was found that the follicles cultured at 37°C in 5% CO_2 by encapsulation in 0.25% alginate grew more optimally than in 1.5% alginate. In addition, the maturation and fertilization rate were 41% after culture in 0.25% calcium alginate culture but only 5% in 1.5% calcium alginate. Moreover, encapsulation of follicles was associated with mechanical damage caused by isolation of the follicle after completion of culture (Xu et al. 2006) may be because degradation of alginate based matrix requires alginate lyase (Brito et al. 2014).

Follicles *in vivo* are organized within an extracellular matrix (ECM). One ECM that is used for *in vitro* culture is Matrigel which is made up of collagen IV, laminin, fibronectin, entactin, heparin sulfate proteoglycans, and a variety of growth factors such as EGF, FGF, IGF-1, PDGF and TGF-b (Oktem & Oktay 2007a; Sigma 2017; Woodruff & Shea 2007). Preantral follicles were isolated from 14-day old mouse ovaries and cultured in a 3D Matrigel and a 2D system on cover slips using serum free culture media for 7 days at 37°C in 5% CO_2. Structural integrity was examined by confocal microscopy and it was found that the 3D structure of follicles was retained in the Matrigel culture system (Oktem & Oktay 2007a). Matrigel promoted follicular growth and survival in comparison to other 2D or droplet culture systems. This improvement was attributed to the presence of the growth factors in the Matrigel as well as the physical interaction between the ECM and the follicles. However the collagen in the Matrigel required enzymatic digestion to extract the follicle after culture, and this was thought to have the potential to cause physical damage (Desai et al. 2010).

Shikanov et al. (2011) used a fibrin-alginate interpenetrating network (FA-IPN) to culture mouse secondary follicles at 37°C with 5% CO_2. FA-IPN was a cell-responsive matrix in which the rigidity of

the matrix was reduced by proteases incorporated into the gel. These were activated by the encapsulated follicles during the initial growth phase. The proteases degraded the fibrin of the IPN and caused a gradual reduction in gel rigidity while the 3D structure was maintained by the alginate remaining in the matrix. Thus this 3D matrix allowed the natural process of follicular maturation *in vivo* (Shikanov et al. 2011) which was indicated through increasing diameters of the follicles measured by Image J from images taken using a light microscope.

To avoid loss of the spherical shape of the follicle, Parrish et al. (2011) used a 3D 0.25 % alginate hydrogel system for culturing mouse follicles at 37°C with 5% CO_2. The 3D culture system contained all the necessary ingredients for the development of follicles. After 12 days culture, the follicles increased in size and developed antral cavities. The morphological development was similar to that seen *in vivo*, but the size of the secondary and antral follicles and the antrums of these follicles were smaller than in follicles developed *in vivo* (Parrish et al. 2011). The alginate hydrogel-based 3D *in vitro* culture system supported growth and development of follicles and oocytes but still not as well as in follicles that had developed *in vivo*. Although the morphological development was similar, gene expression patterns in the *in vitro* and *in vivo* developed follicles were different for some genes but similar in others. 60% of the genes in *in vitro* follicles such as Fgf8, Fshr, Gdf9, Igf1, Inha, Jag1, Kit, Lhcgr, Nlrp5, Nobox, Ddx4, and Zp2 were similar to their corresponding *in vivo* patterns of expression at the secondary follicle stage, and 50% of the above genes had similar expression patterns in COC (Parrish et al. 2011). The main difference between the *in vitro* and *in vivo* developed follicles was that the oocytes did not have the same potential for blastocyst development and production of live pups.

Wang et al. (2012) optimized a hanging drop culture method for groups (3 to 5 follicles) of heterogeneous (90 to 130 µm) preantral mouse follicles by reducing insulin, transferrin, and selenium (ITS) in the media (α-MEM). The advantages of this system were that it allowed the 3D spherical development of the follicles and promoted follicular growth in a cluster. The co-incubated follicles could communicate with each other and synchronize follicular growth. Retinoic acid was added to promote oocyte development. After 14 days, 94% of the oocytes were matured and capable of fertilization.

In a later study, Joo et al. (2016) cultured rat ovarian follicles in a collagen type 1 hydrogel. Collagen type 1 was extracted from rat tail and diluted with media to prepare 1, 3, 5 and 7 mg/mL hydrogel. This hydrogel starts to form gel at 37°C. Isolated follicles were mixed with varying concentrations of

hydrogel and cultured in a 48 well plate with 400 µL media for 12 days at 37°C with 5% CO_2. 5 mg/mL collagen type 1 hydrogel had more than 90% viable follicles when assessed by Live-Dead stain but thereafter follicle viability decreased to 76% in 7% hydrogel (Joo et al. 2016). Although progesterone levels over time were not affected by the hydrogel concentrations, 5mg/mL collagen type 1 hydrogel supported a gradual increase in estradiol production from day 6 till day 12. Similarly, 5mg/mL hydrogel had the highest numbers (88%) of meiotically competent oocytes as estimated by germinal vesicle breakdown (GVB). Collagen is one of the components of ovarian ECM and gives elasticity to the ovary to allow growth of follicles *in vivo* (Salvetti et al. 2003). Therefore, culturing follicles in collagen might have supported survival of follicles and oocyte competence but collagen is derived from animal sources and hence its application for human follicle maturation is restricted by regulations.

Heiligentag and Eichenlaub-Ritter (2018) used 0.25% alginate hydrogel or 0.75% collagen with human menstrual blood mesenchymal stem cells (MenSCs) to encapsulate 3-5 preantral murine follicles before culture in αMEM with FSH and FBS for 12 days. In comparison to collagen, the alginate-based matrix supported more follicle survival and MenSC supported survival and reduced follicle degeneration, whereas collagen supported more antral formation and increase in follicle diameter in comparison to alginate, but similar numbers of oocytes matured in both systems. In both systems, the MenSC supported hormone production may have been because of increased survival (Heiligentag & Eichenlaub-Ritter 2018). Recently, Matrigel and fibrin containing alginate matrices were modified by Sadr et al. (2018) to culture preantral follicles isolated from 13 day old mice. Follicles were encapsulated in Matrigel-alginate (MA) and fibrin-alginate (FA) scaffold and cultured for 12 days. Follicles grew significantly during the 12-day culture, but the Matrigel-alginate based matrix supported better survival and oocyte maturation in comparison to the fibrin-alginate matrix. It was thought that this might be because Matrigel contains ECM components similar to the *in vivo* situation (Sadr et al. 2018; Salvetti et al. 2003).

In general, three-dimensional culture systems preserve the architecture of follicles and a number of 3D culture systems have been examined. Rigid 3D matrixes can restrict increases in follicle diameter, theca formation, antrum formation and estradiol production. 3D matrixes must allow gas exchange and diffusion of nutrients as well as cellular waste removal. This permeation is affected by the porosity of the matrix. Also 3D matrix needs to be elastic to accommodate the growth of follicle in a manner that is comparable to the *in vivo* situation in which collagen in the ovarian matrix makes it fibrous and elastic (Salvetti et al. 2003). Therefore, 3D culture matrixes must be not toxic to the

growing cells and should also allow sustainable growth for the duration of the culture period (Desai et al 2010). To date different types of natural and synthetic gels such as hydrogels like alginate, Matrigel, Fibrin Alginate, Matrigel-alginate and Fibrin-alginate have been used to maintain the spherical structure of the follicle (Table 1. 6) (Heiligentag & Eichenlaub-Ritter 2018; Kreeger et al. 2006; Kreeger et al. 2005; Parrish et al. 2011; Sadr et al. 2018; Shikanov et al. 2011; Xu et al. 2006). All of these gels, as well as collagen or matrixes that contain collagen, allowed the expansion of follicle up to the pre-antral phase (Desai et al. 2010; Heiligentag & Eichenlaub-Ritter 2018; Joo et al. 2016) but the collagen gels shrank over time and failed to support further follicular expansion. It was also difficult to examine follicles embedded in collagen.

Many advances have been made to optimize *in vitro* culture systems for follicles, but still no method has been established as proven and effective for consistent follicular development *in vitro.* However, the Matrigel culture system holds promise as an *in vitro* follicular culture system if it is modified to allow non-destructive isolation of the follicle from the gel. The fibrin-alginate interpenetrating network (FA-IPN) follicle culture system also has potential, but this method still needs experimental evidence that it promotes correct morphological development. Many *in vitro* culture systems have been applied to follicles (Table 1. 6), but each culture system has a range of advantages and disadvantages. 2D *in vitro* culture systems were associated with loss of physical structure that affected the follicular development while although 3D culture systems supported the follicular spherical structure, there are still problems with obtaining good quality follicles after *in vitro* culture. None of these follicle culture systems have been used to examine the effects of chemotherapy *in vitro*. Therefore, it is important to develop an appropriate culture system that supports the 3D follicular structure and enables culture of good quality follicles to assess the effect of chemotherapy on follicular cell viability.

Table 1. 6: In vitro follicle culture systems

Publication	Type	Culture media	Culture period	Outcome
Eppig and Schroeder (1989)	2D	5% FBS, 0.23 mM pyruvic acid, 0.01% phenol red, 50 mg/L streptomycin sulfate, and 75 mg/L penicillin G potassium salt	10 days	Oocytes from *in vitro* cultured follicles were significantly smaller than freshly isolated follicles
Telfer, Torrance and Gosden (1990)	3D, Collagen gel	Medium 199 and Medium 199 supplemented with ITS	5 days	Small preantral follicles only grew to large preantral stages (Not to antral stage) but there was no theca cell layer developed
Spears et al. (1994)	2D, Droplet	20 µL droplet of MEMα media under 50 µL oil	6 days	Follicles doubled in size after 6-day culture
Spears, De Bruin and Gosden (1996)	2D	100 µL MEMα under oil in each well of a 96 well flat bottom plate where follicles were either allowed to grow side by side touching each other or not touching each	6 days	Dominant follicle may have suppressive effect on the neighbouring follicles
Eppig and O'Brien (1996)	2D	COCs were then cultured on Coster Transwell-COL membrane inserts in Coaster six-well cluster dishes containing 4 mL Waymouth medium	10-16 days	Oocytes did not grow to the same size of equivalent age of 22 days
Cortvrindt, R, Smitz, J and Van Steirteghem, A (1996)	2D, droplets in culture dishes	20 µL MEM supplemented with recombinant follicle stimulating hormone (100 mIU/mL)	16 days	Spherical shape of the follicles was lost
O'Brien, Pendola and Eppig (2003)	COCs	MEMα without ribonucleosides and deoxyribonucleosides and supplemented with 3 mg/mL BSA, antibiotics, ITS (insulin, transferrin, and selenium) and fetuin. EGF (epidermal growth factor) or FSH was added after 6 days of culture period.	8 days	Modified MEMα was better that Waymouth media in terms of oocyte maturation while Waymouth media was supportive for early follicular development
Telfer et al. (2008)	2D, in wells of V-bottom 96 well	300 µL McCoy's 5a media	6 days	Follicles did not grow, and hormone production reduced

Wang et al. (2011)	2D, droplet culture system	αMEM with FCS, ITS, Penicillin and streptomycin and FSH	12 days	Follicle degenerated and extruded oocyte
Kreeger et al. (2005)	3D alginate-based culture matrix	1.5% alginate-0.2 mg/mL collagenase or 1.5% alginate-based matrix	8 days	Alginate based culture matrix promoted the follicular growth and retained the three-dimensional structure
Kreeger et al. (2006)	3D alginate-based culture matrix with ECM	Alginate matrix by incorporating ECM such as Laminin or RGD (Arg–Gly–Asp)	8 days	RGD helps in increasing the granulosa cell adhesion and spreading as well as increase the production of progesterone and estradiol
Xu et al. (2006)	Alginate hydrogel-based 3D culture system	0.25% calcium alginate culture and 1.5% calcium alginate	8 days	Higher the concentration of alginate lowered the growth and maturation of oocytes
Oktem and Oktay (2007a)	Matrigel	Collagen IV, laminin, fibronectin, entactin, heparin sulfate proteoglycans, and growth factors such as EGF, FGF, IGF-1, PDGF and TGF-b	7 Days	Promoted follicular growth and survival
Shikanov et al. (2011)	Encapsulation	Fibrin-alginate interpenetrating network (FA-IPN)	8 days	Allow spatial follicular development
Parrish et al. (2011)	3D hydrogel	0.25 % alginate hydrogel	12 days	Sizes of secondary and antral follicles and antrum of these follicles were smaller than follicles developed *in vivo*
Wang et al. (2012)	Hanging drop culture	Reducing insulin, transferrin, and selenium (ITS) in the MIF media (α-MEM)	14 days	Allowed the 3D spherical development
Tagler et al. (2013)	3D, alginate hydrogel	α-MEM/F12 supplemented fetuin, ITS and FSH	14 days	Supported the growth of secondary follicles but the survival rate was low
Laronda et al. (2014)	3D, human primordial follicles encapsulated in 0.5% and	1:1 α-MEM and F-12	3 days	Primordial follicles did not survive in *in vitro* culture

	2% alginate beads			
	3D, human secondary follicles in Matrigel	α-MEM with HSA	21 days	Secondary follicles grew significantly with antrum formation
Xiao et al. (2015)	3D, encapsulated human follicles in 0.5% alginate beads	1:1 α-MEM and F-12	30 days	Promoted oocyte maturation to GVB Synthesis of hormone (E_2, P_4 and AMH) increased with follicle size
Joo et al. (2016)	3D, collagen type 1 hydrogel	α-Minimal Essential Medium	12 days	Collagen supported survival of follicles and oocyte competence
Heiligentag and Eichenlaub-Ritter (2018)	3D, 0.25% alginate hydrogel and 0.75% collagen	αMEM with FSH and FBS	12 days	Collagen supported antral formation and follicles growth
Sadr et al. (2018)	Matrigel-alginate (MA) and fibrin-alginate (FA) scaffold	αMEM	12 days	Matrigel-alginate based matrix supported better survival and oocyte maturation

1.10.3 Effect of Oxygen on *In Vitro* Culture of Follicles

The follicles cultured in different concentrations of oxygen behave differently. The outcomes of follicles cultured in low (5%) and high (20%) oxygen concentration are listed in Table 1. 7. Smitz, Cortvrindt and van Steirteghem (1996) compared the effect of 5% O_2 and 5% CO_2 in air (20% O_2) in a 16-day *in vitro* culture of murine secondary follicles in a droplet (2D) culture system as described by Cortvrindt, R, Smitz, J and Van Steirteghem, AC (1996). 30% and 75% of follicles cultured in 5% O_2 and 5% CO_2 in air were viable. Follicles cultured in 5% O_2 did not form antrums and had higher numbers of extruded oocytes (45% vs 5%) in comparison to 5% CO_2 in air but 83% of the oocytes underwent germinal vesicle breakdown. Also, the E2 production was compromised under 5% O_2 (Smitz, Cortvrindt & van Steirteghem 1996).

Xu et al. (2011) demonstrated the effect of oxygen concentration on macaque secondary follicle growth in a 40-day culture in an alginate-based 3D culture system. Survival of follicles on day 30 was higher in the 5% O_2 cohort than in 20% O_2. In addition, lower oxygen supported oocyte maturation

and follicle growth to antral stage. Also, addition of FSH stimulated AMH, estrogen and progesterone production in 5% O_2 (Xu et al. 2011).

In a later study Gook et al. (2013) investigated the effect of oxygen concentration on follicle growth, morphology, metabolism and viability of whole follicles using a 2D culture system. Primary and secondary murine follicles were mechanically isolated and cultured for 8 days in 700 µL αMEM with penicillin and streptomycin, human serum albumin, ITS, ascorbic acid and FSH under oil in a 4 well plate at 37°C either in 5% oxygen with 6% carbon dioxide or 20% oxygen with 5% carbon dioxide in nitrogen with or without FBS. At the beginning 97% follicles were viable after Live-Dead staining and 89% and 93% follicles cultured in 5% and 20% O_2 respectively retained their 3D structure, but follicles cultured in media with FBS lost their spherical structure. After an 8-day culture period, 59% and 8% of follicles cultured in 5% and 20% O_2 were viable. Follicles grew by 62.9±5.7 µm and 15.8±3.8 µm in 5% and 20% O_2 respectively and the number of GC layers increased to more than 6, which reflected the cell proliferation and metabolic use of glucose and amino acids when cultured in 5% O_2 (Gardner & Wale 2013; Gook et al. 2013). This study suggested that use of 5% O_2 instead of 20% was better for murine follicle maturation through maintaining follicle viability, growth and metabolic pattern.

In another study, Yin et al. (2016) cultured human preantral follicles in an alginate bead (3D system) either in 5% or 20% O_2 with FSH. Follicle survival rate after 7 days culture was 80% in 5% O_2 in comparison to 66% in 20% O_2. Follicles that survived for 7 days were subjected to culture for longer periods (>30 days) and at the end of culture, 6 and 4 follicles out of 15 and 19 follicles from 5% O_2 and 20% O_2 respectively had extruded oocytes (40% vs 20%). Also, 33% and 58% of follicles grew fast in 5% O_2 and 20% O_2 respectively.

Except for Smitz, Cortvrindt and van Steirteghem (1996), all other study results aligned on the finding that 5% O_2 was more favourable for follicle growth (Gook et al. 2013; Xu et al. 2011), oocyte maturation (Eppig & O'Brien 1996; Eppig & Schroeder 1989; Xu et al. 2011), survival of follicle (Gook et al. 2013; Yin et al. 2016), metabolic functions (Gook et al. 2013) and reduced oocyte extrusion (Yin et al. 2016) (Table 1. 7). This might be because higher oxygen concentration may contribute to the production of reactive oxygen species (ROS) that leads to the death of GCs through apoptosis. However, the impact of oxygen on follicle growth, survival and oocyte maturation is still inconclusive because in previous studies follicles and mature oocytes that produced live pups were grown in 20% O_2 (Eppig & Schroeder 1989; Parrish et al. 2011; Spears, De Bruin & Gosden 1996; Wang et al. 2011).

Table 1. 7: Effect of oxygen concentration in follicle culture

Reference	Species	Culture condition	Outcome
Eppig and Schroeder (1989)	COC from isolated murine antral follicles	2D, 5%O_2+5% CO_2 in 90% N_2	Oocyte grew from 56±0.29 to 68±0.23 µm, addition of FSH stimulated oocyte maturation as indicated by germinal vesicle break down
Eppig and O'Brien (1996)	COCs isolated from primordial follicles	2D, 5%O_2+5% CO_2 in 90% N_2	Oocytes grew from 5.3 to 2.1 times but not comparable to equivalent aged mice
Smitz, Cortvrindt and van Steirteghem (1996)	Murine secondary follicles	2D, 5% O_2+5%CO_2+90%N_2 and 5% CO_2 in air	5% CO_2 supported follicle viability, oocyte maturation and E_2 production
Xu et al. (2011)	Secondary macaque follicle	3D, 5% and 20% O_2	5% O_2 increased follicle viability, AMH, E2 and P4 production
Gook et al. (2013)	Primary and secondary murine follicle	2D, 5%O_2+5% CO_2 in 90% N_2 and 20% O_2	5% O_2 supported follicle growth, viability and cell proliferation
Yin et al. (2016)	Human preantral follicles	3D, 5% and 20% O_2	Low O_2 contributed to oocyte extrusion and slow growth of follicle

1.11 Hypotheses

- Exposure of KGN carcinoma-derived GCs and breast cancer MCF-7 cells to clinically relevant doses of cyclophosphamide and doxorubicin singly and in combination will reduce the numbers of viable cells.

- Use of collagenase before hyaluronidase will isolate higher numbers of viable GCs from murine ovarian follicles than collagenase alone.

- 3D culture system will support follicle integrity, growth, granulosa cell viability and steroidogenesis *in vitro.*

- Exposure of murine follicles to the combination of doxorubicin and cyclophosphamide (AC) will kill more follicle cells than exposure to each chemotherapeutic alone.

- The antioxidant properties of gToc will support follicular cell proliferation and steroid hormone production.

- The addition of gToc to the two chemotherapeutic agents (AC) will inhibit follicular ROS generation and extend hormone synthesis.

1.12 Aims

The aims of this project are –

- To examine the effects of doxorubicin and 4-hydroxycyclophosphamide (4OHCYC) on breast cancer (MCF-7) and ovarian cancer cells (KGN) and to determine the IC values of these commonly used chemotherapeutics *in vitro*.

- To examine the effect of antioxidants (alpha, gamma and delta tocopherol and Q10) co-administered with doxorubicin and 4OHCYC on KGN and follicle cell viability and hormone synthesis.

- To develop a technically simple and inexpensive method to disaggregate follicles and produce a single cell suspension suitable for assessment in a modified Trypan Blue Exclusion assay.

- To determine the number of GCs in freshly isolated murine follicles to use as a baseline control in subsequent studies to examine the effects of chemotherapeutics on follicles in vitro.

- To conduct a preliminary study to culture murine secondary follicles *in vitro* in a 2D culture system.

- To establish a 3D culture system for murine ovarian follicles that can be applied to examine the effects of chemotherapeutics used to treat breast cancer.

- To expose murine ovarian secondary follicles to doxorubicin, 4OH-cyclophosphamide and gamma tocopherol at EC25 doses for MCF-7 breast cancer cells to examine their effect on follicle morphology, growth, cell viability and hormone production in a 3D culture system.

2. CHAPTER TWO: CHARACTERISATION OF THE KGN GRANULOSA CELL LINE AND SELECTION OF TEST CONCENTRATIONS OF CHEMOTHERAPEUTICS

2.1 Introduction

The study of follicle cell viability and reproductive hormone synthesis is key to understanding female reproductive health. Granulosa and theca cells are the steroid producing cells in the ovary. The granulosa cell layers within the follicles are avascular whereas the theca cell layers are vascularised and connect to the ovarian stroma. GCs are separated from theca cells by a basal lamina. These two types of ovarian cells produce a number of hormones including estrogen, progesterone and AMH (Baarends et al. 1995; Broekmans et al. 2008; Havelock, Rainey & Carr 2004) and respond to GnRH, FSH and LH produced by the hypothalamus and pituitary gland (De Kretser et al. 2004; Findlay et al. 2000; Groome et al. 1996; Makanji et al. 2009). These hormones regulate fertility by their effect on follicular maturation, menstruation, menopause and sex drive. The diagnosis of female reproductive health problems commonly starts with blood tests to measure these hormones. Therefore, study of the production and regulation of these hormones *in vitro* is important to examine the effects of chemotherapy and other pathologies. Although IVF derived human GCs (hGC) (Alouf et al. 1997; Kollmann et al. 2015; Wickings, Hillier & Reichert 1986; Yong et al. 1992) and primary-derived human GCs from ovarian follicles have been used to study hormone production (McNatty et al. 1979; Pellatt et al. 2007), these cells are not suitable for extended culture and it is difficult to obtain adequate numbers of cells for experiments (Nishi et al. 2001a). In order to study the effects of chemotherapeutics on ovarian steroidogenesis, an appropriate *in vitro* cell line is necessary. The KGN cell line is derived from a carcinoma of the ovary and has granulosa-like cells with a proliferation rate of 46.4h (Nishi et al. 2001a). In addition, KGN cells express FSH receptors and have CYP450 aromatase activity, although the activity is approximately 50 times lower than primary-derived GCs (Nishi et al. 2001a). The advantage of this cell line is that these cells possess most of the physiological activities of human follicular GCs and have similar patterns of steroidogenesis.

Breast cancer is the most commonly diagnosed cancer among young adult women of reproductive age (DeSantis et al. 2014; Ferlay et al. 2015; Tao et al. 2015) and doxorubicin and cyclophosphamide are most commonly used to treat breast cancer (Legha et al. 1982; Morgan et al. 1997; Oktay & Oktem 2007). The MCF-7, human epithelial breast adenocarcinoma cell line is suitable for examining the effects of breast cancer chemotherapeutics on cell viability and the KGN granulosa cell line can

be used to examine the effects of doxorubicin and cyclophosphamide on cell viability and hormone production.

2.2 KGN Cell Line and Its Hormone Production

In women the production of estradiol varies with follicular stage and ranges from 50-60 pg/mL in small early stage follicles and from 250-400 pg/mL in larger late stage follicles in which 95% of estradiol is produced by the GCs of dominant follicle and the corpus luteum (Becker, 2001, p. 930). *In vitro*, the synthesis of estrogen by KGN cells was stimulated by androstenedione from 103.7±6.5 to 1165±2 pg/10^6 cells and by cAMP from 170.7±3.5 to 4060±170 pg/10^6 cells after a 72 hour exposure (Nishi et al. 2001a). *In vivo*, the synthesis of estrogen from androstenedione is mediated by CYP450 aromatase in GCs (Boon & Simpson 2012; Miller & Auchus 2011; Pruitt 2016; Smyth et al. 1993). Follicle stimulating hormone acts via cAMP in GCs to increase the aromatase activity and this may also have role in increasing the production of estrone (E1) or estradiol (E2) by KGN cells *in vitro* (Nelson & Bulun 2001).

GCs start to produce progesterone just before ovulation and primary-derived unstimulated GCs aspirated from follicles of 5 to 20 mm (that were obtained from normally cycling women) produced progesterone spontaneously, and FSH stimulated this progesterone production 10-fold (Erickson, Garzo & Magoffin 1991). The concentration of progesterone in the antral fluid of those follicles was 0.18 ng/mL, and the concentration of androstenedione was 10 times higher than progesterone (Erickson, Garzo & Magoffin 1991) suggesting of degradation of progesterone to 17 alpha hydroxyprogesterone or androstenedione (Nishi et al. 2001a). Also, granulosa cell luteinisation increases the production of progesterone by 10-40 mg per day and in the mid-luteal phase plasma progesterone levels can be 5 – 25 ng/mL (Becker 2001). This is because LH stimulates the production of progesterone by up-regulating 3 beta HSD (hydroxysteroid dehydrogenase) in luteinised GCs (Pelletier et al. 2001). Similar to primary-derived GCs, KGN cells were able to produce progesterone after 24h culture and stimulation by cAMP (Nishi et al. 2001a). The basal synthesis of progesterone by 10^6 KGN cells was 0.24±0.05 and 0.26±0.05 ng/mL after 24 and 48 hours culture respectively (Nishi et al. 2001a).

The determination of serum concentrations of AMH is important for fertility treatment and reproductive research because AMH is produced only by the GCs of growing follicles (La Marca et al. 2005; Visser et al. 2006). GCs collected during oocyte retrieval for IVF were cultured for 48 hours or 6 days with or without FSH. The basal AMH production by 10,000 GCs was 12.9±1.2 ng/mL and

18.1±2.9 ng/mL after 48 hours and 6 days respectively. Stimulation by 1IU FSH increased this to 47.4±4.2 ng/mL and 20.9±4.0 ng/mL i.e. more AMH produced in the first 48h (Kollmann et al. 2015). In another study, GCs were aspirated from follicles of 2 to 10mm that were isolated from ovaries removed from women undergoing transabdominal hysterectomy for benign gynaecological conditions. The GCs were cultured at a density of 5 x 10^4 per well in 200 µL M199 media in a 96 well plate for 48 hours (Pellatt et al. 2007). GCs from different size follicles were cultured in different wells. The isolated GCs produced AMH from 0.025 to 1.7 ng/mL (measured by ELISA from Diagnostic Systems Laboratories, Oxon, UK) and the production of AMH was reduced with increasing size of follicles (Pellatt et al. 2007). Also, the concentration of AMH was measured in the follicular fluid from 14 follicles of 3 to 12 mm and it ranged from 42 to 2240 ng/mL (Pellatt et al. 2007). However, the capacity of KGN cells to produce AMH has not been examined or reported.

It is rarely possible to study the effects of chemotherapeutics directly on ovaries *in vivo*. Therefore, either primary-derived ovarian GCs or granulosa-like cell lines are good experimental options. Although cell lines are not perfect models for the *in vivo* situation, they are easy to use, do not require ethical approval, are easy to access, and are cost-effective. Moreover, cell lines provide pure populations of cells which give consistent and reproducible results during experiments. In contrast, primary-derived cells are often not well-characterized, have slow proliferation rates and short life spans. Therefore, the KGN cell line is suitable for use in a study to examine the effects of chemotherapeutics as long as it is well characterized and it manifests the functional features of the targeted primary cells (Kaur & Dufour 2012). It is therefore necessary to establish the basal production of AMH and other hormones by the KGN cell line in our laboratory. This study aims to determine KGN cell doubling time, and to measure basal hormone production for later comparison with primary-derived GCs exposed to chemotherapeutics.

2.3 Materials and Methods: Characterising KGN Cells

2.3.1 Cell Culture

The KGN granulosa cell line (Nishi et al. 2001a) was kindly donated by Theresa Hickey, Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, and maintained in DMEM/F12 supplemented with insulin (5ug/mL), transferrin (5ug/mL) and selenium (5ng/mL, ITS) and 10% foetal calf serum (FCS, DKSH, Melbourne, AUS). The cell line was maintained in 75cm2 sterile tissue culture flasks (NUNC, Thermo Fisher Scientific) at 37oC in a humidified atmosphere with 5% CO_2 and sub-cultured every 2-3 days as required. The adherent KGN

cell line was detached from 75cm2 flasks in their exponential growth phase at 80% confluence using sterile Trypsin-EDTA, pooled together and centrifuged at 375g (1500rpm). The cell pellet was re-suspended in complete cell culture medium and viable cells were counted on a haemocytometer with trypan blue.

2.3.2 KGN Cell Doubling Time

KGN cell numbers after 24, 48 and 72 hours of incubation were examined to determine cell doubling time. For each incubation period triplicate 25cm^2 culture flasks were seeded with 2.5x10^5 cells (1.0x10^4 cells/cm^2) in 8 mL complete cell culture medium. Flasks were incubated for their prescribed incubation period after which cells were again detached from flasks and the viable cell number was determined by trypan blue exclusion assay. Cell proliferation rate was then determined using initial and final cell numbers. Doubling times for each incubation period were calculated using the doubling time equation presented below. Doubling times are expressed as the mean ± standard deviation.

$$\text{Doubling time } (t_d) = t \times \frac{\log 2}{\log \frac{N_t}{N_o}}$$

In this equation,

t=time of incubation in hours

N_o=initial number of cells

N_t=final number of cells

2.3.3 Basal Hormone Production

KGN cells (0-60,000 cells per well) in six replicate wells were seeded into sterile 96-well flat bottom plates in a final volume of 0.2mL per well of complete cell culture medium and incubated at 37°C with 5% CO_2 for 24h for KGN cells (Jaaskelainen et al. 2009) to allow cell adherence to plates. All outside wells of plates contained 0.1mL of sterile PBS to prevent evaporation in the inside treatment wells. After the initial cell adherence period, the spent medium was removed from all wells and stored (-20°C) for the measurement of AMH, progesterone and estradiol by immunoassay. The cells in the wells were then rinsed with sterile 1x PBS, and cell viability was determined by crystal violet assay (Reid et al. 2015). 50 µL of 0.5% crystal violet in 50% methanol was added to the cells for 10 minutes, during which it bound to the adherent live cell's DNA and protein. 50 µL of 33% acetic acid

was added to de-stain the cells before measuring the absorbance at 630 nm (Feoktistova, Geserick & Leverkus 2016). The Mean±SD optical density values are shown.

2.3.3.1 Estradiol Enzyme Immunoassay (EIA)

Conditioned media were examined in a competitive Estradiol EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti rabbit IgG, and an acetylcholinesterase estradiol tracer. The manufacturer reports a detection limit of 15pg/mL, and an intra-assay coefficient of variation (CoV) of 7.8 to 18.8%. For this study, the estradiol standard was diluted in the DMEM/F12 cell culture medium supplemented with 10% FCS and ITS to give concentrations that ranged from 6.6 to 4000 pg/mL.

2.3.3.2 Progesterone Enzyme Immunoassay (EIA)

Conditioned media were examined in a competitive progesterone EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse monoclonal anti rabbit IgG, and an acetylcholinesterase progesterone tracer. The manufacturer reports a detection limit of 10pg/mL, an intra-assay coefficient of variation (CoV) of 7.5%, and an inter-assay CoV of 2.9%. For this study, the progesterone standard was diluted in the DMEM/F12 cell culture medium supplemented with 10% FCS and ITS to give concentrations that ranged from 7.18 to 1000 pg/mL.

2.3.3.3 AMH Enzyme Immunoassay (EIA)

Conditioned media were examined in a two-immunological step sandwich type EIA (Immunotech, Marseille Cedex, France) that uses an anti-AMH monoclonal antibody for capturing AMH, and biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH in the wells. The manufacturer reports a detection limit of 1pg/mL, an intra-assay coefficient of variation (CoV) of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standard was diluted in the DMEM/F12 cell culture medium supplemented with 10% FCS and ITS to give concentrations that ranged from 0 to 150 pM.

2.3.4 Results

2.3.4.1 KGN Doubling Time

After applying the doubling time equation over the entire 72-hour incubation period, the KGN cell doubling time was calculated to be 31.71 ± 0.98 hours, but when only the 24 to 48-hour incubation period was factored into the equation, the doubling time was much faster at 15.52 ± 1.23 hours. This indicated that the KGN doubling time decreased with time in culture (

Table 2. 1).

Table 2. 1: Cell doubling time of KGN cells (hours)

Incubation time (Hours)	Doubling time (Hours)
0-24	49.83±4.83
0-48	23.60±1.43
0-72	31.71±0.98
24-48	15.52±1.23
48-72	89.35±9.14

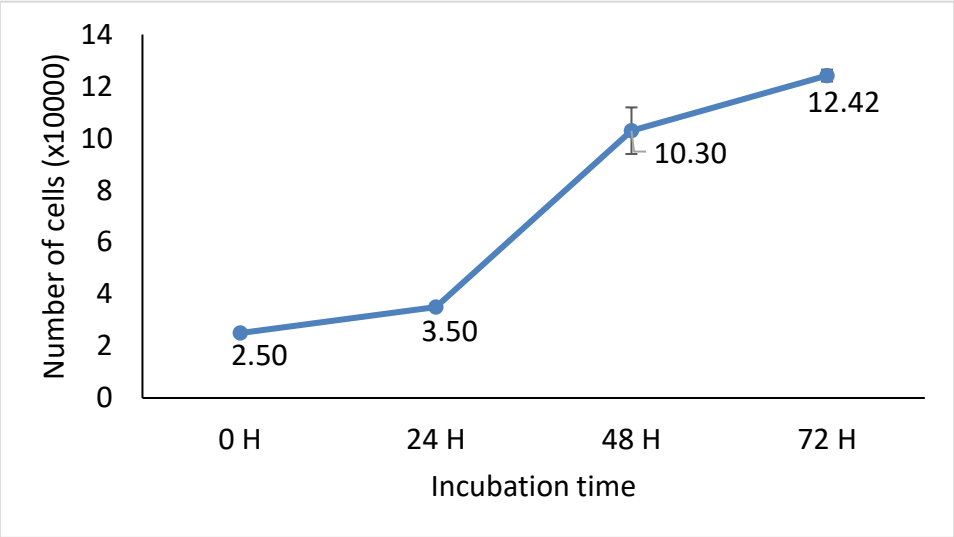


Figure 2. 1: KGN cell doubling time.

25 cm^2 flasks were seeded with 2.5x10^5 KGN cells per well*. Cells were incubated for 24h, 48h and 72h before counting the viable cell numbers by trypan blue assay. Viable cell numbers (x10,000) were plotted against culture period and the KGN cell doubling time in hours was calculated using the equation, td = t x (log2 ÷ logN_t/N_0). The experiment was repeated on three different occasions (n=3), mean±SD shown.*

2.3.4.2 Basal Hormone Production by KGN

KGN cells started to produce estrogen at a detectable limit at a density of 5,000 cells per well and estrogen production gradually increased up to densities of 20,000 cells per well (R^2=0.93) (Figure 2. 2 A). Similarly, progesterone synthesis was linearly related to KGN cell densities (R^2=0.99) (Figure 2. 2 B). However, 24h culture of KGN cells did not produce any detectable levels of AMH.

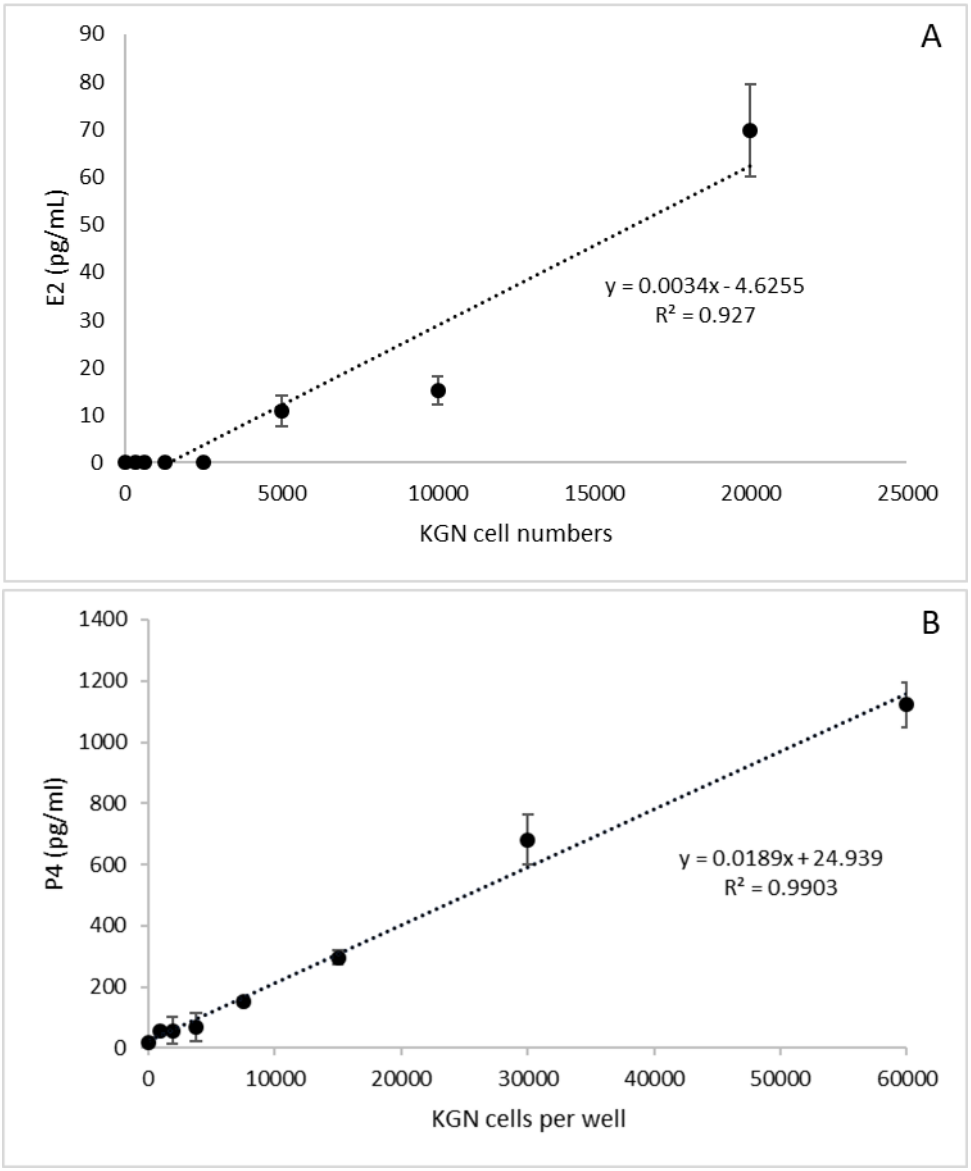


Figure 2. 2 Basal hormone produced by KGN cells in vitro

KGN cells were cultured in triplicate wells of 96 well plate at a density of 0 to 20,000 for E2 and 0 to 60,000 cells per well for P4 and AMH synthesis for 24 hours. Conditioned media after 24-hour culture were then subjected to estrogen, progesterone and AMH ELISA. The experiment was repeated on three separate occasions (n=3). Mean±SD of hormone produced by KGN cells at different densities presented.

2.4 Summary

The KGN cell line was derived from an ovarian adenocarcinoma, and not from healthy ovarian cells but nevertheless the KGN cell line possesses many of the characteristics of primary-derived GCs. In this study, the KGN cell doubling time was approximately 50 hours, similar to that originally reported by Nishi et al. (2001a), 46.4h. The basal production of E2 and P4 suggested that these cells may have similar steroidogenic capacities as normal GCs. *In vivo*, androstenedione is a substrate for the CYP450 aromatase enzyme (Becker 2001; Nelson & Bulun 2001). However, it was previously reported that KGN do not produce detectable levels of androstenedione even after stimulation with cAMP for 24 hour (Nishi et al. 2001a), although KGN cells did show significant aromatase activity while cultured in controlled media (Nishi et al. 2001a). The absence of androstenedione, or the low concentration of androstenedione in 10% FCS probably affected the production of estradiol (E2) by KGN cells. If KGN cells are cultured with androstenedione, it is likely that E_2 synthesis would be increased (Nelson & Bulun 2001).

There was no AMH synthesised by KGN cells. This might be because *in vivo*, AMH is produced by GCs in a tightly regulated pathway mediated by autocrine and paracrine factors deriving from and acting on the theca cells, GCs and oocyte, but KGN cells are immortalized ovarian adenocarcinoma cells which may have compromised AMH synthesis (Knight & Glister 2006; Zec et al. 2011) and also lack the other components of the follicle. However, the production of estrogen and progesterone without stimulation by gonadotrophins or need for precursor substrate is an indication of the estrogenic and progestogenic ability of the KGN cell line. Therefore, KGN cells are suitable for examining the cytotoxic and gonadotoxic effects of commonly used breast cancer chemotherapies.

2.5 Selection of Test Concentrations of Chemotherapeutics

2.5.1 KGN Extended Dose

Breast cancer patients are commonly treated with doxorubicin and cyclophosphamide. After IV infusion of doxorubicin at a dose of 60mg/m^2 and cyclophosphamide at a dose of 600mg/m^2 (therapeutic dose ratio: 1:10), the plasma concentration reached 1.8±0.4µM (Swenson et al. 2003) and 0.2µM (Struck et al. 1987) respectively in 24h after administration (plasma concentration ratio: 9:1).

Vitamin E includes 8 forms including 4 tocopherols and 4 tocotrienols. As a broad class, vitamin E has been used for its antioxidant properties but tocopherols are better absorbed and have higher biological activity than tocotrienols (Brigelius-Flohé et al. 2002; Dimitrov et al. 1991; Mangialasche et al. 2013). It has been reported that the normal plasma levels of α, β, γ and δ-tocopherol are 29.26, 2.3, 1.88 and 0.27 µmol/L without supplementation (Mangialasche et al. 2013). Also, it has been demonstrated that deficiency of vitamin E is related to pathological conditions (Dimitrov et al. 1991; Legha et al. 1982; Mangialasche et al. 2013; Myers, McGuire & Young 1976).

Q10 is a coenzyme that helps enzymes to produce energy for cell proliferation. In addition, it is a potent antioxidant and inhibits apoptosis (Crane et al. 1994; Crane 2001; Muta-Takada et al. 2009; NIH 2015). Q10 also has some cell protective functions such as acceleration of production of cell membrane components such as collagen IV and VII and laminin. *In vitro*, study also suggest that Q10 promoted fibroblast cell proliferation by reducing cell death and protecting against reactive oxygen species (Muta-Takada et al. 2009).

Treatment with doxorubicin and cyclophosphamide is associated with reactive oxygen species (ROS) generation leading to side-effects (Ganz et al. 2017; Guo et al. 2011; Legha et al. 1982; Rogalska et al. 2011; Singh et al. 2017; Swenson et al. 2003). Among various antioxidants, alpha, gamma, and delta tocopherol, and co-enzyme Q10 were short listed based on their antioxidant properties, cytotoxicity and bioavailability (Table 1.4).

Initially KGN cells were exposed to concentrations of chemotherapeutics and antioxidants found in patient's plasma *in vivo* but this concentration of 4OHCYC did not kill KGN cells in comparison to controls. Therefore, the concentration of doxorubicin that killed 50% of KGN cells was combined with a 4OH-cyclophosphamide concentration (0.5µM) that maintained the same ratio as that found

in patient's plasma (10:1) (section 2.6). The aim was to expose KGN and MCF-7 cells to concentrations of test reagents to examine cell viability, and to select a suitable antioxidant to co-expose with doxorubicin and 4OH-cyclophosphamide.

2.5.1.1 Materials and Methods

5.5.1.1.1 Preparation of Chemotherapeutics

Doxorubicin (Dox) (Sigma, Sydney, Australia) and 4OHCYC (active metabolite of cyclophosphamide) (ThermoFisher Scientific, Victoria, Australia) were added to DMEM/F12 medium to make stock solutions of 100 µM and 1000 µM respectively. These were stored at 4°C and -20°C respectively (Hoffman et al. 1979; Ulukaya et al. 2008). Immediately before use they were diluted further to the desired concentrations in DMEM/F12 with 10% FCS and ITS.

2.5.1.1.2 Preparation of Tocopherols

α-Tocopherol (aToc), γ-tocopherol (gToc), delta tocopherol (dToc) (Sigma, Sydney, Australia) and Q10 (Sapphire Bioscience, NSW, Australia) were dissolved in DMSO to obtain stock solutions of 1000µM, and stored at 4°C. Immediately before use they were diluted further to the desired concentrations in DMEM/F12 with 10% FCS and ITS, such that the concentration of DMSO that cells were exposed to was 0.25 to 0.8% DMSO. There was no significant difference in cell viability to this range of DMSO concentrations.

2.5.1.1.3 KGN Cell Culture

The KGN granulosa cell line was maintained in DMEM/F12 supplemented with insulin (5ug/mL), transferrin (5ug/mL) and selenium (5ng/mL, ITS) and 10% foetal calf serum (FCS, DKSH, Melbourne, AUS). The cell line was maintained in 75cm^2 sterile tissue culture flasks (NUNC, Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2 and sub-cultured every 2-3 days as required. The adherent KGN cell line was detached from 75cm^2 flasks in their exponential growth phase at 80% confluence using sterile Trypsin-EDTA, pooled together and centrifuged at 375g (1500rpm). The cell pellet was re-suspended in complete cell culture medium and viable cells were counted on a haemocytometer with trypan blue before seeding into a 96- well plate.

2.5.1.1.4 MCF-7 Cell Culture

The human epithelial breast adenocarcinoma cell line (MCF-7, ATCC® HTB-22™) was obtained from the America Type Culture Collection (ATCC) and maintained in RPMI media, supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin and 10mg/mL streptomycin. Media were replaced every 2-3 days and cells were harvested with 0.1% trypsin EDTA solution and sub-cultured when

80% confluent. Cell culture flasks containing cells in exponential growth phase were used for all experiments. A 96 well plate was seeded with 20,000 cells per well and incubated for 24 hours to allow cell adherence condition at 37°C and 5% CO_2.

2.5.1.1.5 Exposure to Test Reagents

For the examination of chemotherapeutics and antioxidants, a 96 well flat bottom plate was seeded with 25,000 (for aToc and gToc) and 10,000 (for dToc, Q10, doxorubicin and cyclophosphamide) KGN cells per well. 0-60,000 cells/well were added to another plate, with each cell density in 6 replicate wells, to form a standard plot for the MTT assay. After a 24h adherence period, the MTT was applied to the standard plot plate, and chemotherapeutics or antioxidants were added to the treatment plates along with an assay negative control (medium only), and a vehicle control for the tocopherols (0.8% DMSO). There were two treatment plates on each occasion. The exposure durations are shown in Table 2. 2. Test reagents were added at concentrations shown in Table 2. 3. Cell viability was assessment by MTT assay. Each concentration was examined in three replicate wells. This experiment was repeated on 3 separate occasions (n=3).

Table 2. 2: Exposure to chemotherapeutics with/without antioxidants

Treatment plate	Exposure to test reagents	Duration of cell culture without test regents
Plate 1 (24h+)	24h	0
Plate 2 (24h+24h-)	24h	24h

Table 2. 3: Concentrations of antioxidants and chemotherapeutics

Reagents	Test reagent concentrations					
aToc	50µM	75µM	100µM			
gToc	50µM	75µM	100µM			
dToc	25µM	50µM	100µM	200µM	400µM	
Q10	6.25µM	12.5µM	25µM	50µM	100µM	
Doxorubicin	0.2µM	0.4µM	0.8µM	1.6µM	3.2µM	6.4µM
4OH-cyclophosphamide	1.25µM	2.5µM	5µM	10µM	20µM	40µM

2.5.1.1.6 The MTT Cell Viability Assay

This assay quantified mitochondrial succinate dehydrogenase conversion of MTT to formazan (Young, FM, Phungtamdet, W & Sanderson, BJ 2005). Before adding MTT, the media containing chemotherapeutics, antioxidants and all dead unattached cells were removed. The remaining adherent cells had 0.5 mg/mL MTT added for 24h at 37 °C and 5% CO_2. The cells and formazan were solubilised by the addition of 20% SDS in 0.02 M HCl for 24h. The absorbance was measured at 570 nm, with reference absorbance 630 nm, using an automatic spectrophotometer and KC Junior software. The numbers of viable cells were determined by comparison with the standard curve generated using KGN or MCF-7 cells from the same experimental replicate.

2.5.1.1.7 Determination of Inhibitory Concentrations (IC values)

It was considered that the concentrations that reduced breast cancer cell line (MCF-7) viability should be used to model the effects of breast cancer chemotherapy on ovarian follicles or cells. Hence, the breast cancer cell line MCF-7 was used for determining the inhibitory concentrations of test reagents. Cells were exposed to incremental concentrations of test reagents for 24h and cell viability was examined in a MTT assay. IC25 and IC50 values were calculated using a non-linear regression analysis generated by GraphPad Prism (Version 5.00, San Diego, California, USA). The experiment was repeated on 3 separate occasions.

2.5.1.2 Results

2.5.1.2.1 Cytotoxic Effects of Antioxidants and Chemotherapeutics

Among the antioxidants, aToc and gToc were not cytotoxic to KGN cells up to 100µM concentration (Figure 2. 3). dToc caused a dose dependent decrease in KGN cell viability and it interfered with cell division at its highest concentration (400µM). Q10 increased the viability of cells until 25µM but it decreased cell viability both after 24h+ and 24h+24h- exposure at concentrations higher than 25µM (Figure 2. 3). Doxorubicin was more cytotoxic to KGN cells after a 24h+24- exposure while cyclophosphamide did not kill any cells during a 24h+ exposure but increased viability at 1.25 and 2.5 µM after a 24h+24- exposure.

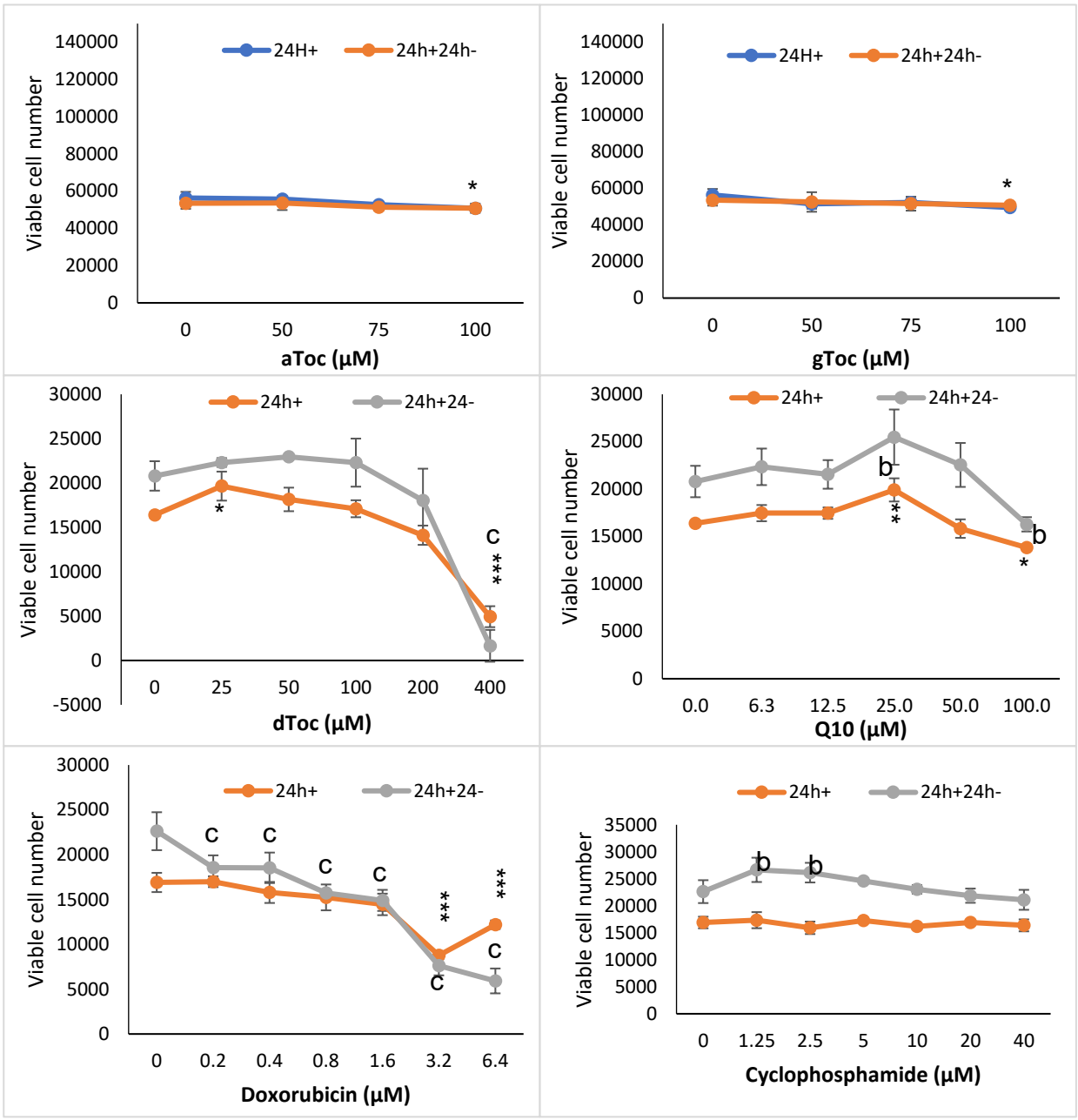


Figure 2. 3 Extended KGN dose response to antioxidants and chemotherapeutics

*KGN cells were exposed to antioxidants or chemotherapeutics (doxorubicin and cyclophosphamide) at increasing doses for either 24h or 24h with subsequent culture in media for 24h. Cell viability was determined by MTT assay. Mean±SD viable cell numbers are shown. Data were analysed by applying one-way ANOVA with Tukey's multiple comparison test. Significant difference, * p<0.5, ** p<0.01 and *** p<0.001 in comparison to control at same culture duration. Data were subjected to two-way ANOVA with Bonferroni post-test, significant difference, a p<0.5, b p<0.1 and c p<0.001 from 24h+ at same dose.*

2.5.1.2.2 IC50s of Doxorubicin, Cyclophosphamide and Gamma Tocopherol

Doxorubicin and cyclophosphamide are commonly used to treat breast cancer and hence the IC50s were calculated using the epithelial breast adenocarcinoma cell line (MCF-7). Mean IC25s and IC50s are shown (Table 2.4).

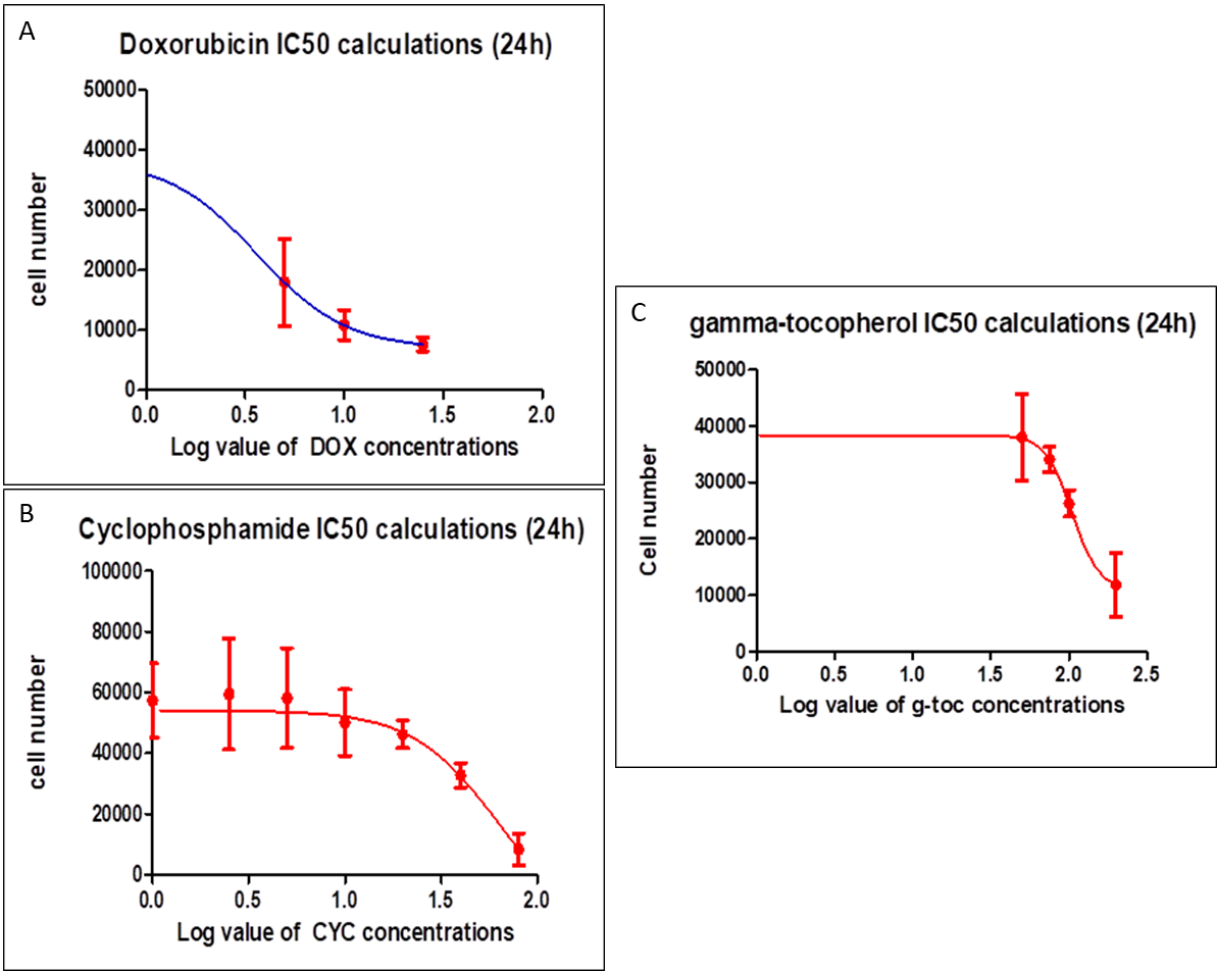


Figure 2. 4: IC25s of treatments against MCF7 cells

A 96 well plate was seeded with 20,000 MCF-7 cells per well and incubated for 24 hours to allow cell adherence and cells were exposed to incremental concentrations of test reagents for 24h at 37°C and 5% CO_2. Cell viability was examined in a crystal violet assay. IC25 and IC50 values were calculated using a non-linear regression analysis generated by GraphPad Prism (Version 5.00, San Diego, California, USA). Mean inhibitory concentrations of 3 experimental replicate presented.

Table 2. 4: IC25s of doxorubicin, cyclophosphamide and gToc against MCF7 cells.

Treatments	IC 25 (µM)	IC 50 (µM)
Doxorubicin (Dox)	1.21	3.63
Cyclophosphamide (Cyc)	21.23	63.69
Gamma Tocopherol (gToc)	35.1	105.3

2.5.1.3 Summary

Among the antioxidants, dToc was the most cytotoxic toward KGN cells but it has the least antioxidant activity in comparison to aToc and gToc (Kamal-Eldin & Appelqvist 1996). On the other hand, in this study Q10 stimulated KGN cell viability. Hence, due to higher cytotoxicity and lower antioxidation activity dToc was not selected for use with the chemotherapy regimen. Also, the cell proliferation supporting activity of Q10 may pose a risk to the cancer patient by promoting cancer cell proliferation.

Between aToc and gToc, although gToc has less antioxidant activity than aToc (Kamal-Eldin & Appelqvist 1996), it is the most abundant form of vitamin E in human diet (Lu, J et al. 2010). Moreover, gToc has demonstrated anti-carcinogenic properties against large cell lung carcinoma cells (Lu, J et al. 2010), colon and prostate cancer (Campbell et al. 2003; Smolarek, AK & Suh, N 2011) and breast cancer (Smolarek, AK & Suh, N 2011). It is therefore anticipated that the ROS produced by doxorubicin and cyclophosphamide during treatment of breast cancer will be reduced by gToc but that may additionally augment cancer cell death. In this study it was promising that the gToc IC25 for MCF-7 cells, 35 µM, had no effect on and was not cytotoxic to the ovarian carcinoma derived KGN granulosa cell line.

Doxorubicin reduced viable KGN cell numbers significantly ($p<.001$) at 0.2µM after 24h exposure with subsequent 24 culture which was suggestive of interference with KGN cell replication because cell doubling time of KGN cells is 46.4 hours (Nishi et al. 2001a). Although cyclophosphamide was not cytotoxic after 24h exposure, it increased cell numbers at a concentration of 1.25 to 2.5 µM but thereafter reduced cell viability which indicated that cyclophosphamide is not cytotoxic at lower concentration. Therefore, it was necessary to determine the IC50s of doxorubicin, cyclophosphamide and gToc *in vitro* by exposing them to cancer cells (breast cancer cells). IC50s

determined in this study were higher than the plasma concentrations of respective test agents (Table 2. 5)

Table 2. 5: IC50s of test agents and plasma concentrations

Test agent	IC50 (µM)	IC25 (µM)	Plasma concentration (µM)	Therapeutic dose
Doxorubicin	3.63	1.21	1.8±0.4	60mg/m^2
4OHCYC	63.69	21.23	0.2	600 mg/m^2
gToc	105.3	35.1	-	
Doxorubicin: 4OH-cyclophosphamide	1:18	1:18	9:1	1:10

IC50s and IC25s of doxorubicin and cyclophosphamide suggest that the effective inhibitory concentrations were different from that of *in vivo* concentrations. Later studies using follicles applied the IC25s determined in this *in vitro* study.

2.5.1.4 Concluding Remarks

Initial data using the KGN cell line is exciting since it suggests there is potential for an improved ovarian friendly chemotherapy. Since the ultimate aim is to test effects of chemotherapeutics on whole follicles, need to find the best or correct concentrations to use in follicle studies. Based on the MCF7 and KGN data it was decided not to use clinically relevant patient serum concentrations, but instead to use *in vitro* derived EC25's (Table 2. 5).

2.6 Effect of Chemotherapeutics and γ-Tocopherol on MCF-7 Breast Adenocarcinoma and KGN Ovarian Carcinoma Cell Lines *In Vitro*

Some replication of content (introduction and background) follows because thesis section 2.6 has been submitted as a manuscript for publication. The datasets presented in section 2.6 are distinct and different from previous datasets.

2.6.1 Introduction

In the United States, approximately 180,000 women are diagnosed with breast cancer each year (DeSantis et al. 2014) and it is the second leading cause of cancer-related death in women (Key, Verkasalo & Banks 2001). In 2008, 6.6% of all breast cancer cases were diagnosed in women younger than 40 years of age, 2.4% in women younger than 35 (Assi et al. 2013). In 2012, 15,050 women were diagnosed with breast cancer in Australia, with a risk of diagnosis before the age 50 of 2.2% or 1 in 46 (Ferlay et al. 2015). In Malaysia, Singapore and other Asian countries breast cancer patients are up to a decade younger than in developed countries, and this premenopausal cohort <35y forms approximately 25% of all breast cancer patients (Tiong et al. 2014). Up to 90% of breast cancer patients survive for 5-years following diagnosis (Ferlay et al. 2015) but find that chemotherapy-induced premature ovarian failure and infertility reduce their quality of life (Baucom et al. 2006; Ganz 2005; Ganz et al. 2003; Ganz et al. 1998; Mariotto et al. 2007; Mor, Malin & Allen 1994; Morgan et al. 2012).

Best practice treatment for women with primary breast cancer involves systemic therapy with hormonal agents, combined chemotherapeutic regimens, or both (Connolly & Stearns 2013). The combination of the chemotherapeutic agents doxorubicin (Adriamycin) and cyclophosphamide (AC) is well-established (Bray et al. 2010), and is also used as part of multidrug schedules for treating advanced breast cancer (Joerger et al. 2007). In phase III randomised trials, an AC regimen showed a response rate of 50% in advanced cancer patients, and complete remission in 7% (Nabholtz et al. 2003). Breast cancer patients are commonly administered an infusion of doxorubicin intravenously (60mg/m^2) then an infusion of cyclophosphamide (600mg/m^2) (Dees et al. 2000; Jones et al. 2006), and these result in plasma concentrations of 1.8±0.4μM doxorubicin within the first 24h after infusion (Swenson et al. 2003). The administration of 600mg/m^2 cyclophosphamide to a mixed cohort of oncology patients resulted in serum concentrations of 4-hydroxycyclophos-phamide that ranged from 0.23 to 1.08 μg/h/m^2 in the 24h period after administration. The serum concentrations of 4-hydroxycyclophosphamide were highest 2-4h after the administration of cyclophosphamide (Struck et al. 1987), approximately 0.5 μg/mL (0.02uM).

2.6.1.1 Chemotherapeutics Mechanism of Action

Cyclophosphamide is an inactive prodrug that requires metabolic activation to release the active phosphoramide mustard (Emadi, Jones & Brodsky 2009). Enzymatic activation begins in the liver through hydroxylation of cyclophosphamide by cytochrome P450 to form 4-hydroxycyclophosphamide and aldophosphamide, which coexist and diffuse freely into the cells. Intracellular aldophosphamide is metabolized into phosphoramide mustard and acrolein (Boddy & Yule 2000; Emadi, Jones & Brodsky 2009). Phosphoramide mustard intercalates within the DNA and causes intra-strand and inter-strand cross-links, thus interfering with DNA replication (Dong et al. 1995). Because the parent cyclophosphamide molecule has no anti-cancer activity and requires hepatic activation, a synthetic compound, 4-hydroperoxycyclophosphamide (4HPCYP) and its metabolite 4-hydroxycyclophosphamide (4OHCYC) have been used to examine the effects of cyclophosphamide *in vitro* (Dees et al. 2000; Ozer et al. 1982) and *in vivo* (Teicher, BA et al. 1996; Yuksel et al. 2015)

Doxorubicin, an anthracycline agent, causes cytotoxicity to cancer cells by intercalating into DNA at double strand DNA breaks in a topoisomerase-II dependent manner, which results in the inhibition of DNA replication, synthesis and mitosis (Tewey et al. 1984; Thorn et al. 2011). Doxorubicin also induces the production of reactive oxygen species (ROS) which cause lipid peroxidation and damage to cell membranes (Gewirtz 1999). Doxorubicin is oxidised to an unstable metabolite or semiquinone. Doxorubicin-semiquinone re-oxidation releases reactive oxygen species, which in addition to causing lipid peroxidation and oxidative stress, also trigger apoptosis and cell death (Doroshow 1986). It remains unclear whether these mechanisms occur simultaneously or as a chain reaction.

2.6.1.2 Adverse Side-effects of Doxorubicin and Cyclophosphamide

The administration of doxorubicin and cyclophosphamide to breast cancer patients can induce a number of acute side-effects including granulocytopenia, fever and infection, emesis, mucositis and diarrhoea (Legha et al. 1982), Cyclophosphamide prevents suppresser T-cell activation (Ozer et al. 1982) whereas doxorubicin induces acute and chronic cardiotoxicity (Legha et al. 1982), which may lead to cardiac dysfunction, cardiomyopathy and eventually to heart failure and death (Wallace 2003; Yeh & Bickford 2009). Despite numerous studies (Chatterjee et al. 2010; M., S. & S. 2003; Takemura & Fujiwara 2007), the precise mechanism for doxorubicin-induced cardiotoxicity is not completely understood (Zhang et al. 2009). It's believed that ROS-generated oxidative stress plays

a pivotal role cardiomyocyte death, as doxorubicin treatment increases oxidative stress and disrupts cytosolic calcium homeostasis (Zhang et al. 2009).

2.6.1.3 Effect of Doxorubicin and Cyclophosphamide on Ovarian Follicles

The chemotherapeutic combination of doxorubicin and cyclophosphamide causes premature ovarian failure in premenopausal breast cancer patients (Meirow et al. 2010; Morgan et al. 2012). Doxorubicin and cyclophosphamide as single agent target dividing GCs in growing follicles (Downs & Utecht 1999; Zhao et al. 2010). These GCs produce anti-Müllerian hormone (AMH) that regulates the rate of follicle activation (Durlinger et al. 1999). The loss of growing follicles and consequent decrease in AMH is thought to result in an increased recruitment of quiescent primordial follicles to activate and grow (Morgan et al. 2012). Therefore, the repeated cycles of chemotherapy may substantially reduce the amount of primordial follicles in the resting pool (Morgan et al. 2012). The effects of the combined regimen on the GCs of the ovary have not been described. In animal studies, doxorubicin alone and cyclophosphamide alone caused a significant loss of ovarian follicles (Morgan et al. 2012; Yucebilgin et al. 2004). The administration of cyclophosphamide to rodents caused a dose-dependent loss of primordial and primary follicles (Meirow et al. 1999; Petrillo et al. 2011). In another study, ovaries collected from 4 day old mice were exposed *in vitro* to 0 – 30μM 4-hydroxycyclophosphamide, and this resulted in significantly fewer healthy primordial and small primary follicles (Desmeules & Devine 2006) with DNA double strand breaks in the oocytes (Petrillo et al. 2011). An immunodeficient mouse model in which human foetal ovary pieces were xenografted into the mice then treated with a single dose of 200mg/kg of cyclophosphamide, found a 93% reduction in primordial follicle density with cell death by apoptosis after 48h (Oktem & Oktay 2007b). It was also found that oocytes showed evidence of apoptotic cell death earlier than the follicular GCs (Oktem & Oktay 2007b). In a subsequent study, ovarian cortical pieces from cancer patients were assessed to quantify the impact of chemotherapy on primordial follicle counts and patients who had previously received any kind of chemotherapy had significantly fewer primordial follicles than the control group of patients who hadn't received chemotherapy (Oktem & Oktay 2007c). Those who were treated with cyclophosphamide as part of their treatment regimens had fewer primordial follicles than patients who were treated with non-alkylating agents (Oktem & Oktay 2007c).

Doxorubicin caused apoptosis in mature murine oocytes (Jurisicova et al. 2006; Perez et al. 1997). Mice administered 7.5 or 10 mg/kg doxorubicin IP had significantly fewer primordial and secondary follicles 1 month later, and levels of ovarian apoptosis increased (Ben-Aharon et al. 2010a). Roti et

al (2012) administered 20mg/kg (twice the human equivalent dose) doxorubicin IP to 4-week old mice and found doxorubicin in the ovarian stromal cells 2h later. The accumulation of doxorubicin was related to the location of follicles in the ovary; it was found in the larger centrally located follicles soon after administration and in the peripherally located primary and primordial follicles 6h later. More apoptosis occurred in granulosa cell populations than the stromal cells, and oocyte DNA fragmentation was first detected 10 hours post injection (Roti et al. 2012). In another *in vitro* study, human ovarian cortical pieces were exposed to different doses of doxorubicin. After 24h doxorubicin caused dose-dependent double-strand DNA breaks in primordial follicles, oocytes and GCs (Soleimani et al. 2011). Doxorubicin also caused apoptosis in a dose-dependent manner in cultured ovarian cortical tissues with a significant increase in the percentage of apoptotic follicles after exposure to 100µg/mL of doxorubicin. These findings were confirmed in an *in vivo* model that exposed SCID mice xenografted with human ovarian tissue to 10mg/kg of doxorubicin (Soleimani et al. 2011).

It is clear that cyclophosphamide alone, or doxorubicin alone, have adverse effects on the follicular GCs of the ovary, but there are no reports describing the gonadotoxic effects of the combined regimen that is used to treat breast cancer patients.

2.6.1.4 KGN Granulosa Cell Line

The human KGN granulosa cell line was established from an invasive granulosa cell carcinoma (Nishi et al. 2001b). KGN are an adherent cell line that maintain most of the physiological activities observed in normal GCs including the expression of Follicle-stimulating hormone (FSH) receptor, aromatase activity, and oestrogen synthesis. Nishi et al., (2001) found that KGN cells also secrete pregnenolone and progesterone in culture and the production of these hormones can be increased by stimulating the cells with dibutyryl cAMP (Nishi et al. 2001b). The KGN granulosa cell line can therefore be used to examine some of the gonadotoxic effects of a combined regimen of doxorubicin and cyclophosphamide *in vitro*.

2.6.1.5 Vitamin E and Cancer

Histological features of doxorubicin-induced cardiomyopathy are similar to those caused by vitamin E deficiency (Legha et al. 1982; Myers et al. 1977), and this observation suggested that both were a result of ROS-induced membrane lipid peroxidation. Vitamin E prevents the propagation of ROS (Lu, G et al. 2010) and this led to the proposal that supplementation of chemotherapy with vitamin E might prevent doxorubicin-induced cardiotoxicity. Early studies suggested that vitamin E decreased

the toxicity of doxorubicin without reducing its effectiveness as chemotherapeutic agent (Geetha et al. 1990; Herman & Ferrans 1983; Krivit 1979; Lubawy, Whaley & Hurley 1979; Milei et al. 1986; Myers, McGuire & Young 1976). The doxorubicin-induced ROS damage was significantly lower in mice receiving higher doses of vitamin E (Thabrew et al. 1999). Additionally the *in vivo* administration of a water-soluble prodrug of vitamin E reduced doxorubicin (15mg/kg) induced liver lipid peroxidation in mice in a dose-dependent manner (Nagata et al. 1999).

Vitamin E consists of eight structurally distinct compounds synthesised by plants, that are classified as tocopherols (alpha, beta, gamma and delta) and tocotrienols (alpha, beta, gamma and delta) (Brigelius-Flohe et al. 2002; Lu, G et al. 2010; Smolarek, A & Suh, N 2011). Gamma tocopherol is the prominent form in the human diet and is found in some vegetable oils (Brigelius-Flohe & Traber 1999). Tocopherols have antioxidant activity against ROS-induced lipid peroxidation (Traber 2007; Traber & Atkinson 2007).

The administration of α-tocopherol to 21 breast cancer patients, prior to chemotherapy, elevated serum concentrations of α-tocopherol to 8.5mg/mL; 6 to 8 times higher than baseline level. Despite this, there were no effects; the αT did not augment efficacy of the chemotherapeutics, and did not decrease toxic side-effects (Legha et al. 1982). Other clinical trials have had varying results. Dietary supplementation with αT, selenium and β-carotene for 5.5y reduced the incidence of cancer in one study (Day & Bingham 1994) and the incidence of prostate and bowel cancer, but not stomach cancer, in another cohort of male smokers (Heinonen et al. 1998). A larger, more recent study however found that dietary supplementation with αT for 7 to 12 years increased the incidence of prostate cancer (Klein et al 2011).

Although there are conflicting results about the anti-cancer properties of alpha tocopherol, recent studies support the chemotherapeutic potential of other tocopherols. Delta and gamma tocopherol increased the levels of pro-apoptotic proteins, whilst inhibiting expression of anti-apoptotic proteins *in vivo* (Smolarek, A & Suh, N 2011) and inhibited the proliferation of human breast cancer cells *in vitro* (Lee et al. 2009; Smolarek, A & Suh, N 2011). Gamma tocopherol had anti-tumour activity in animal models of colon and prostate cancer (Smolarek, A & Suh, N 2011) and delayed the formation of breast cancer tumours in rodent models (Smolarek, A & Suh, N 2011). Gamma tocopherol induced apoptosis in breast cancer cells via up-regulation of DR5 expression (Klein et al. 2011), and through activation of pro-apoptotic JNK/CHOP/DR5 mediated events.

We hypothesised that the combination of doxorubicin and cyclophosphamide would be more cytotoxic to the human breast cancer cell line MCF-7, and the human cancer-derived granulosa cell line KGN, than each chemotherapeutic agent alone. Both alpha and gamma tocopherol are antioxidants with the potential to reduce chemotherapeutic-induced ROS damage and consequently reduce cytotoxicity, but gamma tocopherol additionally has anticancer activity. We therefore hypothesised that gamma tocopherol, but not alpha tocopherol, would augment the cytotoxic activity of the combined AC regimen *in vitro*.

2.6.2 Materials and Methods

2.6.2.1 Chemicals and Reagents:

All chemicals and reagents used in this study were obtained from Sigma-Aldrich (Australia), unless specified otherwise.

2.6.2.2 Preparation of Solutions:

Stock solutions of doxorubicin and 4-hydroperoxycyclophosphamide (ThermoFisher Scientific, Victoria, Australia) were prepared in RPMI media and 10% foetal calf serum (FCS) (DKSH, Victoria, Australia) for MCF-7 cells or in DMEM/F12 media and 10% FCS for KGN cells at concentrations of 100µM and 1000µM respectively. These solutions were kept at 4°C and -20°C respectively for a maximum of 3 months and were diluted immediately before use. Stock solutions of alpha and gamma tocopherol were prepared by diluting the compounds in DMSO to yield solutions of 1000µM. These were stored for a maximum of 3 months at 4°C. Further dilutions in the appropriate cell culture medium were prepared immediately before use, such that the concentration range of DMSO that cells were exposed to was 0.25 to 0.8% DMSO. The 0.5% crystal violet stain was prepared in a 50% methanol (99.9% pure). 100% acetic acid was diluted to 33% with demineralised water, to be used as a destain solution in the crystal violet assay.

2.6.2.3 Cell Culture

The MCF-7 human epithelial breast adenocarcinoma cell line was obtained from the America Type Culture Collection (ATTC) and maintained in RPMI media, supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10mg/mL streptomycin. Media were replaced every 2-3 days and cells were harvested with 0.1% trypsin/EDTA solution. The KGN human granulosa carcinoma cell line was maintained in DMEM/F12 supplemented with insulin (5µg/mL), transferrin (5µg/mL) and selenium (5ng/mL, ITS), 10% FCS and 1% of 10,000 units/mL penicillin + 10mg/mL streptomycin.

Media were replaced every 2-3 days and both cell lines were sub-cultured twice a week. Cell culture flasks containing 80% confluent cells in exponential growth phase were used for all experiments.

2.6.2.4 Effect of Doxorubicin, 4-hydroperoxycyclophosphamide, α and γ Tocopherol on MCF-7 and KGN Cell Viability

MCF-7 cells (20,000 cells per well) and KGN cells (25,000 cells per well) were added to 96-well microplates. After a 24h adherence period, supernatants were removed, and cells were exposed to 100µL of chemotherapeutics or tocopherols (Table 2. 6). The chemotherapeutic doses selected for this *in vitro* study, bracket the clinical, *in vivo* serum concentrations of doxorubicin (Swenson et al. 2003) and 4-hydrocyclophosphamide (Struck et al. 1987) (Table 2. 6). Cells were exposed to chemotherapeutics and tocopherols according to four different schedules: 24h exposure, 24h exposure + 24h culture in media, 24h exposure + 48h culture in media, or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h. After exposure to chemotherapeutics and tocopherols, media containing reagents were collected and frozen, and the cell viability was assessed by the crystal violet (CV) assay. Each concentration of chemotherapeutics was examined in three replicate wells and the experiment was repeated on 3 separate occasions (n=3) for each of the two cell types.

Table 2. 6: Concentrations of chemotherapeutics and tocopherols. Dox: Doxorubicin, 4HPCYP:4-hydroperoxycyclophosphamide, α-Toc: α-Tocopherol, γ-Toc: γ-Tocopherol.

Single agents	**Concentrations (µM)**
Doxorubicin	0.5, 10, 25
4-hydroxyperoxycyclophosphamide	0, 0.5, 1, 2.5
α-Tocopherol	0, 50, 75, 100
γ-Tocopherol	0, 50, 75, 100

Combined agents		**Concentrations (µM)**
Doxorubicin + 4-hydroxyperoxycyclophosphamide	Low	10 (Dox) + 1 (4HPCYP)
Doxorubicin + 4-hydroxyperoxycyclophosphamide	High	25 (Dox) + 2.5 (4HPCYP)
Doxorubicin + 4-hydroxyperoxycyclophosphamide + α-Tocopherol	Low	10 (Dox) + 1 (4HPCYP) + 75 (α-Toc)
Doxorubicin + 4-hydroxyperoxycyclophosphamide + α-Tocopherol	High	25 (Dox) + 2.5 (4HPCYP) 75 (α-Toc)
Doxorubicin + 4-hydroxyperoxycyclophosphamide + γ-Tocopherol	Low	10 (Dox) + 1 (4HPCYP) + 75 (γ-Toc)
Doxorubicin + 4-hydroxyperoxycyclophosphamide + γ-Tocopherol	High	25 (Dox) + 2.5 (4HPCYP)+ 75 (γ-Toc)

2.6.2.5 Crystal Violet Cell Viability Assay

Supernatants were replaced with 50µL of Crystal Violet (CV) stain (0.5%). The cells were stained and fixed for 10min at room temperature. Excess stain was rinsed away with demineralised water, and cells were left to air dry overnight. 50µL of destain solution was added for 10min. The optical density was read at 570nm with correction at 630nm (Reid et al. 2015). A crystal violet standard plot was produced in each replicate experiment in which cell densities ranged from 0 – 80,000 cells per well in replicates of 6 for each cell density. Absorbance readings were plotted against cell densities with an average linear correlation of $R^2 = 0.99$ (n=3) replicate experiments for MCF-7 cells and $R^2 = 0.97$ (n=3) replicate experiments for KGN cells. Numbers of viable cells after exposure to chemotherapeutics and/or tocopherols were determined by comparison with the CV standard curve.

2.6.2.6 Estradiol Enzyme Immunoassay (EIA)

Supernatants from each separate KGN culture experiment (n=3) were examined in a competitive Estradiol EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. Detection ranges from 6.6 to 4000 pg/mL, and the intra-assay coefficient of variation (CoV) ranges from 7.8 to 18.8%. For this study, the oestradiol standard was diluted in the DMEM/F12 cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. A separate standard plot was constructed for each experimental replicate (n=3) and the lowest R^2 value was 0.99. Concentration of estrogen was determined by comparison with the standard curve. Estrogen/cell concentration was calculated by dividing pg/mL of estrogen by the numbers of viable cells in the corresponding well.

2.6.3 Statistical Analysis

To examine the dose-dependent effect of chemotherapeutics and/or tocopherols a one-way ANOVA with Tukey HSD and Bonferroni post-hoc was conducted. To examine the effect of the four different exposure schedules on cell viability, an ANOVA was conducted that used the periods of culture as independent factors. Statistical significance was assessed by Tukey HSD and Bonferroni post-hoc tests. A one-way ANOVA with Tukey HSD post-hoc was conducted to examine estrogen production. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Australia). Statistical significance was set at $p \leq 0.05$. All experiments were performed as three independent replicates, and all data expressed as mean ± standard deviation.

2.6.4 Results

KGN (25,000) and MCF-7 (20,000) cells were used to seed each well, and after 24h adherence and 24h culture in control conditions, cells had proliferated to 113,600 ± 15,600 KGN cells/well and 38,100 ± 4400 MCF-7 cells/well (Figure 2.5 A).

Doxorubicin caused death of both MCF-7 and KGN cells in a dose- dependent manner. The number of viable MCF-7 cells decreased significantly when treated with 5, 10 or 25µM doxorubicin compared to the control (Figure 2.5 A). Similarly, KGN cell numbers after 24h exposure to 5µM doxorubicin was 73,500±11,200 cells/well; significantly lower than the medium controls. A 24h exposure to 5µM doxorubicin was more cytotoxic to the MCF-7 cell than the KGN cells, killing 54 and 35% of the cells respectively. Exposure to doxorubicin followed by 24h or 48h culture with medium alone did not result in cell recovery in either cell line (Figure 2.5 B and C), and there was no significant difference in the number of viable cells that were exposed for 24h then cultured for another 48h, and those that were exposed to doxorubicin continuously for 72h (Figure 2.5 C and D).

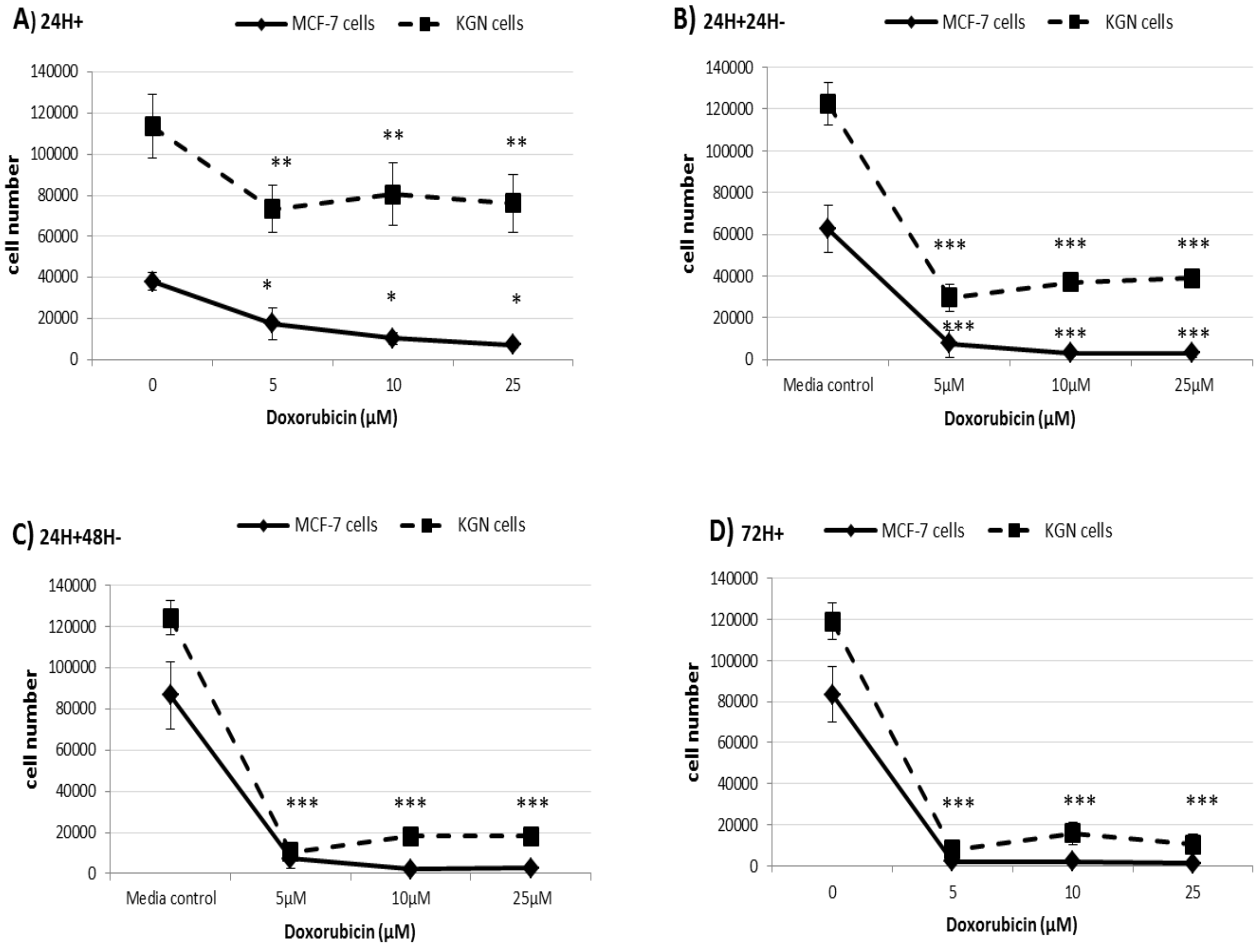


Figure 2.5: Doxorubicin-induced cytotoxicity.

*MCF-7 and KGN cells were exposed to Doxorubicin 0, 5, 10, 25µM for A) 24h (24H+), B) 24h exposure and 24h culture with medium (24H+24H-), C) 24h exposure and 48h culture with medium (24H+48H-), or D) 72h continuous exposure (72H+). Complete RPMI (MCF-7) or DMEM/F12 (KGN) without doxorubicin (0µM) was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control.*

4-hydroperoxycyclophosphamide (4HPCYP) had no effect on KGN cell viability. No significant difference was noted in cells treated with 0.5, 1 or 2.5µM 4-hydroperoxycyclophosphamide compared to the control. Likewise, different exposure and culture schedules had no significant effect on KGN cell numbers (Figure 2.6). Similar to KGN, no significant differences in MCF-7 cell viability were observed when cells were exposed to increasing concentrations of the drug, however after 72h exposure a significant decrease in viability (p=0.012) was detected in cells treated with 2.5µM 4-hydroperoxycyclophosphamide (Figure 2.6).

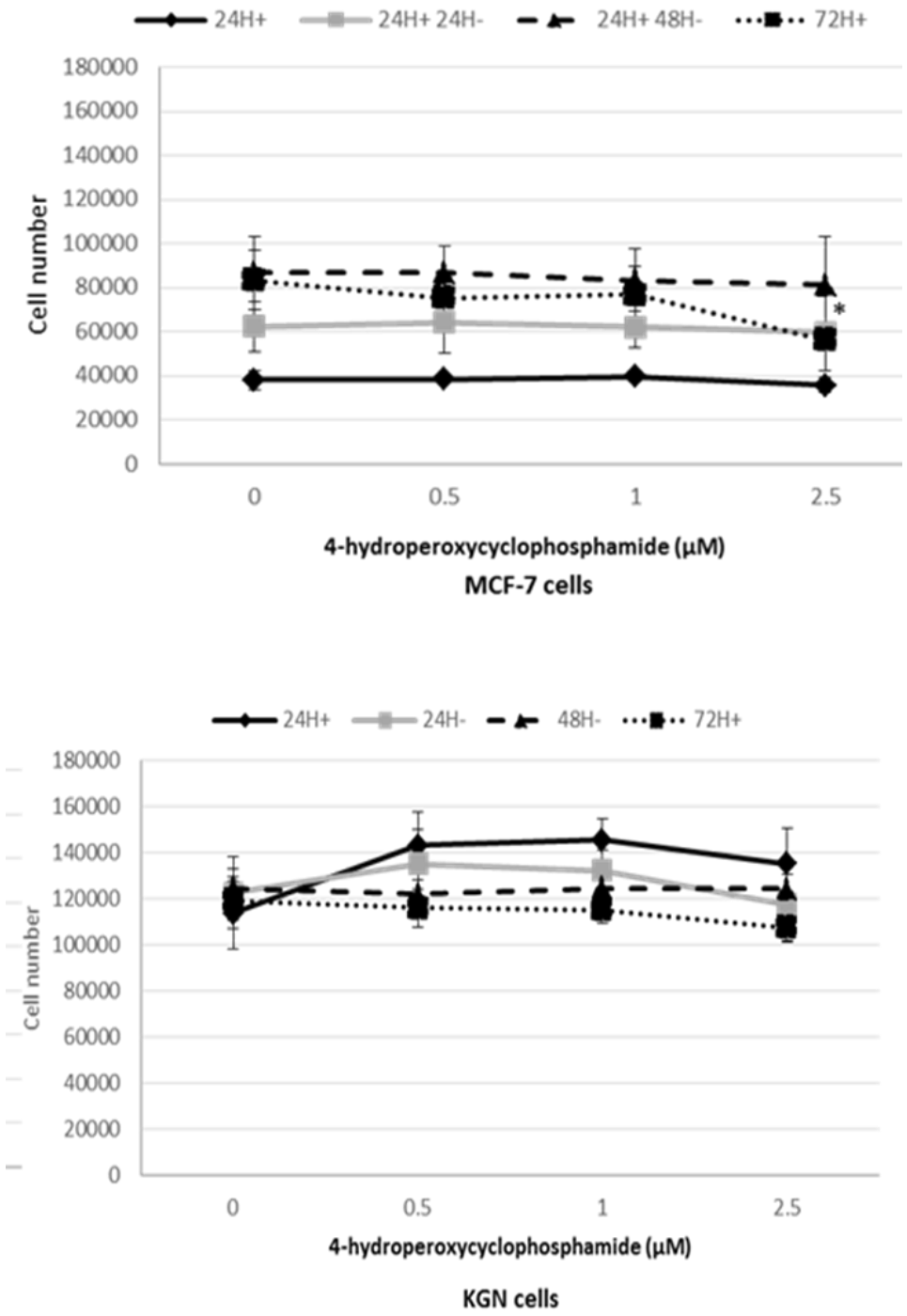


Figure 2.6: Effect of 4-hydroperoxycyclophosphamide on cell viability.

*MCF-7 and KGN cells were exposed to 4-hydroperoxycyclophosphamide 0, 0.5, 1, 2.5µM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Complete RPMI or DMEM/F12 without 4-hydroperoxycyclophosphamide (0µM) was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control.*

Exposing MCF-7 cells to α-Tocopherol did not affect cell viability (Figure 2.7) in any of the exposure or culture schedules tested. Similarly, α-Tocopherol did not cause a significant increase in KGN cell viability.

On the other hand, γ-Tocopherol was significantly more cytotoxic to MCF-7 cells than to KGN cells (Figure 2.8). A dose-dependent decrease in MCF-7 cell viability was observed after 24h and 72h continuous exposure. Further culture without γ-Tocopherol did not result in a recovery or increase in the numbers of viable MCF-7 cells (Figure 2.8)

Increasing concentrations of γ- Tocopherol did not cause any significant changes in KGN cell viability compared to the vehicle control (Figure 2.8). Moreover, the number of viable KGN cells after 24h exposure to 100μM γ- Tocopherol was 111,500±21,400 cells/well; similar to the number of viable cells after exposure to the same concentration of α-Tocopherol (114,000±11,650 cells/well).

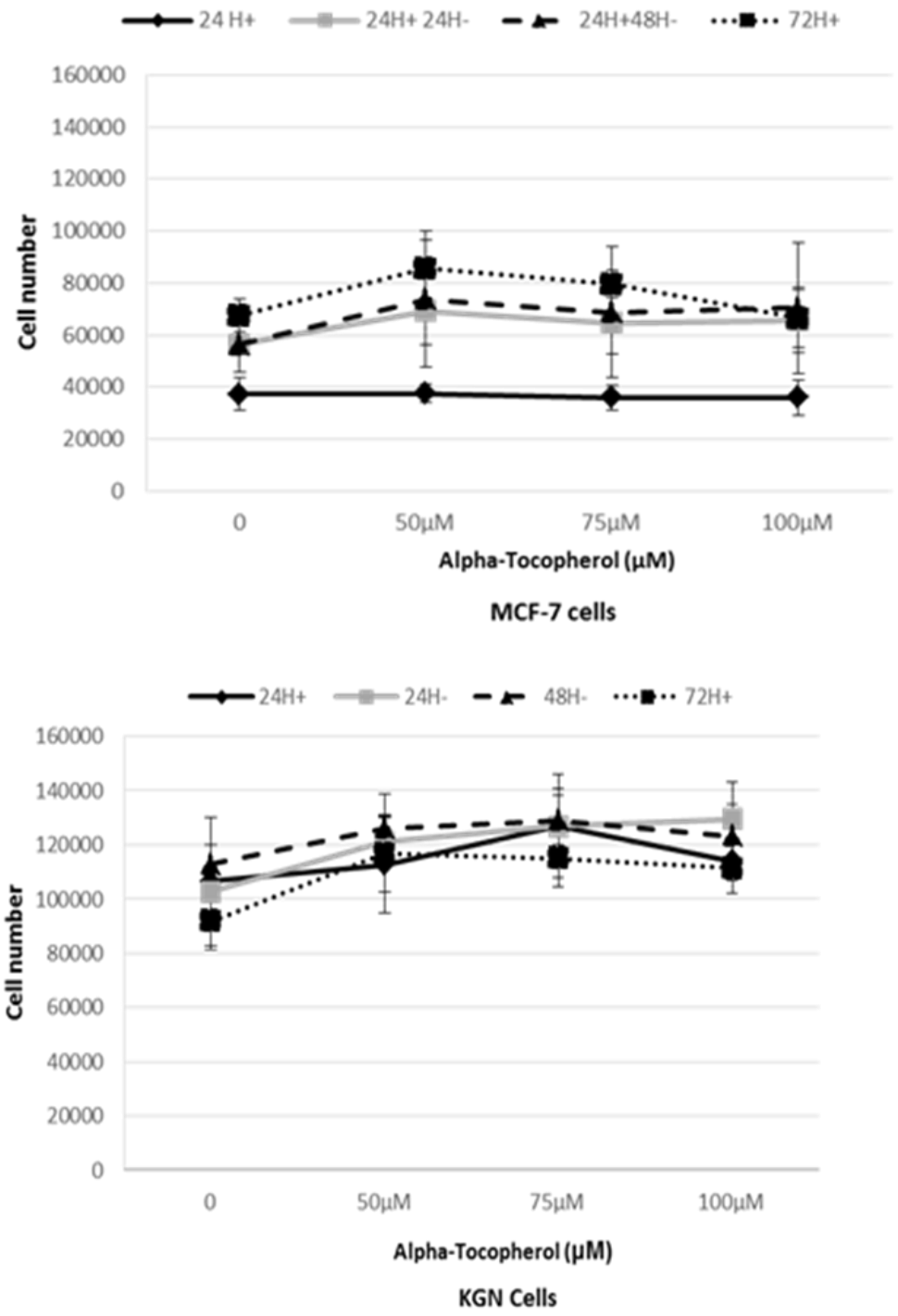


Figure 2.7: Effect of α-Tocopherol on cell viability.

*MCF-7 and KGN cells were exposed to α-Tocopherol 0, 50, 75, 100µM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Culture media containing 0.8% DMSO in was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control.*

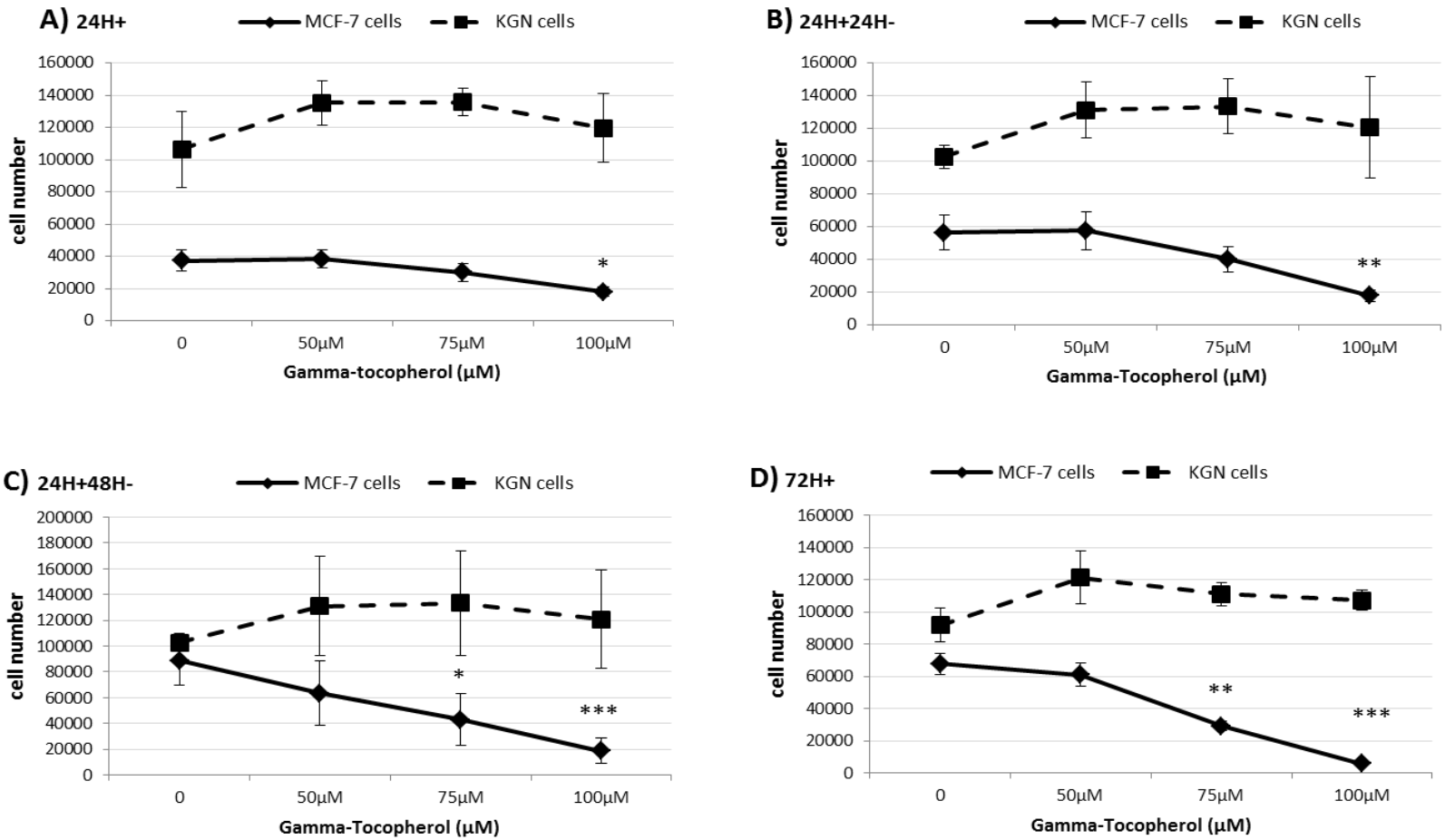


Figure 2.8: Effect of γ-Tocopherol on cell viability.

*MCF-7 and KGN cells were exposed to γ -Tocopherol 0, 50, 75, 100µM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+).).8% DMSO in Complete RPMI or DMEM/F12 was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control.*

The combination of doxorubicin (10µM) and 4-hydroperoxycyclophosphamide (1µM) caused a significant decrease in MCF-7 and KGN cell viability similar to that observed with doxorubicin alone, i.e. 54% of the MCF-7 cells were killed in the first 24h exposure period. The combined regimen with higher concentrations of doxorubicin (25µM) and 4-hydroperoxycyclophosphamide (2.5µM) had the same effect as doxorubicin alone (Figure 2.9). Adding α-Tocopherol to the combined drug regimen had no effect on cell viability. The addition of γ- Tocopherol (75µM) to the combination significantly decreased MCF-7 cell viability after 24h exposure compared to doxorubicin alone (Figure 2.9 A) or to 4-hydroperoxycyclophosphamide alone (data not shown), or to the combination of doxorubicin and 4-hydroperoxycyclophosphamide. γ- Tocopherol augmented the cytotoxic activity of the combination of doxorubicin and 4-hydroperoxycyclophosphamide in MCF-7 after 24h continuous exposure (Figure 2.9 A).

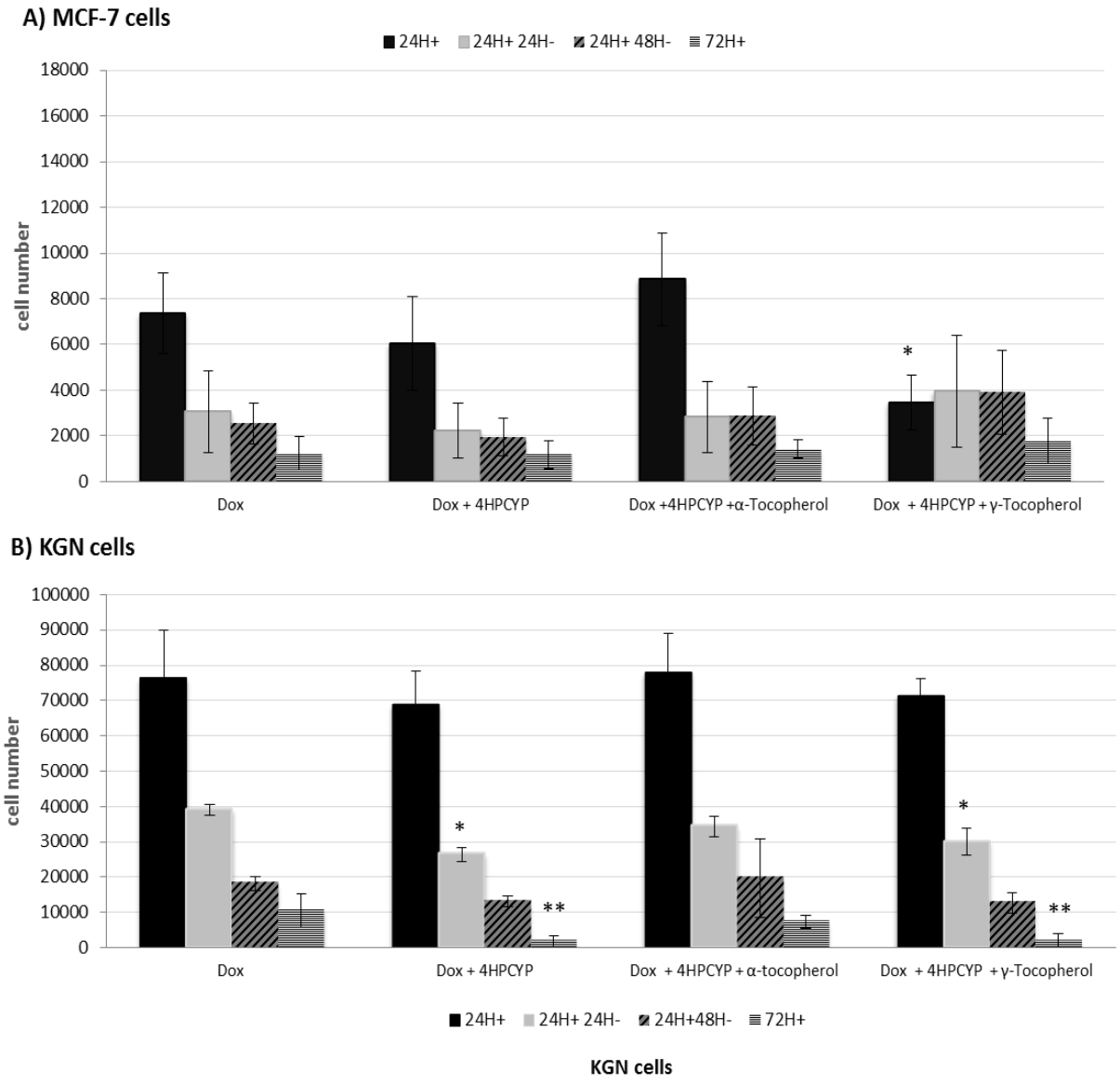


Figure 2.9: Cytotoxicity of combined chemotherapeutic regimen.

*A) MCF-7 and B) KGN cells were exposed to a combination of chemotherapeutics (25µM Doxorubicin + 2.5µM 4HPCYP), or a combination of chemotherapeutics + 75µM α-Tocopherol, or a combination of chemotherapeutics + 75µM γ-Tocopherol for 24h (24H+); 24h exposure and 24h culture with media (24H+24H-); 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control same concentration of Doxorubicin alone (25µM).*

The addition of γ- Tocopherol to the combination of chemotherapeutics did not affect KGN cell viability compared to the combination of doxorubicin and 4-hydroperoxycyclophosphamide (Figure 2.9 B); γ- Tocopherol was more cytotoxic towards MCF-7 than to KGN cells in the first 24h of exposure.

After 24h culture in complete DMEM/F-12 medium, KGN cells produced 1.2 ± 0.1 pg/cell of estrogen; which was not significantly different to the estrogen produced by KGN cells in the last 24h of a 72h culture under control conditions (0.8 ± 0.08 pg/cell) (Figure 2.10). A dose dependent decrease in estrogen per cell production was observed after 24h exposure to 4-hydroperoxycyclophosphamide; on the other hand, a 24h exposure to doxorubicin had no effect on estrogen production (Figure 2.10). However, a continuous 72h exposure to doxorubicin, where fresh reagents in culture media were added to KGN cells every 24h, increased estrogen per cell production significantly. Cells exposed to 25µM doxorubicin after 72h produced 13.1 ± 3.4 pg/cell of estrogen, which was significantly higher than the control (0.8 ± 0.08 pg/cell) (Figure 2.10). Neither α-Tocopherol (50-100µM) nor γ-Tocopherol (50-100µM) had an effect on estrogen production after 24h exposure or 24h exposure + 24h culture in medium, or 24h exposure + 48h culture in medium (Figure 2.10); but estrogen production per cell after 72h continuous exposure to either α-Tocopherol (50-100µM) or γ-Tocopherol (50-100µM) was significantly lower than the control (Figure 2.10 C + 6D).

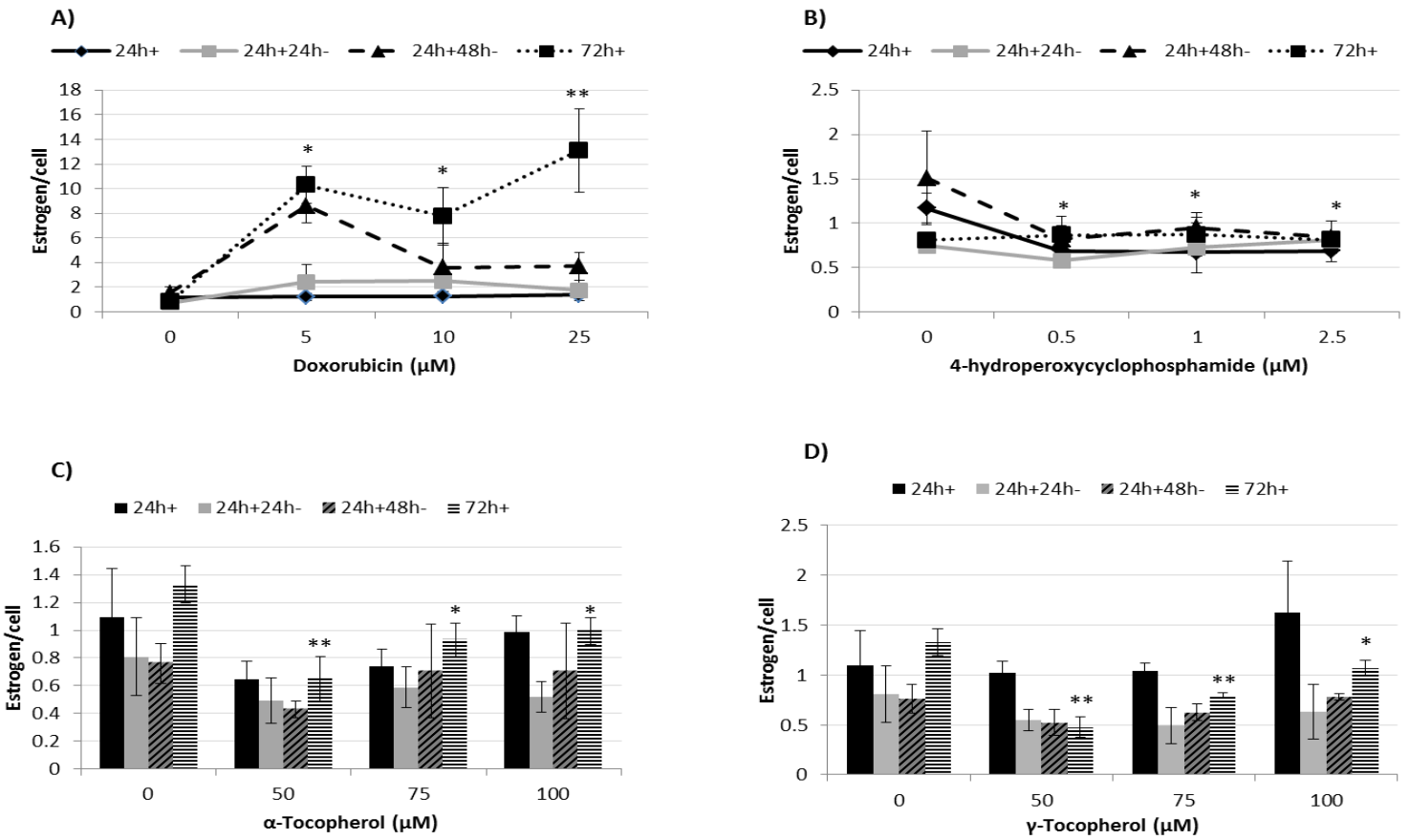


Figure 2.10: Effect of chemotherapeutics and tocopherols on estrogen production.

*KGN cells were exposed to doxorubicin (0,5,10,25µM), 4-hydroperoxycyclophosphamide (0,0.5,1,2.5µM), α-Tocopherol (0,50,75,100µM) or γ-Tocopherol (0,50,75,100µM) for 24h exposure (24H+), 24h exposure and 24h culture with fresh DMEM/F-12 complete medium (24H+24H-), 24h exposure and 48h culture with DMEM/F-12 complete medium (24H+48H-), or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h (72H+). Estrogen production was assessed in supernatant at the end of each time schedule by a competitive Estradiol EIA, in which concentration of estrogen (pg/mL) was obtained by comparison with a standard curve, and estrogen/cell concentration was calculated by dividing pg/mL of estrogen by the cell numbers. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control same concentration of Doxorubicin alone (10µM).*

The addition of α-Tocopherol or γ-Tocopherol to the combination of chemotherapeutics did not affect estrogen production after 24h exposure, or 24h exposure + 24h culture in medium, or 24h exposure + 48h culture in medium (Figure 2.11). But α-Tocopherol appeared to reduce estrogen production (p=0.176) after a 72h exposure in the presence of doxorubicin and 4-hydroperoxycyclophosphamide, whereas γ-Tocopherol maintained estrogen production (Figure 2.11). Estrogen production per KGN cell was higher after continuous exposure for 72h to the combination of chemotherapeutics.

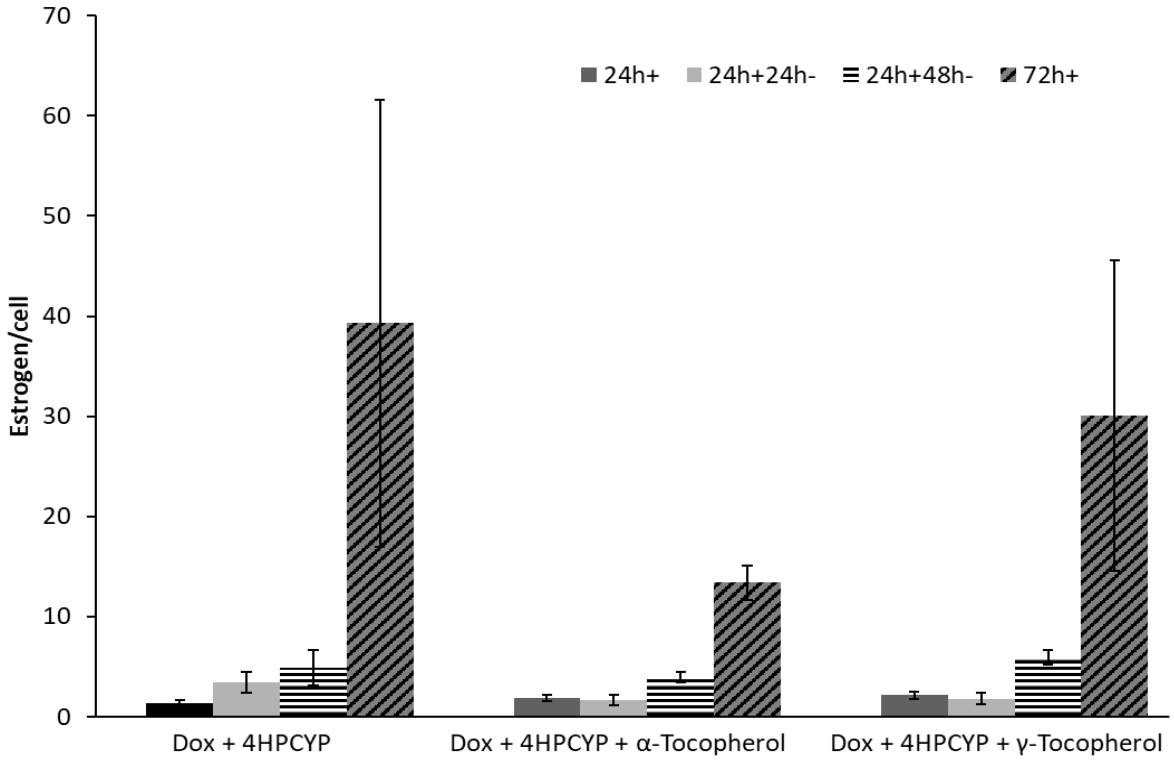


Figure 2.11: Effect of chemotherapeutics and tocopherols on estrogen production.

*Combined chemotherapeutic regimen (10μM Doxorubicin /1μM 4HPCYP), combined regimen + 75μM α-Tocopherol, or combined regimen + 75μM γ-Tocopherol for 24h exposure (24H+), 24h exposure and 24h culture with fresh DMEM/F-12 complete medium (24H+24H-), 24h exposure and 48h culture with DMEM/F-12 complete medium (24H+48H-), or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h (72H+). Estrogen production was assessed in supernatant at the end of each time schedule by a competitive Estradiol EIA, in which concentration of estrogen (pg/mL) was obtained by comparison with a standard curve, and estrogen/cell concentration was calculated by dividing pg/mL of estrogen by the cell numbers. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to control same concentration of Doxorubicin alone (10μM).*

2.6.5 Discussion

The combination of doxorubicin and cyclophosphamide as a regimen has been used as a standard chemotherapy option for breast cancer patients since 1975 (Younis, Rayson & Skedgel 2011). Despite the numerous side-effects associated with each of the two chemotherapeutic agents, there appear to be no studies that report on the effects of the combined doxorubicin/4-hydroperoxycyclophosphamide regimen. In the present study, exposing MCF-7 breast cancer cells to the combination of doxorubicin and 4-hydroperoxycyclophosphamide caused a significant decrease in cell viability; but the numbers of viable cells were similar to those of cells exposed to doxorubicin as a single agent, suggesting that in this *in vitro* model the addition of 4-hydroperoxycyclo-phosphamide did not potentiate the *in vitro* effect of doxorubicin. Exposure to 2.5µM 4-hydroperoxycyclophosphamide for continuous 72h significantly decreased MCF-7 viability, suggesting that the 4-hydroperoxycyclophosphamide had toxic activity, but that the concentrations the cells were exposed to were too low to cause cell death.

It was surprising that after 24h, 2.5µM 4-hydroperoxycyclophosphamide had no effect on MCF-7 cells *in vitro* when 0.02µM 4-hydrocyclophosphamide was found in patient serum 2-24h after administration of cyclophosphamide *in vivo*. There has been an extensive analysis of the pharmacokinetics of cyclophosphamide (de Jonge, Milly E. et al. 2005; Grochow & Colvin 1979; Moore 1991); but much less is known about the kinetics of the cyclophosphamide active metabolites. 4-hydroxycyclophosphamide, the hepatic metabolite of cyclophosphamide, is non-toxic but highly unstable and it has a half-life in plasma of only a few minutes (de Jonge, Milly E. et al. 2005). 4-hydroxycyclophosphamide undergoes spontaneous alteration into phosphoramide mustard; which is a bi-functional DNA alkylating agent (Boddy & Yule 2000; Emadi, Jones & Brodsky 2009); however, unspecific oxidation of 4-hydroxycyclophosphamide can also result in production of inactive metabolites such as carboxyphosphamide and dechloroethylcyclophosphamide (de Jonge, Milly E. et al. 2005). Furthermore, circulating phosphoramide mustard may be ionised at physiological pH and consequently may not enter the cell and therefore not contribute to cytotoxicity (de Jonge, Milly E. et al. 2005). It has also been suggested, that some cells might develop *in vitro* resistance to 4-hydroperoxycyclophosphamide (McGown & Fox 1986). Therefore, the clinically relevant dose of cyclophosphamide necessary to treat breast cancer patients and consequent serum concentrations of 4-hydroxycyclophosphamide might differ from the *in vitro* effective concentration.

The gonadotoxic effect of doxorubicin has been extensively reviewed (Meirow et al. 2010; Morgan et al. 2012), and it has been suggested that treatment with doxorubicin increases the risk of developing amenorrhea in 20-80% of women (Oktay & Sönmezer 2008). Similarly, alkylating agents such as cyclophosphamide have shown to be highly ovo-toxic (Morgan et al. 2012) and to cause ovarian failure in approximately 42% of women (Meirow et al. 1999). Similar to the results observed for MCF-7 breast cancer cells, KGN cell viability was significantly reduced in a dose-dependent manner after exposure to doxorubicin as a single agent, but not after exposure to 4-hydroperoxycyclophosphamide as a single agent. It was therefore unexpected that the longer 72h exposure to the combination of doxorubicin and 4-hydroperoxycyclophosphamide was more cytotoxic to KGN cells than exposure to doxorubicin alone. In early animal studies, the combination of doxorubicin and cyclophosphamide showed a synergistic effect (Corbett et al. 1975; Tobias et al. 1975). It might be possible; therefore, that the continuous addition of 4-hydroperoxycyclophosphamide potentiates the effect of doxorubicin on KGN cells even at small doses. It is also likely that KGN cells are more sensitive to the effect of 4-hydroperoxycyclophosphamide when co-administered with doxorubicin; however, any mechanism of interaction between these two drugs requires evaluation.

Although there are conflicting reports regarding the role of α-Tocopherol in the treatment for breast cancer (Brigelius-Flohe et al. 2002), its potent antioxidant activity led us to propose that α-Tocopherol could play a role in reducing non-specific ROS-induced cytotoxicity. In the present study, exposure to α-Tocopherol (50-100μM) did not affect MCF-7 or KGN cell viability. Furthermore, the addition of α-Tocopherol to the combination of doxorubicin and 4-hydroperoxycyclophosphamide did not reduce chemotherapeutic-induced cytotoxicity compared to the combined regimen alone, suggesting that the levels of chemotherapeutic-induced ROS exceeded the antioxidant capacity of α-Tocopherol.

Apart from its antioxidant activity, γ-Tocopherol has shown cytotoxic effect in breast cancer cells by enhancing the transactivation of PPAR-γ and possibly inhibiting cell cycle progression and inducing apoptosis (Lee et al. 2009). In our study, a dose-dependent decrease in cell viability was observed in MCF-7 cells exposed to γ-Tocopherol while no significant effect in viability was observed in KGN cells exposed to the same doses of γ-Tocopherol.

γ-Tocopherol has also shown anticancer activity in numerous cancer models, including colon (Campbell et al. 2006), prostate (Jiang, Wong & Ames 2004), and lung cancer (Li et al. 2011). To our

knowledge, this is the first study that evaluates the effect of γ-Tocopherol on an ovarian carcinoma cell line. Our results indicate that KGN cells are resistant to cytotoxic activity of γ-Tocopherol, and that γ-Tocopherol does not interfere with KGN cell cycle and proliferation.

The addition of γ-Tocopherol to the combined chemotherapeutic regimen, potentiated the cytotoxic effect of doxorubicin in MCF-7 cells after 24h exposure, but did not add any significant effect when KGN cell were exposed to the same regimen, validating our hypothesis that γ-Tocopherol could possibly augment the cytotoxic anticancer activity on MCF-7 cells in the combined regimen.

A single dose of chemotherapy is not enough to kill all cancer cells; chemotherapy schedules for breast cancer require the infusion of multiple cycles every few weeks, which increases adverse effects on ovary and the likelihood of premature ovarian failure. *In vivo* anti-cancer efficacy is also confounded by selection for chemotherapeutic-resistant cells, which can give rise to recurrence of the cancer. The exposure and culture schedule used in this study was an attempt to model the *in vivo* selection of resistant cells. Our results showed that only 54% of MCF-7 and 35% of KGN cells were killed in the first 24h of exposure, suggesting that the initial infusion of AC given to women *in vivo* may be similar. In our *in vitro* model, surviving cells did not proliferate, and if this reflects the *in vivo* situation, would explain why subsequent cycles of AC chemotherapy are effective in eradicating the cancer. The *in vitro* data demonstrated that a longer, continuous 72h exposure killed 98% of the MCF-7 cells. It may not be possible to increase the duration of infusions for patients, but an alternative suggested by this *in vitro* data is that the addition of γ-Tocopherol as an adjuvant to the AC regimen might increase the efficacy of each cycle and kill more breast cancer cells than AC alone. Since the *in vitro* data showed that AC, and AC + γ-Tocopherol was more cytotoxic to MCF-7 than to KGN cells, it suggests that fewer more effective cycles might improve breast cancer treatment whilst simultaneously reducing adverse effects to the ovary and therefore reducing primordial follicle depletion and premature ovarian failure.

KGN cells are capable of producing estrogen (Ishiwata et al. 1984b; Ryan & Petro 1966). When incubated with androstenedione (substrate for estrogen) for 72h, significant amounts of estrogen were produced and secreted into the culture medium indicating that the mechanism of steroidogenesis in KGN is similar to that in human GCs (Nishi et al. 2001b). Our results showed that after 24h culture in complete DMEM/F-12 medium the amount of estrogen produced by KGN cell was 1.2 ± 0.1 pg/cell, after prolonged 48h or 72h incubation periods, with culture medium

replenished every 24h, estrogen production per cell did not increase. Exposure to 4-hydroperoxycyclophosphamide (0.5-2.5μM) for 72h significantly reduced estrogen production, despite not causing significant KGN cell death. On the other hand, exposure to doxorubicin for 72h caused a significant increase in estrogen production per KGN cell. Doxorubicin was the only reagent that was cytotoxic to KGN cells, and the only reagent that elevated estrogen/cell production. In our *in vitro* model, KGN cells were replenished not only with the chemotherapeutic agents and/or tocopherols every 24h for 72h but also with foetal calf serum (FCS), which is rich in fatty acids and cholesterol, the substrate for steroidogenesis (Miller & Bose 2011). Fatty acids, like arachidonic acid (AA), also play an essential role in StAR protein expression (Wang et al. 2000). AA inhibition in MA-10 mouse leydig tumor cells inhibited LH-dibutyryl cyclic AMP–induced progesterone production and StAR protein expression; but addition of AA into the cell culture reversed these effects and increased progesterone synthesis and StAR protein (Wang et al. 2003). It is possible therefore, that the KGN cells that survived after exposure to doxorubicin could have maintained metabolic activity and increase the production of estrogen.

In COV434 GCs a cyclophosphamide-dependent increase in ROS induced apoptosis and co-treatment with the antioxidants glutathione ethyl ester (GEE), dithiolethreitol (DTT) and ascorbic acid prevented cyclophosphamide-induced apoptosis (Tsai-Turton, Miyun et al. 2007). It has been proposed that the antioxidant properties of dietary forms of vitamin E may reduce cancer risk (Smolarek, A & Suh, N 2011). α-Tocopherol and γ-Tocopherol play an important role in protecting cells from oxidative stress by removing ROS (Smolarek, A & Suh, N 2011). In the present study, exposure to α-Tocopherol or γ-Tocopherol as single agents had no effect on estrogen production per KGN cell; moreover, adding α-Tocopherol or γ-Tocopherol to the combination of doxorubicin and 4-hydroxyperoxycyclophosphamide did not enhance estrogen production compared to the combined regimen alone. It is likely that the concentration of α or γ-Tocopherol required to neutralise intracellular ROS in KGN cells is higher than the ones tested in this study; therefore, more experiments are needed in order to examine the potential of tocopherols to protect GCs from chemotherapeutic-induced ROS and preserve production of estrogen and other ovarian hormones.

2.6.6 Concluding Remarks

Doxorubicin is equality cytotoxic to breast cancer and ovarian cancer cells (MCF-7 and KGN) but 4OHCYC was not cytotoxic to ovarian cancer cells (KGN). When used in combination, additive reduction in cell viability was observed and addition of γ-Tocopherol to this combination (AC) potentiated the cytotoxic effects on breast cancer cells but did not show any effect on ovarian

carcinoma (KGN) cell viability. This was supported by the evidence that γ-Tocopherol was cytotoxic to breast cancer cells in a dose dependent manner but did not affect the KGN cell viability. This suggest that addition of gamma tocopherol to doxorubicin and cyclophosphamide may reduce the number of chemotherapy cycles to treat breast cancers.

3. CHAPTER THREE: DEVELOPMENT OF METHODS TO MEASURE EFFECTS OF CHEMOTHERAPEUTICS ON FOLLICLE

3.1 Introduction

Follicles are isolated from ovarian tissue for many reasons (Aerts et al. 2008; Campos et al. 2011; Chen et al. 2014; Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Gook et al. 2003; Gook et al. 2013; Green & Shikanov 2016; Hornick et al. 2012; Hornick et al. 2013; Lenie et al. 2004; Li et al. 2016; Lierman et al. 2015; Lunardi et al. 2012; McLaughlin, Marie et al. 2014; Merdassi et al. 2011; Nandi et al. 2009; Park et al. 2013; Paulini et al. 2016; Picton, H et al. 2008; Rosendahl et al. 2011; Telfer, Torrance & Gosden 1990; Telfer, Evelyn E & Zelinski, Mary B 2013; Vanacker et al. 2011) including *in vitro* maturation (IVM) to produce mature fertilizable oocytes (Eppig & O'Brien 1996; Eppig & Schroeder 1989; Gook et al. 2003; Green & Shikanov 2016; Jin et al. 2010; Wang et al. 2011). Isolated follicles are cultured *in vitro* in a number of different systems including 3D matrices (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989; Kreeger et al. 2006; Kreeger et al. 2005; Kreeger, Woodruff & Shea 2003; Oktem & Oktay 2007a; Parrish et al. 2011; Shikanov et al. 2011; Xu et al. 2011; Xu et al. 2006). These methodologies often assess follicle growth *in vitro*, usually by measuring diameter, and occasionally by assessing the number and viability of GCs (GC) within the follicle (Anderson et al. 2013; Eppig & O'Brien 1996; Eppig & Schroeder 1989; Gook et al. 2013; Gook et al. 2005; Lundy et al. 1999; Oktem & Oktay 2007a; Parrish et al. 2011; Shikanov et al. 2011; Xu et al. 2006). However, there are few reports describing reliable and accurate methods for assessing the number of viable GC within an intact isolated follicle (Bagger et al. 1993; Griffin, Jeanine et al. 2006; Lundy et al. 1999; van Wezel, Ingrid L & Rodgers, Raymond J 1996), and the use of fluorescent markers of cell viability such as Live-Dead stain or CMXRos (Aerts et al. 2008; Campos et al. 2011; Li et al. 2016; Lunardi et al. 2012; Merdassi et al. 2011; Sanfilippo et al. 2011; Young et al. 2017) to accurately quantify GC numbers requires careful optimization. Since the plan of this project is to measure the effects of chemotherapeutics on whole follicles, and it is hypothesized that the chemotherapeutics will kill GCs (GCs), it is necessary to develop robust and reliable methods for quantifying viable GCs within whole follicles. Hence, I had to perform the next two studies.

3.2 Ovarian Follicle Disaggregation to Assess Granulosa Cell Viability

Follicles are the functional unit of ovary and are comprised of an oocyte surrounded by GCs. The numbers of GC increase as the follicles grow and mature (Gook et al. 2001; Gougeon 1986; Lintern-Moore & Moore 1979; Pedersen & Peters 1968). Fixed haematoxylin and eosin-stained sections of murine ovaries were used to calculate the numbers of GC in primary (9-59), early to mid-secondary (60 – 185), late secondary to incipient antral (187 – 392) and antral (>395) follicles (Griffin, Jeanine et al. 2006). The basal laminae of murine follicles are composed of laminins (α1, β2 and γ1), collagens, particularly type IV, nidogen and perlecan (Gook et al. 2003; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; Van Wezel, Rodgers & Rodgers 1998). In addition to these, the extra cellular matrix (ECM) within the follicle also contains hyaluronic acid (Rodgers, Irving-Rodgers & Russell 2003). The molecular structure of a follicle suggests that a single cell suspension of GC might be obtained by digesting follicular basal lamina with collagenase IV, and the intra-follicular ECM with hyaluronidase.

The Trypan Blue (TB) Exclusion assay is based on the principle that viable cells have intact membranes that prevent the uptake of the dye, whereas ruptured and non-functional cell membranes are permeable to the dye and hence non-viable cells stain blue (Avelar-Freitas et al. 2014; Lunardi et al. 2012; Mascotti, McCullough & Burger 2000; Merdassi et al. 2011; Piccinini et al. 2017; Strober 2001). After staining with Trypan Blue, cells are assessed using an objective lens light microscope and a haemocytometer (Stoddart 2011b). The Trypan Blue Exclusion assay is an established and reliable direct estimation technique for the differentiation and enumeration of live and dead cells and was reported to be more reliable than Live-Dead stain for estimating the viability of fresh and thawed follicles (Sanfilippo et al. 2011).

Piccinini et al. (2017) found no difference in viability when cells retrieved from two- dimensional (2D) or three-dimensional (3D) culture systems were assessed by TB. Cell viability and density measures varied by 5% and 20% respectively, with percent error in estimating cell numbers higher for 3D systems (21%) than for 2D systems (17%) (Piccinini et al. 2017). Other studies reported coefficients of variation (CoV) for estimating cell numbers using TB from 4.5% (Eillipore 2018) to 6.9% (Allison & Ridolpho 1980). It has been noted that the viability of cells decreases as the time after adding the dye increases, and also after cells have been harvested using trypsin (Altman, Randers & Rao 1993; Tennant 1964; Tsaousis et al. 2013). The TB assay does not differentiate between metabolically active and non-active cells (Black & Berenbaum 1964).

In general practice, an accurate and precise TB estimation requires $0.4x10^6$ to $1x10^6$ cells per mL of cell suspension. Hence, suspensions with densities lower than $1x10^5$ cells per mL cannot be assessed accurately using existing TB assay protocols (Lonza 2012; Louis & Siegel 2011). The number of GCs in a single follicle; approximately 400 in an incipient antral follicle (Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Pedersen & Peters 1968) is too low for accurate assessment using TB.

The aim of this study was to develop a technically simple and inexpensive method to disaggregate follicles and produce a single granulosa cell suspension which can be assessed in a modified Trypan Blue Exclusion assay. To do this, a preliminary study using human KGN, T47D and 184B5 cell lines was conducted to determine centrifugation conditions applicable to low cell densities, and to determine the limit of sensitivity of a standard TB Exclusion assay. Thereafter, the optimal centrifugation conditions were applied to the human granulosa KGN cell line to assess the reproducibility of the TB assay protocol. The resulting low cell-density TB Exclusion assay was then used to optimize follicle disaggregation methods before being applied to the characterization of GCs obtained from disaggregated murine follicles. The TB evaluation was compared to follicle-derived GC that were cultured in a 96-well plate format before assessing their viability by using a crystal violet assay (Reid et al. 2015).

3.2.1 Materials and Methods

All the reagents used in this study were purchased from Sigma unless otherwise stated.

3.2.1.1 Cell Lines and Culture Media

184B5 (ATCC® CRL-8799™) and T47D (ATCC® HTB-133™) cell lines with passage numbers of 11 and 14 respectively were used for this experiment. The cells were cultured in RPMI with 10% foetal calf serum (FCS, DKSH, Melbourne, AUS) and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) at 37°C with 5% CO_2, and sub-cultured when 80% confluent. The KGN granulosa cell line derived from a human granulosa cell carcinoma (Ishiwata et al. 1984a; Nishi et al. 2001a) was maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 1% insulin (5ug/mL), transferrin (5ug/mL) and selenium (5ng/mL, ITS) and 10% FCS and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) and sub-cultured every 2-3 days as required using trypsin ethylene diamine tetra-acetic acid (EDTA) to detach cells.

3.2.1.2 Effect of Centrifugation on Cell Retrieval

A preliminary study used the KGN granulosa cell line to identify the optimal centrifugation conditions for maximizing the retrieval of viable cells. A low-density cell suspension containing 600 cells in 500 µL of media was distributed to 12 centrifuge tubes (1.5mL) which were centrifuged at room temperature for 4, 5 or 6 minutes at 1306g, 2040g, 2938g, or 4000g. Supernatants were removed, and the cells were re-suspended in 10 µL of media and 10 µL Trypan Blue. The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. This experiment was repeated on four separate occasions (n=4).

3.2.1.3 Trypan Blue Exclusion Assay Protocol

Cell suspensions were diluted 1:1 with Trypan Blue (2mg/mL Trypan Blue in phosphate buffered saline; PBS) and 10 µL added to each side of a haemocytometer, i.e. onto each of two grids. Cells were viewed using an objective lens microscope at 10x magnification (Leica, Leitz Wetzlar, Germany). The numbers of unstained viable and blue-stained dead cells in all 9 large squares of the haemocytometer (Figure 3. 1) were counted, and the average number of cells per large square determined. These were adjusted by the dilution factor and converted to reflect the number of cells in 10 µL cell suspension using the equations shown in Figure 3.1 (Stoddart 2011a; Strober 2001).

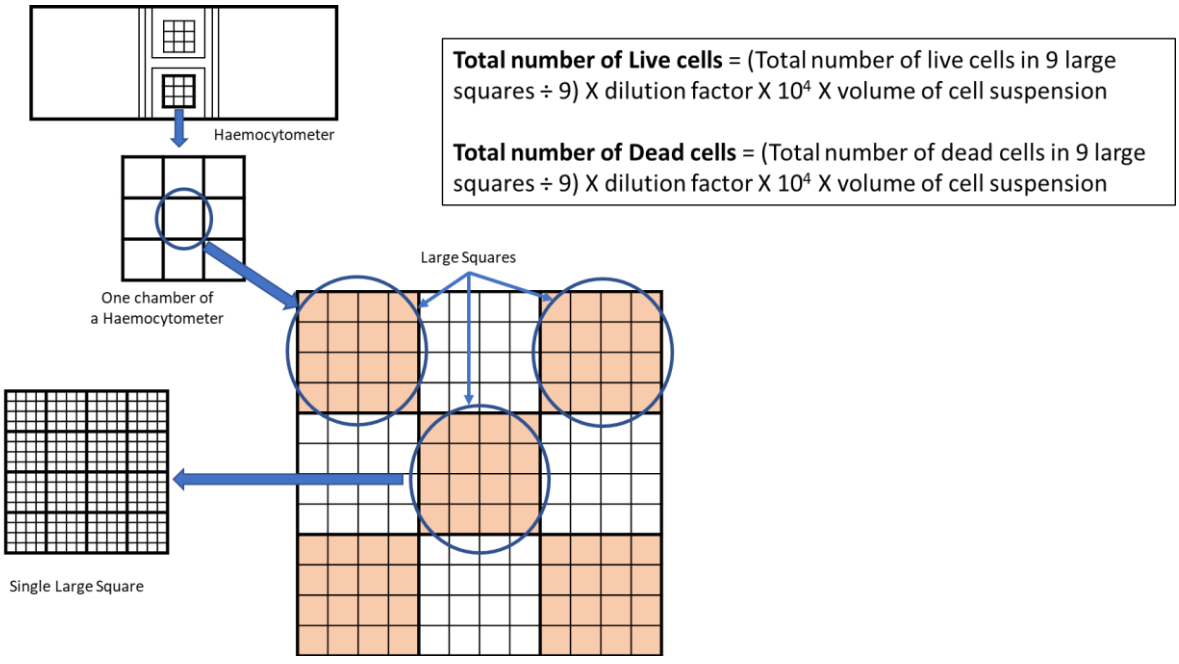


Figure 3. 1: Haemocytometer chamber

3.2.1.4 Accuracy of the Trypan Blue Assay

The ability of the Trypan Blue assay to accurately quantify viable cells was examined using 184B5 and T47D cell lines. The number of viable cells in each parent cell suspension was determined in the Trypan Blue Exclusion assay, and the volumes of parent cell suspension that contained 1250 (184B5), 930 (T47D) or 953 (T47D) viable cells were added to centrifuge tubes and the volume made up to 100 µL with media. Four 1 in 2 dilutions were performed, and three separate dilution series were made from each parent cell suspension. Cells were centrifuged at 2040g for 5 minutes and the supernatant removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The numbers of cells retrieved were determined in the Trypan Blue Exclusion assay.

3.2.1.5 Coefficient of Variation (CoV) of the Trypan Blue Assay

Three single cell suspensions of KGN GCs containing 2500, 1250 and 156 cells per mL of media were produced on four separate occasions (n=4). 500 µL of each cell suspension were added to 10 separate centrifuge tubes, so that they contained 1250, 625 or 78 KGN cells per tube. These were centrifuged at 2040g for 5 minutes at room temperature and the supernatants removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. Coefficients of variation (CoV) were calculated by dividing the standard deviation (SD) by the mean of viable cell numbers for each of the cell densities within each of the experimental replicates (intra-assay CoV) and between the experiments (inter-assay CoV, n=4) and presented as a percentage; CoV (%) = (SD/mean) x 100 (Hanneman et al. 2011).

3.2.1.6 Mouse Ovary Collection

Female mice that were surplus to the College of Medicine and Public Health Animal Facility breeding colony needs were allocated to routine culling procedures. The Flinders Animal Welfare Committee approved our use of cull animals on condition that no requests were made for specific strains or ages. Swiss mice (n=13) aged 6—8 weeks as calculated by their weight (20-25 grams) were used in these studies. Mice were killed by cervical dislocation and the ovaries were isolated by dissection. The ovaries and attached oviducts were placed in warm Hanks Balanced Salt Solution (HBSS) and transported to the laboratory at 37°C. Whole ovaries were dissected free of attached tissues, blotted dry, cut in half and weighed.

3.2.1.7 Follicle Isolation from Ovarian Tissue

Follicles were isolated from ovarian tissue by disaggregation with 0.5 mL of 2mg/mL collagenase IV (Worthington, 295units/mg) in DMEM/F12 for 30 minutes at 37°C in a humidified 5% CO_2 incubator. After incubation 100 µL FCS were added (Ke et al. 2011; Van Luyn et al. 2002) and each half ovary was mechanically disaggregated with 22 gauge needles for 5 minutes. All released follicles were collected and transferred to a 96-well plate containing DMEM/F12.

3.2.1.8 Allocation of Follicles into Size Cohorts

The diameters of follicles collected after ovarian tissue disaggregation were determined by using a camera (Scientific C-Mount Camera, 9MPX) mounted on a dissection microscope to take a microphotograph of the well containing all the follicles from one half ovary. The well diameter was provided by the manufacturer and used to calibrate the Image J software. The area in pixels of each follicle in the well was determined by applying the circle tool to each follicle twice and calculating the average area. The area was used to calculate follicular diameter. The diameters of the follicles were used to allocate follicles to primordial, primary, early to mid-secondary, late secondary to incipient antral, or antral stage cohorts, according to the size ranges described by Griffin, Jeanine et al. (2006).

3.2.1.9 Follicle Disaggregation using Hyaluronidase

Follicles were disaggregated using 50µL hyaluronidase (1228 units/mg) at two concentrations (0.01% and 0.025%). A group of three secondary follicles from one mouse were exposed to 0.01% and another group of 3 secondary follicles from the same mouse were exposed to 0.025% hyaluronidase, for 2 hours at room temperature. 50 µL of FCS were added and the GCs isolated from each group of 3 follicles were centrifuged at 2040g for 5 minutes. The supernatants were discarded, and cells re-suspended in 20 µL DMEM/F12 with 10% FBS, and 20 µL Trypan Blue. This experiment was repeated using secondary follicles from two more mice (n=3, a total of 18 secondary follicles).

3.2.1.10 Follicle Disaggregation Using Combinations of Collagenase IV and Hyaluronidase

Three antral follicles from each half ovary from four mice (n=4) were isolated (i.e. 48 follicles with mean diameter of 303±65 µm from 8 ovaries). Four groups of three antral follicles from each mouse were exposed to collagenase IV (2mg/mL) in a 96-well plate for 15, 30 or 45 minutes before adding 0.025% hyaluronidase. No hyaluronidase was added to the 4th group of follicles which were incubated in collagenase IV for 60 minutes. Follicles in all the treatment groups were incubated at 37°C with one minute shaking every 15 minutes. After 60 minutes incubation, 50 µL of FCS were

added and all the contents of each well were transferred to 1.5 mL centrifuge tubes. Each well was washed with 1X PBS and this was used to make the final volume in the centrifuge tubes up to 500 µL. The GCs were centrifuged at 2040g for 5 minutes. The supernatants were discarded, and the isolated cells were re-suspended in 10 µL of DMEM with 10% FCS. The numbers of viable cells were then determined in a Trypan Blue Exclusion assay as described previously.

3.2.1.11 Crystal Violet (CV) Assay Assessment of GC Viability

GCs were obtained from 250-265 disaggregated follicles from two mice on each of three separate occasions (n=3). Aliquots from each of the three single GC suspensions were assessed in a Trypan Blue Exclusion assay to determine the numbers of viable cells. These primary-derived murine GC were added to sterile 96-well flat bottom plates in a final volume of 0.1mL per well of DMEM/F12 at densities of 0 to 20,000 cells per well, with each cell density in triplicate wells. The primary-derived murine GC were incubated at 37°C with 5% CO_2 for 24h to allow cell adherence (Jaaskelainen et al. 2009). The non-adherent, non-viable cells were removed by withdrawing supernatant from the wells. The supernatant was stored (-20°C) for the later measurement of anti-Mullerian hormone (AMH) and estradiol by Enzyme-linked Immunoassay (EIA). The adherent viable cells in the wells were rinsed with sterile phosphate buffered saline (PBS) before adding 50 µL of 0.5% crystal violet in 50% methanol for 10 minutes, followed by the addition of 50 µL of 33% acetic acid and measurement of the absorbance at 570 nm with correction at 630 nm (Feoktistova, Geserick & Leverkus 2016; Reid et al. 2015).

3.2.1.12 Estradiol Enzyme Immunoassay (EIA)

The conditioned media were examined in a competitive Estradiol (E2) EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. The manufacturer reports a detection range from 6.6 to 4000 pg/mL, and an intra-assay coefficient of variation (CoV) of 7.8 to 18.8%. For this study, the estradiol standard was diluted in the DMEM/F12 cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. All supernatants from three separate primary-derived GC culture experiments were examined in one EIA, in which the standard curve R^2 value was 0.98.

3.2.1.13 Anti-Mullerian Hormone (AMH) Enzyme Immunoassay (EIA)

Conditioned media were examined in a two-immunological step sandwich type EIA (Immunotech, Marseille Cedex, France) that uses an anti-AMH monoclonal antibody for capturing AMH, and a biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH

in the wells. The manufacturer reports a detection limit of 1pg/mL, an intra-assay CoV of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standards were diluted in DMEM/F12 cell culture medium to give concentrations that ranged from 0 to 150 pM. All supernatants from three separate primary-derived GC culture experiments were examined in one EIA, in which the standard curve R^2 value was 0.99.

3.2.1.14 Statistical Analysis

The effect of centrifugation on viable cell retrieval was analysed by 2-way ANOVA with Bonferroni post-test. The expected numbers of T47D, 184B5 and KGN cells were compared to the numbers counted in the Trypan Blue Exclusion assay in a Chi Square analysis.

The numbers of GC obtained by follicle disaggregation using 0.01% or 0.05% hyaluronidase were compared in a Students Paired T-test. The numbers of GC obtained after follicle disaggregation using a combination of Collagenase IV and Hyaluronidase were compared to disaggregation using 0.05% hyaluronidase alone by applying a two-way ANOVA with Bonferroni post-test. Statistical significance was assigned at $p<0.05$.

3.2.2 Results

3.2.2.1 Effect of Centrifugation on Viable Cell Retrieval

The centrifugal force significantly affected cell retrieval ($p=0.0012$ overall effect in a 2-way ANOVA) but the duration of centrifugation had no effect ($p=0.11$). Centrifugation at 2040g for five minutes retrieved the highest number of viable cells (583±14, $p>0.05$) and the highest total number of cells 608±14 ($p>0.05$, Figure 3. 2). Under these conditions, 96% of the cells were viable.

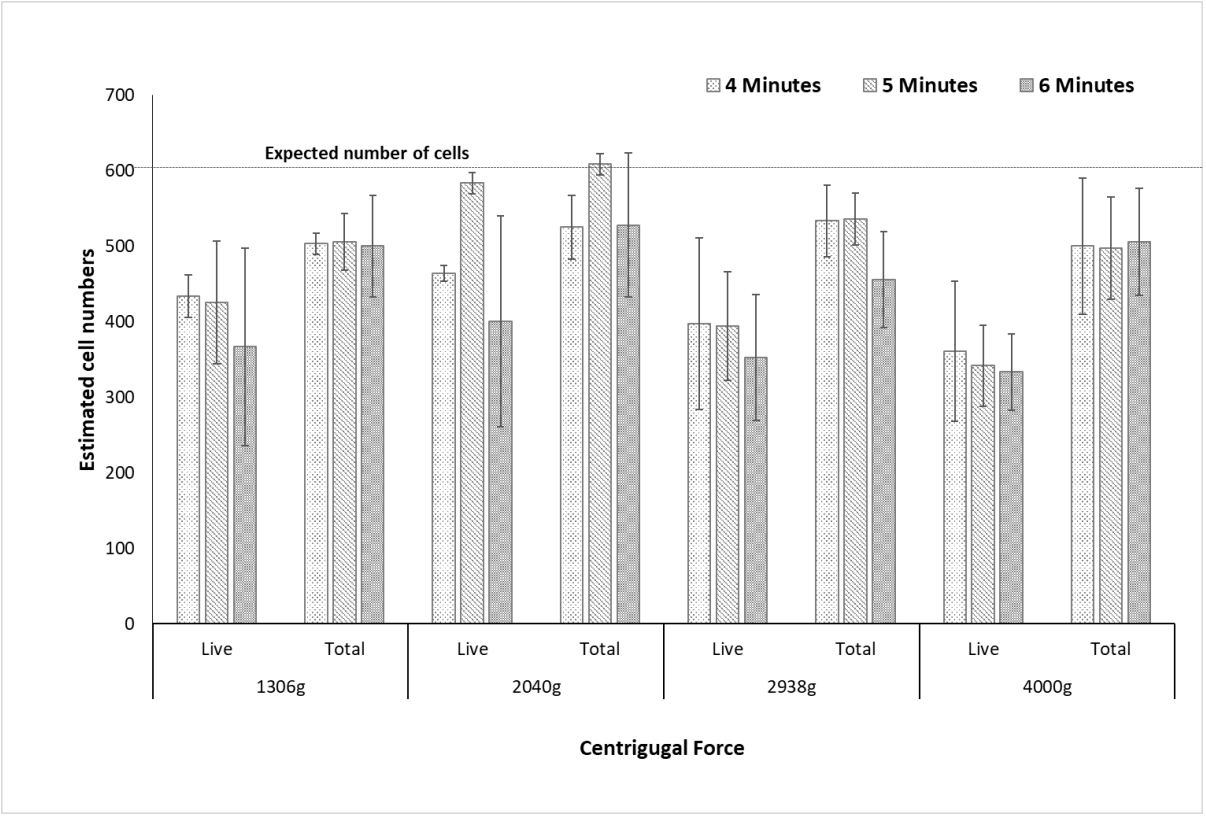


Figure 3. 2: Effect of centrifugation on viable cell retrieval.

KGN cells (600 cells in 500 µL of media) were centrifuged (1306g, 2040g, 2938g and 4000g) for 4, 5 or 6 minutes on four separate occasions (n=4). Supernatants were removed, and cells resuspended in 10 µL culture media and 10 µL of Trypan Blue before counting dead and viable cells using a haemocytometer and 10x objective lens microscope. Mean±SD of cells in each cell suspension shown. Data were analysed by 2-way ANOVA with Bonferroni post-test, significance was assigned at $p<0.05$.

3.2.2.2 Accuracy of the Trypan Blue Assay

There was no significant difference between the numbers of cells calculated to be in a dilution series, and the number of cells counted directly in a Trypan Blue assay (Figure 3. 3).

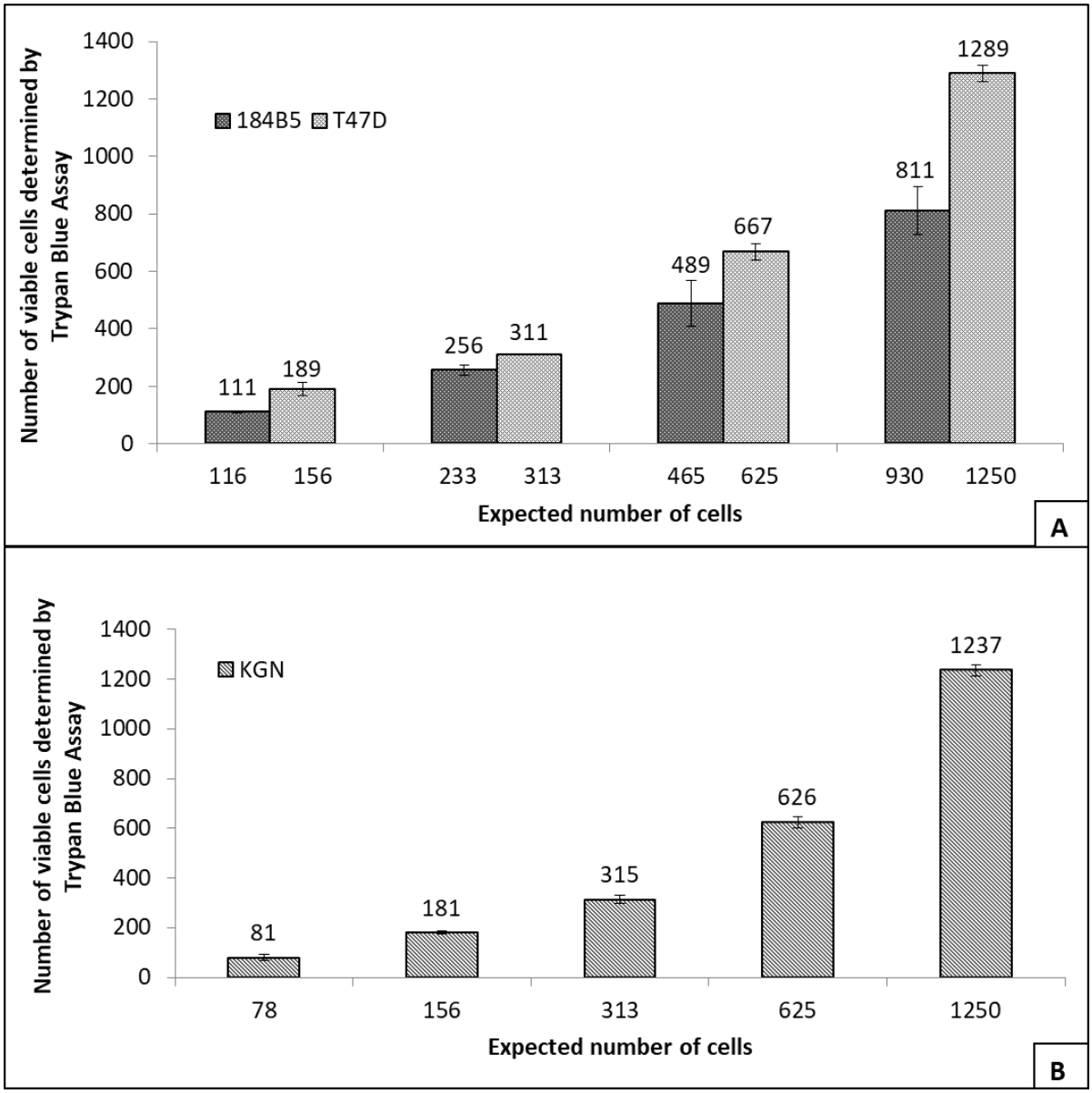


Figure 3. 3: Accuracy of the Trypan Blue Assay.

Three parent cell suspensions of the human cancer cell lines; T47D, 184B5, (A) and KGN (B) were assessed using a standard Trypan Blue Exclusion assay. The volumes of cell suspension that contained 1250 viable 184B5 and KGN cells, or 930 viable T47D cells were made up to 100 µL then subjected to four 1:2 dilutions. The numbers of cells calculated to be in each preparation were designated 'Expected'. The cells were centrifuged at 2040 g for 5 minutes, then re-suspended in 10 µL of media and 10 µL of Trypan Blue was added. Each stained cell preparation was assessed twice using a haemocytometer and 10x objective lens microscope, and the numbers of viable cells in the original 100 µL cell suspension determined. The experiment was repeated on three separate occasions (n=3) and the mean ± SD shown. Data was analysed with Chi Square test.

3.2.2.3 Coefficients of Variation of the Trypan Blue Exclusion Assay

The intra-assay co-efficient of variation decreased from 22.3% to 5.4% as the number of cells in the cell suspension increased, and so did the inter-assay CoV (Table 3. 1).

Table 3. 1: Intra and inter assay Coefficients of Variation (CoV).

1250, 625 or 78 KGN cells per 500 µL of media, with each cell density examined in 10 separate centrifuge tubes, were centrifuged at 2040g for 5 minutes. Supernatants were removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The experiment was repeated on four separate occasions (n=4). The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. CoV (%) = (SD/mean) x 100.

% CoV	78 cells	625 cells	1250 cells
Intra-assay	22.27	7.57	5.38
Inter-assay	6.97	4.14	1.57

3.2.2.4 Follicle Disaggregation using Hyaluronidase

There was no significant difference in the number or viability of GC obtained from secondary follicles disaggregated with 0.01% or 0.025% hyaluronidase (Figure 3. 4). The numbers of viable GC collected after disaggregation with 0.025% hyaluronidase however, were closer to those enumerated previously in H&E stained sections of mouse ovaries (Griffin, J. et al. 2006).

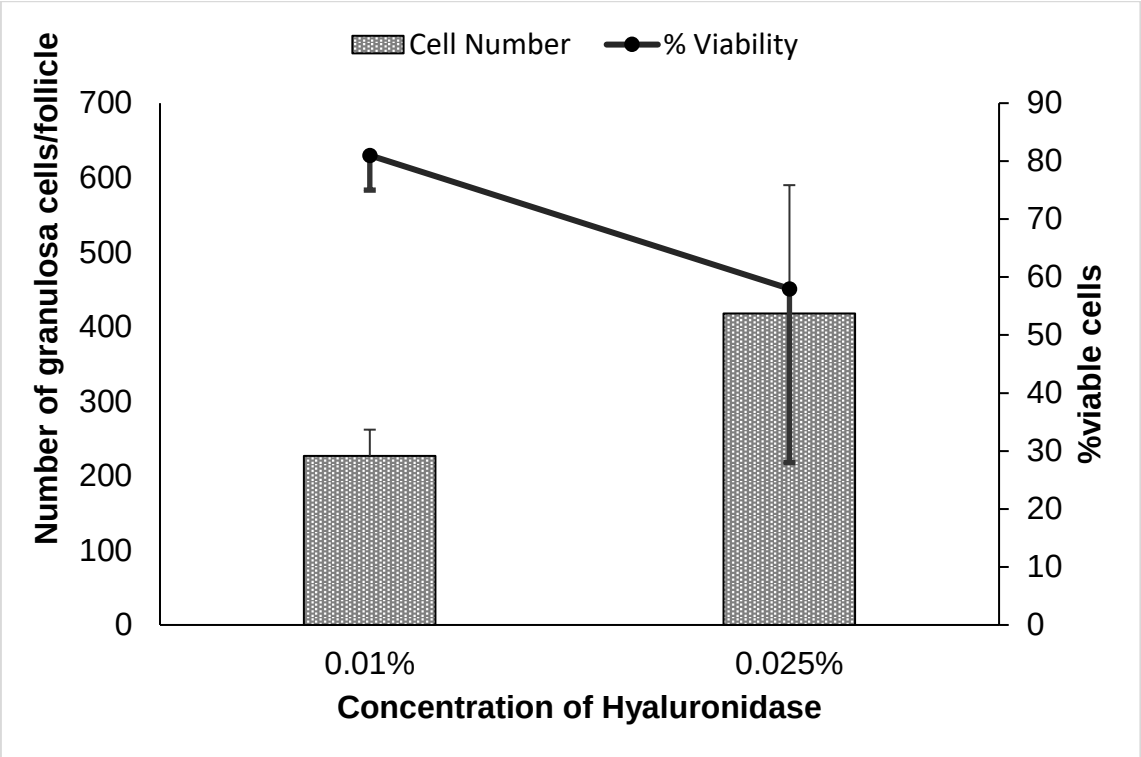


Figure 3. 4: Follicle Disaggregation using Hyaluronidase.

Groups of three secondary follicles were disaggregated with 0.01% or 0.025% hyaluronidase in DMEM/F12 for 2h. The isolated cells were centrifuged at 2040g for 5 minutes then re-suspended in 20 µL DMEM with 10% FBS before viable cell numbers were counted by Trypan Blue Exclusion assay. The experiment was repeated with 3 follicles for each hyaluronidase concentration from one mouse on three separate occasions (n=3) and mean ± SD total cells and % viable cells per secondary follicle shown. Data were analysed using a Students T-test.

3.2.2.5 Follicle Disaggregation Using Combinations of Hyaluronidase and Collagenase IV

Antral follicles that were incubated with 2 mg/mL collagenase IV for 30 minutes then with 0.025% hyaluronidase for 30 minutes yielded the highest total number of GCs per follicle (656±87, $p<0.05$) and the highest number of viable GCs (542±95, $p<0.05$, Figure 3. 5). The percent viability of GCs was 82±5% under these conditions, higher than when follicles were disaggregated with hyaluronidase alone (Figure 3. 4). Follicular disaggregation using only collagenase (without hyaluronidase) resulted in isolation of the lowest number of GC (341±69), and 76% of these were viable.

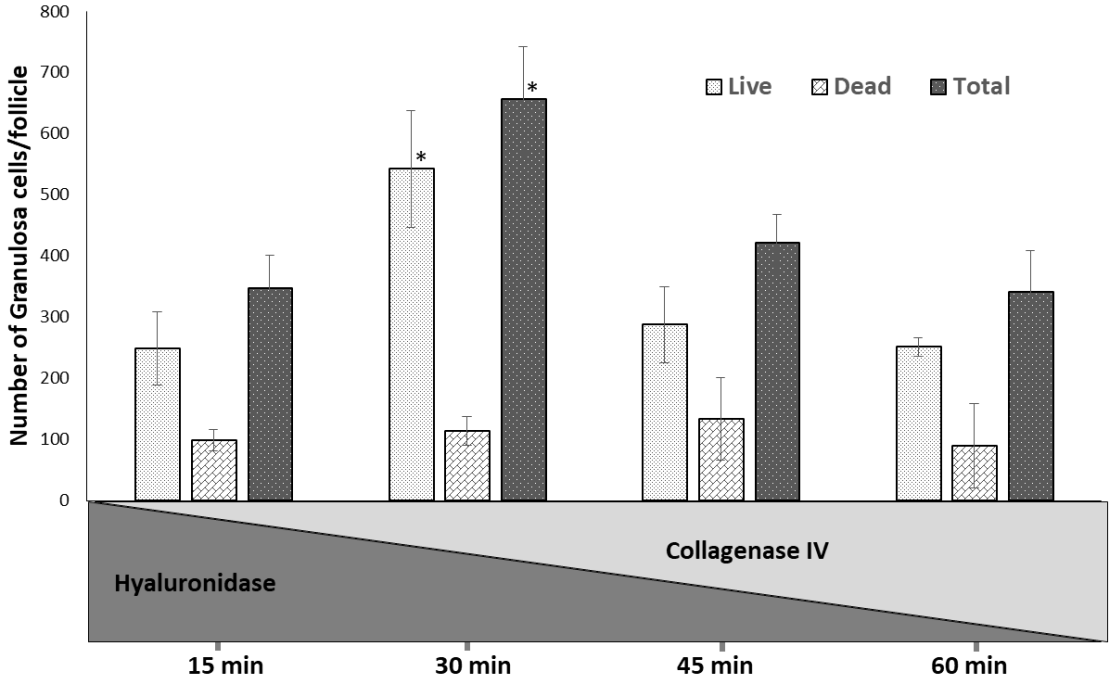


Figure 3. 5: Collagenase IV and Hyaluronidase Disaggregation of Murine Follicles.

*Four groups of three antral follicles (348±26 µm) were incubated in collagenase IV (2 mg/mL) for 15, 30, 45 and 60 minutes (shown on x-axis) before the addition of Hyaluronidase (0.025%) to the 15-45 minutes groups. The isolated GCs were assessed in a Trypan Blue Exclusion Assay. The experiment was repeated using follicles from four mice (n=4). Data analysed by two-way ANOVA with Bonferroni post-test. Mean ± SD GCs per follicle shown. p<0.05 * compared to same cell category.*

3.2.2.6 Primary-derived Granulosa Cell Viability and Hormone Production In Vitro

Primary-derived GC obtained from disaggregated mouse follicles generated a linear correlation (R^2=0.98) (Figure 3. 6 A) between cell density per well and crystal violet absorbance (570nm) after 24h in vitro. Primary-derived GCs did not produce any detectable AMH even at 20,000 viable GCs per well, but there was a density-dependent increase in basal estrogen (E2) production (R^2=0.92) (Figure 3. 6 B).

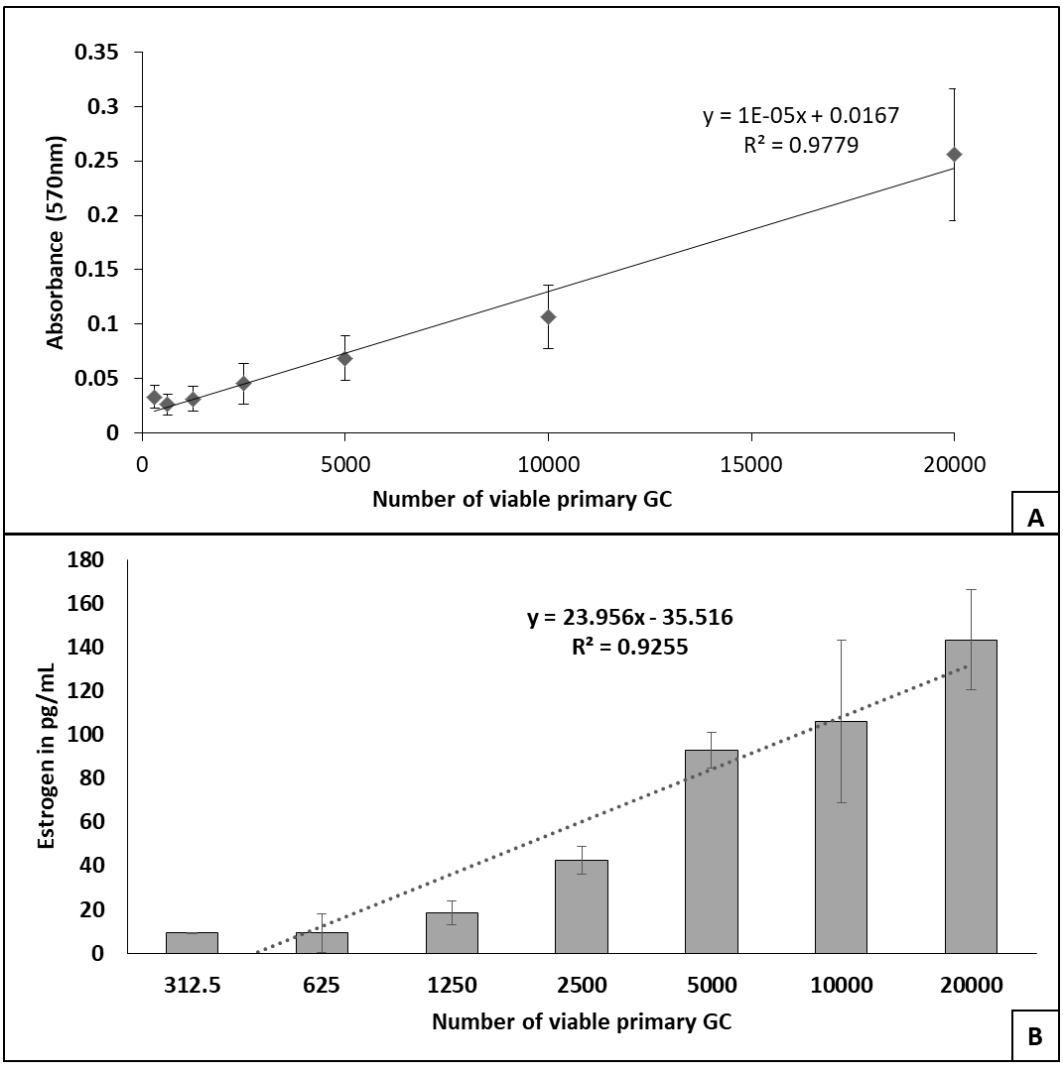


Figure 3. 6: Estrogen produced by primary-derived GCs.

Primary-derived GCs isolated from disaggregated murine follicles were cultured for 24 hours at 37°C with 5% CO_2 at densities of 0 to 20,000 cells per well in a 96-well flat bottom plate on three separate occasions (n=3). After 24h, the numbers of viable cells in each well were determined in a crystal violet assay. Mean±SD (n=3) OD570nm (with correction at 630nm) was plotted against cell densities and fitted to a linear trendline (A). Conditioned media after 24h culture were examined in a competitive estradiol immunoassay and E2 plotted against cell density (B).

3.2.3 Discussion

Here we report a technically simple, highly reliable and inexpensive method for enumerating the number of viable and dead GC in an isolated ovarian follicle. To our knowledge, this is the first report of a straightforward process for disaggregating isolated follicles to produce a suspension of primary-derived GC from naturally cycling unstimulated adult mice, the *in vitro* culture of these GCs in a 96-well plate format, and their production of E2 and AMH.

The standard Trypan Blue protocol requires more than 1x105 cells per mL in the parent cell suspension, or a minimum of 100 cells counted on each grid (Lonza 2012; Louis & Siegel 2011). This limitation directed the development of our protocol. After allowing for dilution of a cell suspension with Trypan Blue dye, a minimum volume of 20uL of cell suspension containing a minimum of 200 cells was required to allow the duplicate assessment of a GC preparation. Primary to mid-secondary follicles only contain 9 – 185 GC (Griffin, Jeanine et al. 2006), therefore follicles were disaggregated in groups of three, to generate sufficient GC for an accurate assessment. The accuracy of this protocol is expected to decrease when applied to small primary follicles, which might only contain 9 GC, and we therefore recommend that the follicle disaggregation group size should be increased accordingly.

Previous reports describing the enumeration of GCs in fixed ovarian sections (Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Pedersen & Peters 1968) were comparable to the numbers of GC counted in this study after follicle disaggregation using 30-minute exposure to collagenase IV followed by 30-minute exposure to hyaluronidase. Under these conditions 82% of the GCs were viable. The loss of 18% viability may be attributed to the enzymes used to disaggregate the follicle (Orimoto et al. 2008), or to the disruption of the tight junctions between GC (Desai et al. 2010), or to mechanical sheer force during centrifugation. However, others have reported that approximately 80% of GCs in intact follicles were viable when assessed by whole follicle Live-Dead staining (Aerts et al. 2008; Campos et al. 2011; Kreeger et al. 2006; Lunardi et al. 2012; Merdassi et al. 2011), which suggests that our follicle collection and disaggregation procedure had no adverse effects on the viability of the GC.

The Trypan Blue Exclusion assay is notorious for low accuracy, poor reproducibility and high variation between counts of the same preparation of cells, thought to be caused by non-specific binding of the TB dye to cellular artefacts (Piccinini et al. 2017). Our optimized TB assay has coefficients of variation similar to, or lower than those reported previously (Allison & Ridolpho 1980;

Eillipore 2018; Piccinini et al. 2017). In the present study, when preparations containing 625 GCs were counted on four separate occasions, the intra-assay CoV of 7.6%, and the inter-assay CoV was 4%. Since the GC were isolated from groups of three follicles, this meant that when there was an average of 209 GCs per follicle (equivalent to a late secondary stage follicle (Griffin, Jeanine et al. 2006), the CoVs for the TB assay were of the same order of magnitude as those reported by the manufacturers of commercially available EIA kits, and the CoVs for cell numbers corresponding to groups of three small primary follicles were within the ranges previously found acceptable for other cell-based assays (Sendak et al. 2002). From this we conclude that we developed an assay protocol with relatively high precision and accuracy.

Although there are numerous reports of primary-derived human GCs being cultured *in vitro* (Chang et al. 2015; Paksy et al. 1997; Sacchi et al. 2017; Tuck et al. 2015; Young, F, Micklem, J & Humpage, A 2008; Young, F et al. 2012; Young, Menadue & Lavranos 2005) and many instances in which GC obtained from the follicles of larger mammals have been cultured (Amsterdam, Knecht & Catt 1981; Bicsak et al. 1986; Hsueh et al. 1984; Ratoosh et al. 1987; Tilly, LaPolt & Hsueh 1992), as far as we know this is the first report describing the *in vitro* culture of GC obtained from the follicles of naturally cycling adult mice in a 96-well format. Cell viability assays that use the high throughput 96-well plate formats benefit from the application of control standard plots, in which known cell densities are related to optical density values (Edwards, Benkendorff & Young 2014; Edwards et al. 2008; Young, FM, Phungtamdet, W & Sanderson, BJS 2005). Previously GCs have been reported as having a doubling rate of 46.4 hours (Nishi et al. 2001a), hence we considered it likely that the murine GC would not proliferate in the first 24h, but would only adhere to the floor of the wells (Paksy et al. 1997). Since this is the first study to examine primary-derived murine GC *in vitro*, we established baseline control conditions, which can be referred to in future studies to examine the effects of gonadotrophins such as follicle stimulating hormone (FSH), and other reproductive parameters, during longer culture periods.

Murine follicles cultured *in vitro* increase E2 production when stimulated by Follicle Stimulating Hormone (FSH) (Adriaens, Cortvrindt & Smitz 2004; Kreeger et al. 2005; Romero & Smitz 2010), and Adriaens, Cortvrindt and Smitz (2004) found that intact follicles containing approximately 20,000 GC produced the equivalent of 125 pg/mL E2 every 48h. We found that 20,000 murine GC in a monolayer *in vitro* produced 143 pg/mL E2 in the first 24h of culture. The difference may be because only 80% of the GC may have been viable in the follicles *in vitro*, whereas it was more likely that all the primary-derived GC were viable in the monolayer. On the other hand, the GC monolayer did not

produce any detectable AMH, even though Kevenaar et al. (2006) reported data suggesting that a follicle containing approximately 100 GC may produce 1.3pM AMH *in vivo*. Since the analytical sensitivity of the AMH kit was 1pM, we were therefore surprised that no AMH was produced by 20,000 GC *in vitro*. There are two key differences between our *in vitro* system and the *in vivo* situation; the *in vitro* system did not include FSH and hence the GC were not stimulated to proliferate (Visser et al. 2006; Visser & Themmen 2005), and follicle disaggregation detaches GC from other GCs and the oocyte which interfered with the intercellular communication essential for the production of AMH (Desai et al. 2010; Gibori 2012; Knight & Glister 2006; Kreeger et al. 2006).

3.2.4 Conclusion

Although the Trypan Blue Exclusion assay is the standard laboratory workhorse for assessing cell viability, it has not previously been applicable to suspensions with low cell densities. This study presents a straightforward and simple method to assess the viability of suspensions with cell densities as low as 7,800 cells per mL (less than 10 cells/µL). There are few reports of follicles isolated from ovarian tissue being disaggregated to produce a single cell suspension (Li et al. 2016; Tajima et al. 2006) but the ability to obtain GC from pools of follicles of defined size cohorts will allow examination of reproductive parameters that are affected by follicular development, such as follicular granulosa cell viability, hormone synthesis or sensitivity to chemotherapeutics. Therefore, the next study aims to quantify viable GCs in murine ovarian follicles of different developmental stages.

3.3 Quantification of Viable GCs in Murine Ovarian Follicles

3.3.1 Introduction

Folliculogenesis is dependent on the proliferation of granulosa cells (GC) surrounding the oocyte, and follicles have therefore been classified according to their diameter and the number of GCs they contain (Table 3.2) (Bagger et al. 1993; Da Silva-Buttkus et al. 2008; Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Pedersen & Peters 1968). Different methods for quantifying the number of GCs in fixed, embedded and serially-sectioned follicles have been reported (Bagger et al. 1993; Griffin, Jeanine et al. 2006; Lundy et al. 1999; van Wezel, Ingrid L & Rodgers, Raymond J 1996). Bagger et al. (1993) used two methods to calculate the total number of GCs in sections of 9 follicles isolated from 2 mice. One of these methods, the nucleator method, depended upon the nucleolus of the oocyte being in focus in the field of view. The focal plane was adjusted to create a 3-dimensional 'column' of 10 µm depth that corresponded to the shape of the follicle. All granulosa cell nuclei in this 3D follicle 'column' were counted, and the distance of the granulosa cell nuclei from the oocyte nucleus determined. These data were entered into an equation which was used to calculate the total number of GCs in the whole follicle. The second fractionater method essentially counted all the GCs in serial sections and was considered the 'true' number of GCs per follicle. Although the nucleator method only assessed about 2% of the GCs in each follicle, the total number calculated for the whole follicle was accurate when compared to the number obtained from the fractionator method. There was a close and linear relationship between the follicle volume and the total number of GCs. The nucleator method was later used to determine the total number of GCs in more than 300 ovine follicles (Lundy et al. 1999). The relationship between the total number of GCs per follicle, and the number of GCs in the section containing the oocyte nucleolus, was described by the regression equation $\log_e X = a+b \log_e Y$ where X is the total number of GCs and Y is either oocyte diameter or GC in a section containing oocyte (Lundy et al. 1999).

Griffin, Jeanine et al. (2006) examined serial sections of formalin-fixed mouse ovaries and human ovarian cortex biopsies to find the section containing the largest cross-section of each follicle. The data from small antral follicles were excluded because the antral fluid appeared to compress the GCs. Large antral follicle data were excluded because many of the follicles were atretic. The remaining data were used to conduct a regression analysis of granulosa cell count as a function of follicle diameter, and this was used to derive equations applicable to the calculation of granulosa cell numbers in follicles of a known diameter (Table 3. 2 and Table 3. 3).

Table 3. 2: Numbers of granulosa cells (GC) in fixed sections of murine ovaries.

Ovaries were fixed, embedded, sectioned and usually stained with haematoxylin and eosin. Follicles were allocated to different size cohorts by using morphological criteria or by measuring the follicle diameters after Griffin et al (2006). GC – GCs. Morphological criteria included; primordial - flattened, squamous pre-granulosa cells with oocytes lacking a zona pellucida, primary - a single uniform layer of cuboidal GCs, secondary follicles - 2, 3 or 4 layers of GC lacking visible spaces between the GC, whereas antral follicles had fluid filled spaces in the mural GC layers.

Reference		**Pedersen and Peters (1968)**	**Lintern-Moore and Moore (1979)**	**Bagger et al. (1993)**	**Griffin, Jeanine et al. (2006)**
Method		Ovarian sections from two mice were reviewed and the sections of each follicle that had the highest number of GC was used. The number of follicles assessed was not provided.	Ovarian sections from two mice were reviewed and the section of each follicle that had the highest number of GC was used.	GC numbers in 9 murine follicles with mean diameter of 154±31 μm were calculated using an algorithm derived from follicular volumes.	GC were counted in fixed mouse ovaries that contained the largest cross-section of each follicle. Data were used to conduct a regression analysis of granulosa cell count as a function of follicle diameter. Granulosa cell numbers calculated using y=0.0078*2 + 0.147x – 0.3863
Number of GCs per follicle	**Primordial 0 – 26 μm**	≤ 20	2-9 n=~4100 follicles		
	Primary 27 – 78 μm	21-60	10 – 100 n = ~728 follicles		9-59
	Early-Mid secondary 79 – 145 μm	61-200	101 -200 n = ~66 follicles		60-185
	Late Secondary-Incipient Antral 146 – 215 μm	201-400	201-400 n=~23 follicles	4656±3308 n=9 follicles	187-392
	Antral > 216 μm	401->600	401->600 n = ~32 follicles		>395

Table 3. 3: Numbers of granulosa cells in human follicles

Lintern-Moore et al. (1974): Histological sections of ovaries from 19 children were used to measure the diameters of follicles, granulosa cell (GC) nuclei and oocytes. GC numbers were counted in the widest cross-section of non-atretic follicles, n=20 follicles of each class.

Tsuji et al. (1985): Ovarian specimens were collected from 90 patients (ages 30-45 yr), healthy oocytes (n=195) were collected from follicles of 3 - 15mm diameter.

Gougeon (1986): Ovaries from 31 women aged 19-49 years were fixed and sectioned, follicle diameter was measured in an average of 3 sections. Density of GCs was measured in five different areas of each follicle. The number of GC in each follicle was calculated by multiplying the volume occupied by the GCs by the cellular density. Class 7 GC per follicle numbers are 47x106 – lower than GC numbers in Class 6.

Gougeon and Chainy (1987): Ovaries (n=9) from 5 women aged 19 – 49y collected after surgery. Number of follicles assessed shown in brackets.

Griffin et al. (2006): Ovaries were collected from 5 women aged 23-45y after oophorectomy for non-neoplastic indications, and ovarian cortex sections used for this study. These tissue samples were therefore biased towards smaller preantral follicles. Ovarian biopsies were fixed, sectioned and stained with haematoxylin and eosin and primordial, primary and secondary follicle and oocyte diameters (n=126) were measured. Application of the polynomial equation derived from assessment of these cortex sections shows average GC numbers for each human follicle size cohort.

	Follicle Size / Class	Lintern-Moore et al., 1974	Tsuji et al., 1985	Gougeon, 1986	Gougeon 1987	Griffin formula $Y=0.0021X^2 + 1.014x - 42.969$
Diameter (µm)	Primordial, flattened GC, Class C	26 - 40		30	35±6	44
	Primary, single layer cuboidal GC, Class C	40 -50		60	46±6	70
	Secondary, Class 1, many GC layers no theca, Class C/D	50-100		120-200	77±11	114
	Incipient Antral, Class 2, Class D1	101-200		200 -400		302
	Early Antral, Class 3, Type D2	200 - 1000		400 -900		889
	Antral, Class 4	1000 - 6000		900 -2000		
	Antral, Class 5		3,000-15,000	2000 -5000		
	Antral, Class 6			5000 -10000		
	Antral, Class 7			10000 -16000		
	Antral, Class 8	<40		16000 -20000		18800
Oocyte Diameter (µm)	Primordial	80				36
	Primary	81-120				42.1
	Secondary	1-14				73
	Early Antral	14-25	114±1.4			76-127
	Antral, Class 8	21-75				
GCs per Follicle	Primordial	8-25			13±6 (408)	6
	Primary	25 - 75			76±27 (153)	50
	Secondary	75 - 300		600-5000	360±185 (30)	100
	Incipient Antral	300 - 500		5000 -15000		455
	Antral, Class 3	$0.0005 – 0.0015x10^6$		$0.015 - 0.75x10^6$		2500
	Antral, Class 4			$0.75 – 3.7x10^6$		
	Antral, Class 5	501-1500		$3.7 – 19x10^6$		
	Antral, Class 6			$19 – 94x10^6$		
	Antral, Class 7			$94 – 47x10^6$		
	Antral, Class 8			$47 - 60x10^6$		760,000

Table 3. 4: Numbers of granulosa cells in murine follicles calculated using follicle and oocyte diameters.

Diameter range in left hand column 'follicle stage' after Griffin et al (2006). References in subscripts.

(1) Pedersen and Peters (1968): 2 mice were the source of ovarian sections. GC were counted in the section of the follicle with the highest number of GC. Number of follicles assessed was not provided

(2) Cortvrindt et al. (1996): 30-40 follicles from a 14-day old mouse. Measured the diameters of denuded oocytes

(3) Liu et al. (2000): New born mice were the source of ovaries that were transplanted into ovariectomised adults. 14 days later, 248 preantral follicles (average diameter 114±14 µm) were harvested from the ovaries

(4) Smitz and Cortvrindt (2002): 14-day old mice were the source of ovaries from which secondary follicles were isolated. The diameters of oocytes and follicles were measured, but the numbers of granulosa cell layers were not provided. For this calculation assume 2 layers of GC

(5) Segino et al. (2005): 3-week-old mice (n=5) were the source of ovaries which were cryopreserved before harvest of 308 preantral follicles (100-130 µm) with 2 to 3 layers of GCs

(6) Griffin et al. (2006): Follicle and oocyte diameters were measured in 104 ovarian sections from 10 mice

(7) Salmon et al. (2004): cumulus oocyte complexes (COCs) from preantral follicles from prepubertal mice (n=3) were cultured for 24 hour and oocyte diameters measured. Data less reliable than other sources, because measurements were obtained from COC's not follicles and after 24-hour in vitro culture. In the absence of other reports, the diameters of prepubertal oocytes after in vitro culture were used for the late secondary calculation, although the oocyte diameter is probably too small, therefore, data from this paper were noted in the Table for comparison, but not used to calculate average values. The diameter of a single granulosa cell (D3; Figure 3. 7) was estimated by subtracting oocyte diameter from follicle diameter and dividing by the number of granulosa cell layers.

The diameters of GCs at different stages of follicular development were used to calculate individual and total granulosa cell volumes. In the case of secondary and antral follicles, the contribution of theca cells was excluded by adding two, three, four or five times the diameter of a single granulosa cell to the diameter of the oocyte respectively. Volume of whole follicle (V1) and oocyte (V2) were calculated by $4/3\ \pi r3$, total volume of GCs surrounding the oocyte =V1-V2. Total number of GCs per follicle were determined by N = (V1-V2)/V3.

Follicle Size (diameter)	Diameter of follicle (µm)	Average diameter of follicle (D1) (µm)	Diameter of oocyte (µm)	Average diameter of oocyte (D2) (µm)	Number of GC layers	Diameter of a GC (D3), (µm)	Vol. of Oocyte + GC (V1) µm³	Vol. of Oocyte (V2) µm³	Vol. of GC (V1-V2) µm³	GC cuboid and close-packed, all volume accounted for 		GC spherical & loose-packed, volume between GC not accounted for 	
										Vol. of a single GC (V3) µm³	Number of GC/follicle N= (V1-V2)/V3	Vol. of a single GC (V3) µm³	Number of GC/follicle N= (V1-V2)/V3
Primary (27 - 78µm)	52.1 (6) 70 (4)	61.05	28.8 (6)	28.8	1	16.13	47,466	1,2505	34,961	4,193	8	2,195	16
Early-Mid Secondary (79 – 145 µm)	92.5(3)	103.83	55(4) 53.8 (6)	54.6	2	12.31	260,219	85,211	175,008	1,864	94	976	179
	104 (6)		41 ± 0.9 (7)										
	115 (4)		55 ±4 (2)										
	114±14 (3)	117.2	63 ±5 (2)	63	3	9.03	382,905	130,900	252,005	737	342	386	653
	120 (5)												
Late Secondary – Incipient Antral (146 – 215 µm)	162(3)	162	56 ± 1.25 (7)	56	4	13.25	677,948	91,935	586,013	2,326	252	1,218	481
Antral (>216 µm)	187 (7)	203.5	67.4 (6)	68.7	5	13.48	1,319,748	169,741	1,150,007	2,449	469	1,282	897
	220 (4)		70 (1)										

Human and murine follicular development is similar until the late secondary stage when the growth of human follicles accelerates to produce follicles up to 20 mm in diameter (Gougeon 1986; Griffin, Jeanine et al. 2006; Telfer, Evelyn E & Zelinski, Mary B 2013). The formation of fluid-filled spaces in the granulosa cell layers confounds estimations of the numbers, and this effect increases with the increased size and antral volume of human follicles. Early secondary human follicles with a diameter range of 50 to 100 μm (Table 3. 3) have been reported as containing 65 - 300 (Lintern-Moore et al. 1974) or 600–5000 (Gougeon 1986) GC per follicle, whereas 30 human follicles of diameter 77±11 μm were found to contain 360±185 GC (Gougeon & Chainy 1987), supporting the lower estimates by Lintern-Moore et al. (1974). The application of the polynomial equation derived by Griffin, Jeanine et al. (2006) to a human follicle with diameter of 77 μm however yielded a value of only 48 GC per follicle. Gougeon and Chainy (1987) reviewed 49 human ovaries, whereas Griffin, Jeanine et al. (2006) reviewed only 5 cortex biopsies, therefore the human regression analysis and polynomial equation proposed by Griffin, Jeanine et al. (2006) may not be broadly applicable to human follicles. Previously Bagger et al. (1993) observed that although there are differences between species with regards to follicle diameter and granulosa cell counts, the highest source of variation came from differences between follicles with similar diameters. Inter-follicle variation may be attributed to polarisation caused by uneven distribution of GCs around the oocyte. When bovine ovaries were sectioned in three planes it was found that 3 sections of 1 follicle, each with the oocyte nucleus in the focal plane, could give quite different granulosa cell numbers (van Wezel, Ingrid L & Rodgers, Raymond J 1996). We reviewed published studies that reported the diameters of follicles and oocytes (Table 3.4), then used these two-dimensional (2D) measurements to calculate the volume of follicle, the volume within each follicle occupied by GCs, and the volume of a single granulosa cell (Figure 3.7). We used the latter two values to calculate the number of GCs per follicle. To do this we assumed that the true number of GCs per follicle lay within a range delineated by the value calculated by allocating a cuboidal close-packing shape to each granulosa cell, and the GC per follicle value calculated by allocating a spherical, loose-packing shape to each granulosa cell (Table 3. 4).

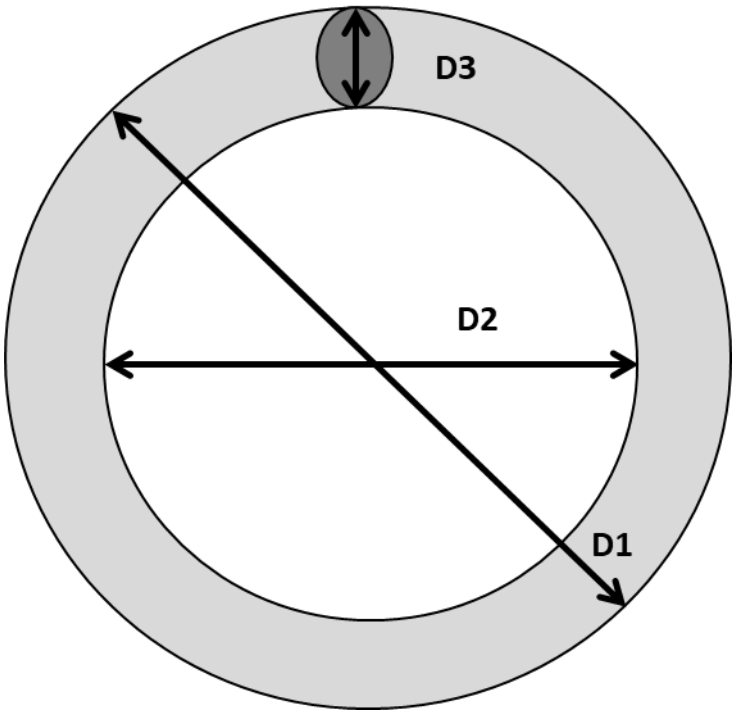


Figure 3. 7: Representative primordial/primary follicle, D1: diameter of follicle, D2: diameter of oocyte and D3= (D1-D2)/2: diameter of a single flattened/cuboidal granulosa cell.

Our review of published reports identified three different ways to determine follicular granulosa cell numbers; direct counting in fixed histological sections (Table 3. 2 and Table 3. 3), application of a formula derived from the assessment of histological sections (Griffin, Jeanine et al. 2006), and the conversion of diameters to volumes (Table 3. 4). In general, the three approaches generated similar values (Table 3. 5); a late secondary-incipient antral murine follicle with diameter 162μm was estimated to contain 201 to 400, or 187 to 392, or 252 to 481 GC by these three methods respectively. The latter method gave the broadest and least accurate values, since they were derived from studies that were not designed to quantify granulosa cell numbers, but their overlap with the other two methods allowed us to select consensus values with a degree of confidence. Additionally, these values were of a similar order of magnitude as GC per follicle estimations from other species. Human follicles of approximately 150μm in diameter contained 300 - 500 GCs (Lintern-Moore et al. 1974), and Lundy et al. (1999) found that smaller 128 μm ovine follicles contained a total of 637 GCs.

Table 3. 5: Consensus values for numbers of murine granulosa cells per follicle.

Left hand column shows range of diameters for different size cohorts of follicles. a) Granulosa cell numbers in fixed and sectioned follicles b) Granulosa cell numbers calculated using $y=0.0078x^2 + 0.147x – 0.3863$ (Griffin et al. 2006). Granulosa cell numbers estimated using follicle and oocyte diameters, and the number of granulosa cell layers d) GCs in disaggregated follicles estimated by Trypan Blue assay e) Number of DAPI-stained GCs per follicle f) Number of Live-Dead stained GCs per follicle and g) number of dead stained GCs per follicle.

	a) Counted in fixed histological sections	b) $y=0.0078x^2 + 0.147x – 0.3863$	c) Estimated using follicle and oocyte diameters	d) Disaggregated follicles, Trypan Blue Estimation	e) Intact DAPI stained follicles	f) Intact Live-Dead Stained follicles	g) Intact Dead Stained follicles
Primary (27-78 µm) Single layer of cuboidal GC	10-100	9-59	8-16	94±43	47±17	52±24	69±31
Early-Mid Secondary (79-145 µm) 2 – 3 layers of GC. Initial formation of theca cell layers	101-200	60-185	94-654	287±135	183±94	131±58	187±81
Late Secondary-Incipient Antral (146-215 µm) 4 – 5 layers of GCs, with some fluid filled spaces. Mature, vascularised theca internal and externa layers	201-400	187-392	252-481	488±125	333±154	253±111	350±64
Antral (> 216 µm)	401-> 600	>395	469-897	636±168	350±116	292±87	466±241

More recent methods for determining granulosa cell numbers per follicle include staining with DAPI (4',6-diamidino-2-phenylindole), a cell permeable fluorescent probe that binds to double stranded DNA of both live and dead cells (Nuttinck et al. 1993; Porter & Feig 1980; Tarnowski, Spinale & Nicholson 1991), and can be used to enumerate GCs in a follicle. Since DAPI may also bind to mitochondrial DNA (Seifer, DeJesus & Hubbard 2002; Van Blerkom & Runner 1984) though, it should not be assumed that discrete DAPI-stained areas correspond to the nucleus of a single granulosa cell. Another stain that could be used is a Live-Dead stain consisting of Calcein AM and Ethidium Homodimer 1 (EthD 1). Calcein AM is a cytoplasmic dye that enters live cells and is metabolised to emit a green fluorescent signal, whereas Ethidium Homodimer 1 (EthD-1) is excluded by intact cell membranes, and can only enter the nuclei of dying cells with damaged membranes (Aerts et al. 2008; Decherchi, Cochard & Gauthier 1997). In this study, we aimed to quantify GCs in whole isolated murine follicles, and to compare these values with the number of GCs previously reported in fixed, embedded and sectioned follicles.

3.3.2 Materials and Methods

Reagents were obtained from Sigma Aldrich unless otherwise stated.

3.3.2.1 Mouse Ovary Collection

The Flinders Animal Welfare Committee approved our use of cull animals. Swiss mice (n=21) aged 15—25 weeks were used in this study. Mice were killed by cervical dislocation and the ovaries were isolated by dissection. The ovaries and attached connective tissues were placed in Hanks Balanced Salt Solution (HBSS) and transported to the laboratory at 37°C. Whole ovaries were dissected free of oviducts and connective tissue, blotted dry, cut in half and weighed.

3.3.2.2 Follicle Isolation from Ovarian Tissue

3.3.2.2.1 Ovarian Enzymatic Disaggregation

Ovaries (that had been cut in half) were disaggregated using 0.5 mL of 2 mg/mL collagenase IV (295 units/mg, Worthington, New Jersey, USA) in DMEM for 30 minutes at 37°C, then mechanically disaggregated with 29-gauge needles for 5 minutes at room temperature. All released follicles were collected and transferred to a 96-well plate containing DMEM with 10% foetal bovine serum (FBS, Bovogen Biologicals Pty Ltd, VIC, Australia) and 1% ITS (insulin 1000 mg/L, transferrin 550 mg/L, selenium 0.67 mg/L, Gibco, New York, USA).

3.3.2.2.2 Ovarian Non-Enzymatic Disaggregation

Six ovaries from 3 mice were mechanically disaggregated and follicles were dissected free using 29-gauge needles. Follicles were collected into a 96-well plate as before.

3.3.2.3 Initial Follicle Size Classification

Diameters of freshly isolated follicles were measured using dissection microscope with a scale graticule in the eye piece. Three diameters were measured for each follicle and the average calculated. The diameters were used to classify follicles as primordial, primary, early secondary, late secondary or antral according to their size (Table 3. 5) and morphological characteristics (Table 3.6). The diameters of stained follicles were also measured using Image J software.

3.3.2.4 Follicle Disaggregation

To produce a single cell suspension that could be assessed in a Trypan Blue assay, primary, early to mid-secondary, late secondary to incipient antral and antral follicles from each mouse were disaggregated in groups of 3 follicles per well, in 96 well plates with 10 µL collagenase IV (12mg/mL) in 50 µL of media (working concentration was 2mg/mL) at 37°C for 30 minutes before 50 µL 0.05% hyaluronidase for 30 minutes. All the contents of each well (i.e. the single cell suspension of GCs from 3 follicles) were transferred to 1.5 mL centrifuge tubes and the volume was made up to 500 µL with phosphate-buffered saline (PBS). The GCs were centrifuged at 2040 g for 5 minutes, supernatants were discarded, and the cells were re-suspended in 20 µL of DMEM media. The follicles from one mouse were examined in groups of 3 follicles, so that from each mouse a total of 12 follicles of 4 different size cohorts were assessed. This experiment was repeated using follicles harvested from enzymatically disaggregated ovaries from three different mice (n=4), and follicles harvested from mechanically disaggregated ovaries from three different mice (n=3) as described previously.

3.3.2.5 Trypan Blue (TB) Exclusion Assay

The GCs harvested from each group of 3 disaggregated follicles were resuspended in 20 µL DMEM, and 20 µL Trypan Blue solution were added to each of these cell suspensions. Two aliquots, each of 10 µL, were assessed by two authors who counted the live and dead cells in 9 large squares of the haemocytometer. The mean±SD numbers of viable or dead GCs were reported for each follicle.

3.3.2.6 DAPI Staining

Primary, secondary and antral follicles were collected from five mice (n=5). Each group of follicles was washed with PBS then stained with 150 µL DAPI (0.04 mg/mL) for 30 minutes at room temperature in the dark. The DAPI solution was discarded, the follicles washed with PBS, then fixed in 100 µL of 4% paraformaldehyde (PFA, BDH Laboratory Supplies, Dorset, England) for 40 minutes in the dark at room temperature. The PFA was discarded and the follicles washed twice with PBS. The DAPI-stained whole follicles were placed onto a poly-L-lysine coated glass microscope slide and the excess PBS removed before adding 5 µL buffered glycerol and a cover slip.

3.3.2.7 Live-Dead Staining

Primary, secondary and antral follicles were collected after enzymatic disaggregation (n=3) of murine ovaries. Each size cohort of follicles was washed with PBS then stained with 150 µL Live-Dead combined stain (10 µM Calcein AM and 20 µM EthD-1, Invitrogen, Paisley, UK) at 37°C for 45 minutes. The follicles were washed with PBS before fixation in 150 µL of 4% PFA for 40 minutes in the dark at room temperature. The Live-Dead stained whole follicles were placed onto a poly-L-lysine coated glass microscope slide with 5 µL buffered glycerol and a cover slip.

3.3.2.8 Ethidium Homodimer-1 'Dead' Stained Follicles

Primary, secondary and antral follicles were collected from mice (n=7). Each size cohort of follicles was incubated with 150 µL 70% ethanol for 30 minutes at room temperature to kill the GCs. Ethanol was discarded and 150 µL combined Live-Dead stain (10 µM Calcein AM and 20 µM EthD-1) stain was added as described previously.

3.3.2.9 Two-dimensional (2D) Fluorescence Microscopy

Images of DAPI and Live-Dead stained whole follicles were captured using a fluorescence microscope (Bright field BX50, Olympus, Japan) with a LED light source (Cool LED) at light intensity from 50% to 90%.

Images were taken at 200 milli-seconds exposure time at either 10x (for larger follicles) or 20x magnification. Images were captured using Micro-Manager (v1.4.13, USA) image capture software. At 10x and 20x magnifications the pixel areas in the images were 0.667 µm and 0.333 µm respectively. These were used to calibrate the scale bars that were embedded in the image and later used to measure diameters of the follicles.

3.3.2.10 Image Analysis

3.3.2.10.1 Determination of Follicular Diameter

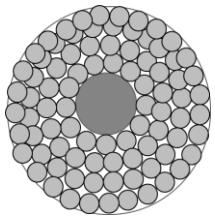
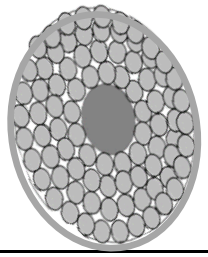
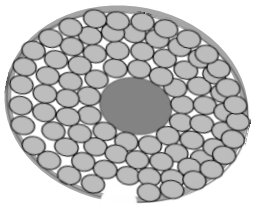
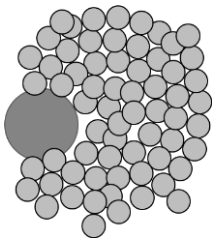
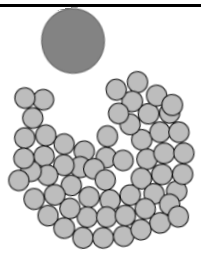
Fluorescence microphotographs of stained follicles were analysed using Image J (1.49v, NIH, USA). Diameters of each follicle were measured by drawing a straight line across the follicle three times then calculating the average. The diameter was used to assign the follicle to a size cohort (Table 3.5). Follicles that were initially classified as primary, secondary, and antral using an eye micrometre and dissection microscope were found to be of the same size cohort when the diameters were measured again using the stained images.

3.3.2.10.2 Morphological Integrity

The morphological integrity of each follicle was scored according to Young et al. (2017) and Dolmans et al. (2006) (Table 3.6). Follicles classified as having intact high quality M1 and M2 grade morphology were used to estimate nuclear and granulosa cell area.

Table 3. 6: Follicle morphology scoring system.

Enzymatically isolated mouse ovarian follicles were classified after Young et al. (2017) (2017), loss of GCs, shape and integrity of basal lamina of follicles were used to classify follicular morphology as shown in the table.

Diagram	**Morphological Classification**	
	M1	No loss of granulosa cells, complete granulosa cell layer, regular and circular shape suggesting a complete and intact basal lamina
	M2	Complete granulosa cell layer but irregular shape, and possibly disrupted basal lamina without obvious loss of granulosa cells
	M3a	90% smooth regular outline with irregular shape and loss of < 5% of granulosa cells
	M3b	90% smooth regular outline with irregular shape and loss of 5-10% of granulosa cells
	M4a	Ragged irregular outline, oocyte visible or its presence apparent. Oocyte can be at surface of follicle. >10% loss of granulosa cells
	M4b	Ragged irregular outline, oocyte separated from granulosa cells, or absent. >10% loss of granulosa cells

3.3.2.10.3 Area of Granulosa Cell Nucleus and Cytoplasm

Six Live-Dead stained M1 or M2 follicles from each of the primary, secondary and antral follicle cohorts from three mice were used to measure the area of clearly defined EthD-1 stained GC nuclei, or Calcein AM stained cytoplasm. Ten nuclear or cytoplasmic areas in each follicle were measured using a circular tool provided in Image J. The green Calcein AM-stained areas included the nuclear area. The average nuclear or cytoplasmic area was determined for each follicle from the ten replicate cell determinations, and the mean±SD value of all follicles in each size cohort shown.

3.3.2.10.4 Objective, Semi-automated Quantification of Granulosa Cell Numbers Using Image Analysis

In each photomicrograph image, there was stain-specific fluorescence and non-specific background fluorescence that was quantified using an Image J generated 'rolling ball' algorithm, which allowed background fluorescence to be subtracted from the whole image. The grey scale image was converted to black and white and adjusted so that every stained area was black. When two or more stained areas overlapped, they were subdivided by inserting a 1-pixel line using an Image J plugin programme 'watershed'. The area of a granulosa cell was set as being larger than 20 μm^2 because previous measurement of Calcein AM stained areas had determined this as being the minimum cytoplasmic area of a GC. Similarly, the DAPI-stained nuclei were defined as being larger than 5 μm^2 because previous measurement of Ethd-1 stained nuclei had determined this as being the minimum area of GC nuclei. The upper limits for cytoplasm or nuclear areas were set to infinity to include any overlapping stained areas that may not have been separated by the 'watershed' programme. It was anticipated that this might lead to an underestimation of granulosa cell numbers, since two or more overlapping cytoplasmic or nuclear areas might be counted as one. A 'Particle analyser' function in Image J was used to count the number of separate stained areas that were larger than 5 or 20 μm^2. The counted 'particles' were outlined to check against the original image, and the estimated numbers were recorded for each follicle.

3.3.2.10.5 Coefficient of variation of DAPI image analysis

10 DAPI-stained follicles from each cohort of primary, secondary and antral follicles were deidentified before being assessed by three co-authors who were supplied with the standard operating procedure for the image analysis of stained follicles using Image J (described above). The co-authors were asked to determine follicle diameters, morphological integrity and grade, and the

number of GCs in each follicle. Coefficient of variation was calculated by stdev/mean and converted into percentage.

3.3.3 Statistical Analysis

Cytoplasmic and nuclear areas of GCs, number of DAPI, Live-Dead and positive control EthD-1 stained GCs per follicle, and number of GCs in disaggregated follicles estimated by Trypan Blue assay, were analysed by 1-way ANOVA with Tukey's Multiple Comparison Test. All tests were conducted using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA) where significance was assigned at $p<0.05$.

Regression analysis of log number of GCs per follicle as a function of the diameter of follicle indicated a linear best fit line for each estimation (Trypan Blue, DAPI, Live-Dead and positive Dead).

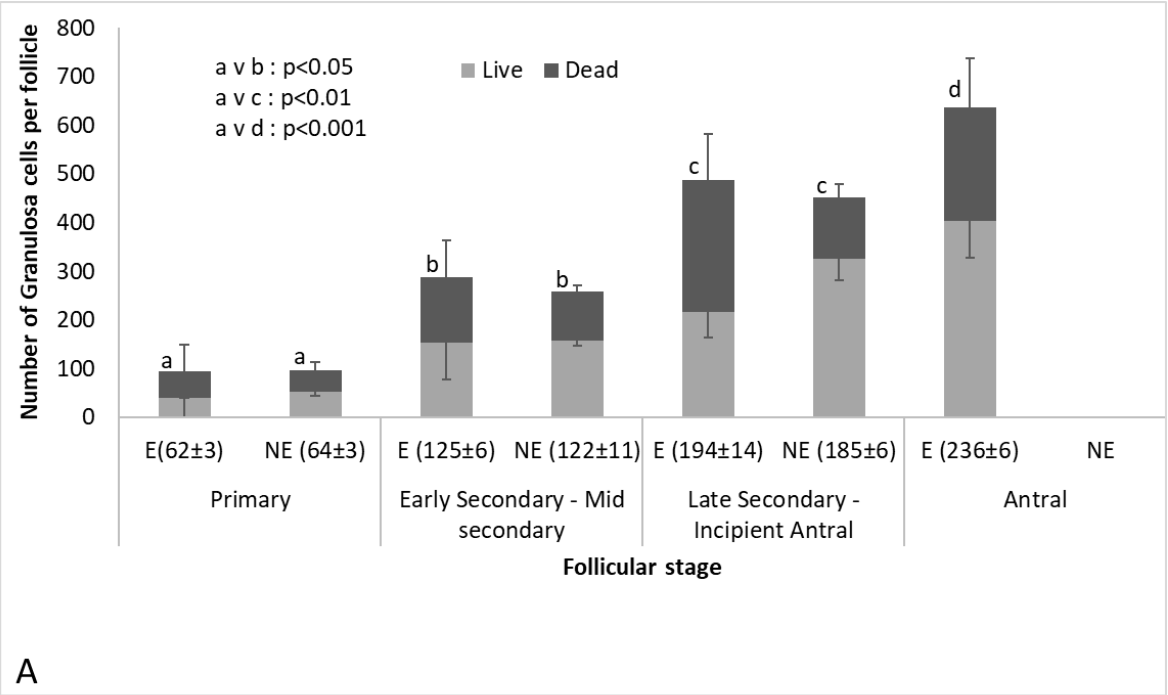
3.3.4 Results

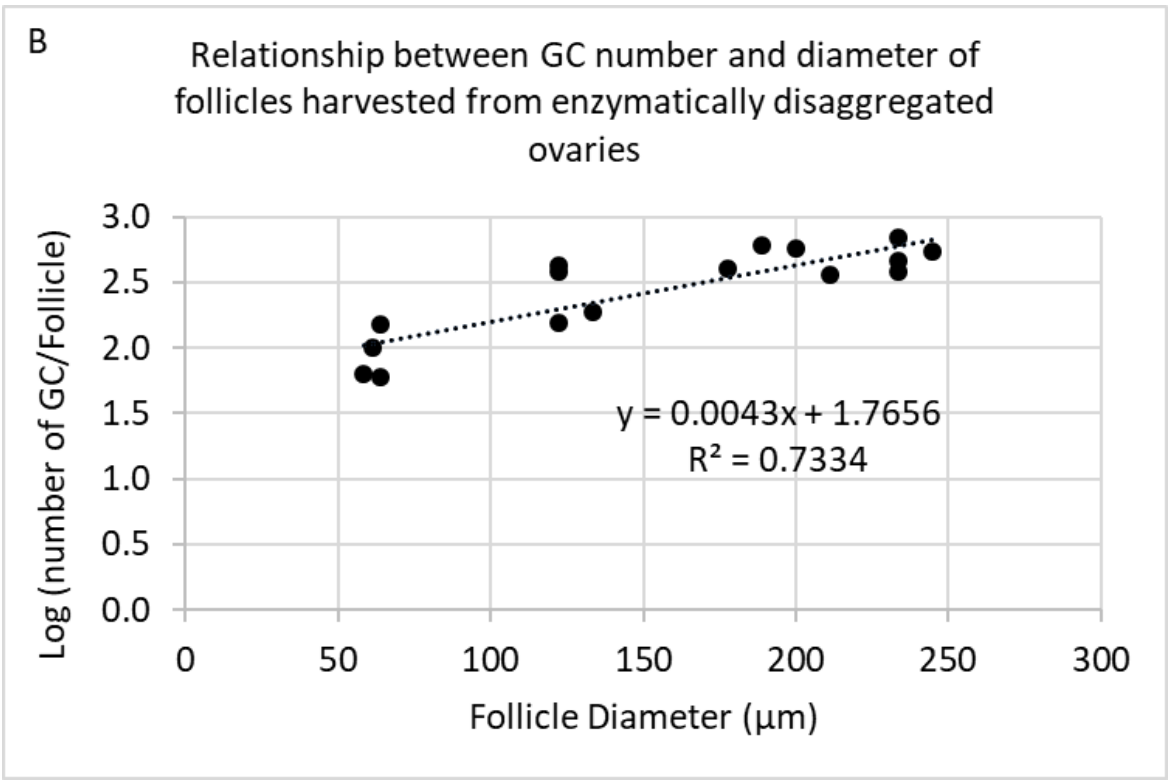
3.3.4.1 Number of GCs in Disaggregated Follicles

When follicles were disaggregated, and the GC counted using a Trypan Blue exclusion assay, granulosa cell numbers were proportional to the size of the follicle (Figure 3. 8) and the proportion of viable GCs per follicle also increased as the follicles grew larger. The numbers of GCs in primary follicles were significantly lower than in late secondary and antral follicles. There were 259 to 488 GCs per secondary follicle.

Although all the follicles had good morphology and were graded as M1 or M2 quality (Young et al. 2017) before disaggregation, approximately half of the GCs in the follicles were not viable. The proportions of viable GCs were 40±11% (primary), 54±16% (early secondary), 45±7% (late secondary) and 64±7% (antral) in the follicles harvested from enzymatically-disaggregated ovaries and 54±5% (primary), 61±3% (early secondary) and 72±7% (late secondary) follicles harvested from mechanically-disaggregated ovaries. However, there were no statistically significant differences in the total numbers of GCs, nor the numbers of viable GCs, in follicles harvested from enzymatic or mechanically-disaggregated ovaries. The total numbers of GC per disaggregated follicle were higher than the highest consensus values (Table 3. 5) for secondary and antral follicles estimated using histological sectioning (method a) and Griffin's equation (method b), and the high end of the ranges estimated using follicle and oocyte diameters (method c). When all the data from enzyme and mechanically-disaggregated ovaries were pooled (Figure 3. 8 B+C), the equation describing the

relationship between follicle diameter and log GC per follicle was y = 0.0045x + 1.76 where y is the log of GC per follicle and x is the diameter of follicle.





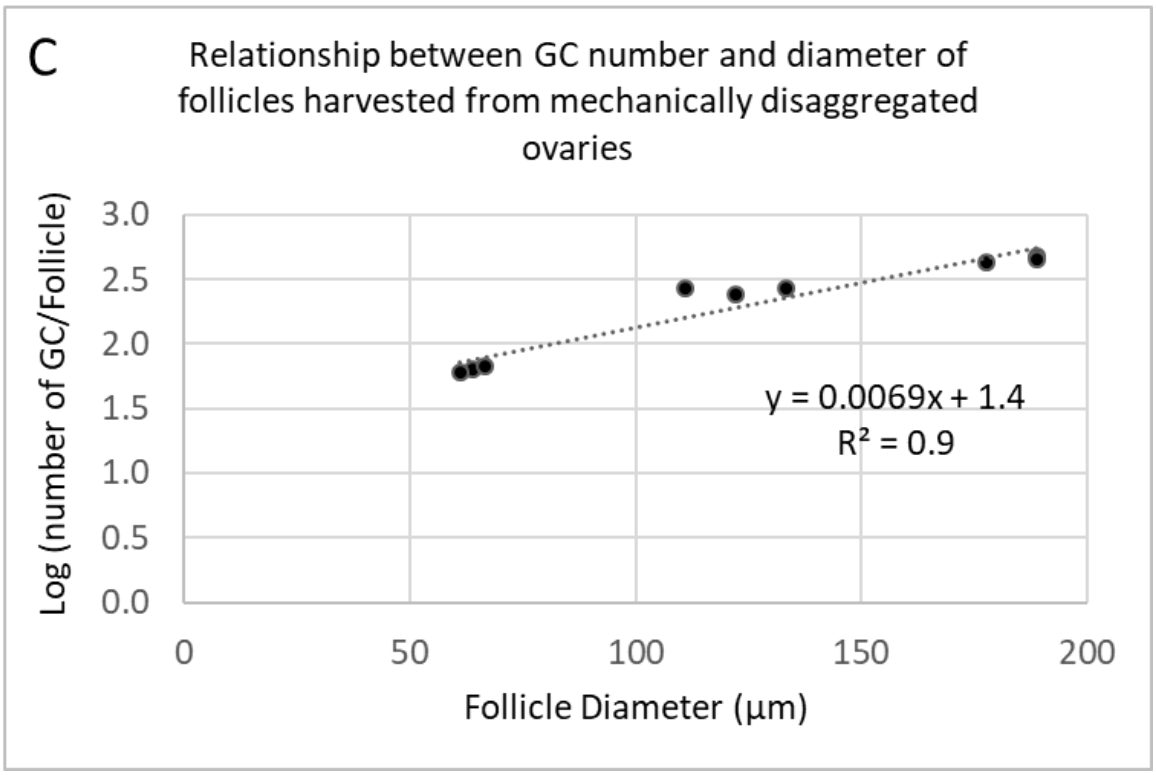


Figure 3. 8: Number of GCs in disaggregated follicles.

Follicles were collected from either enzymatically (E) (n=3 mice) or non-enzymatic mechanically (NE) (n=3 mice) disaggregated ovaries. Mechanical disaggregation of the ovary did not yield any antral follicles. Mean±SD follicle diameters (µm) shown in brackets on x-axis. Follicles allocated to size cohort after Griffin et al (2006). Isolated follicles were disaggregated with collagenase IV and hyaluronidase in groups of 3 follicles from each mouse. The GCs collected from groups of 3 disaggregated follicles were examined in a Trypan Blue Exclusion assay to determine the numbers of viable and dead cells. Data were analysed by 1-way ANOVA and Tukey's Multiple Comparison Test. (A) Mean±SD shown, (B) and (C) Linear Regression analysis of log number of GC/follicle against diameter of follicles from enzymatically and non-enzymatically disaggregated ovaries.

3.3.4.2 Cytoplasmic and Nuclei Areas in Live-Dead Stained GC

There were no significant differences in the areas of individual granulosa cell nuclei or cytoplasmic compartments in primary, secondary or antral follicles (Figure 3. 9). The smallest areas of granulosa cell cytoplasm that were measured were 19.6, 20.9 and 28.5 μm^2, and the smallest nuclei areas were 5.3, 8.9 and 5.3 μm^2 in primary, secondary and antral follicles, respectively. From these it was decided to define the minimum area of stained cytoplasm or nuclei for image analysis as being 20 μm^2 or 5 μm^2, respectively.

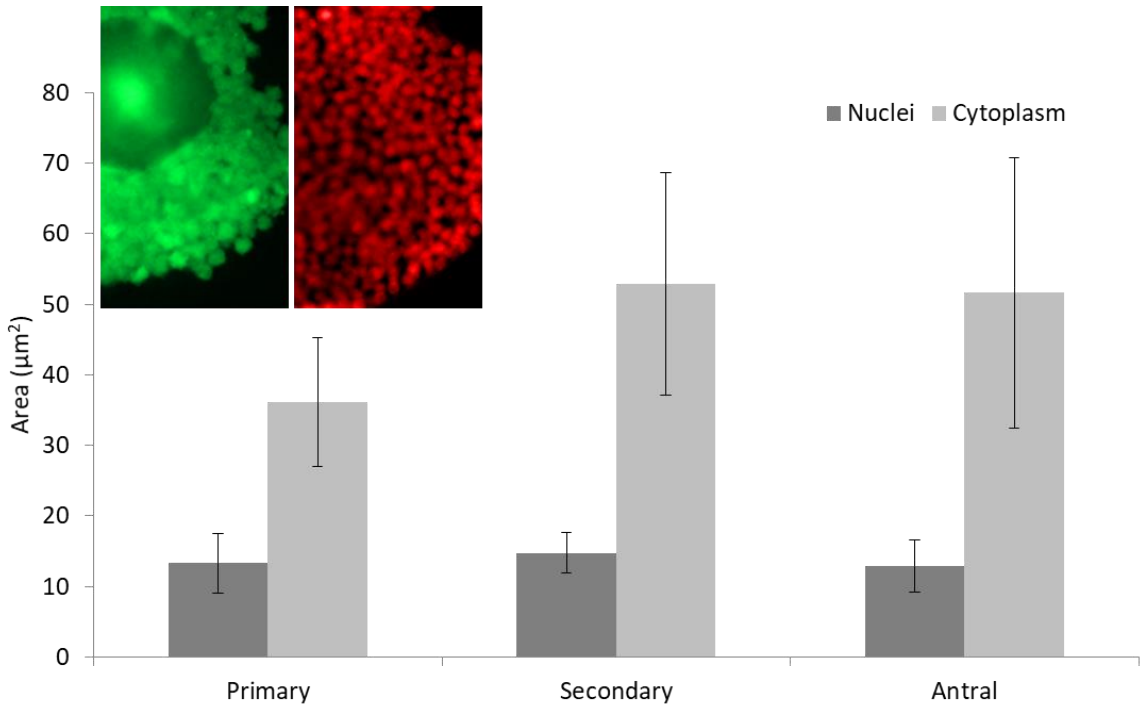


Figure 3. 9: Area of granulosa cell nuclei and cytoplasm in intact follicles.

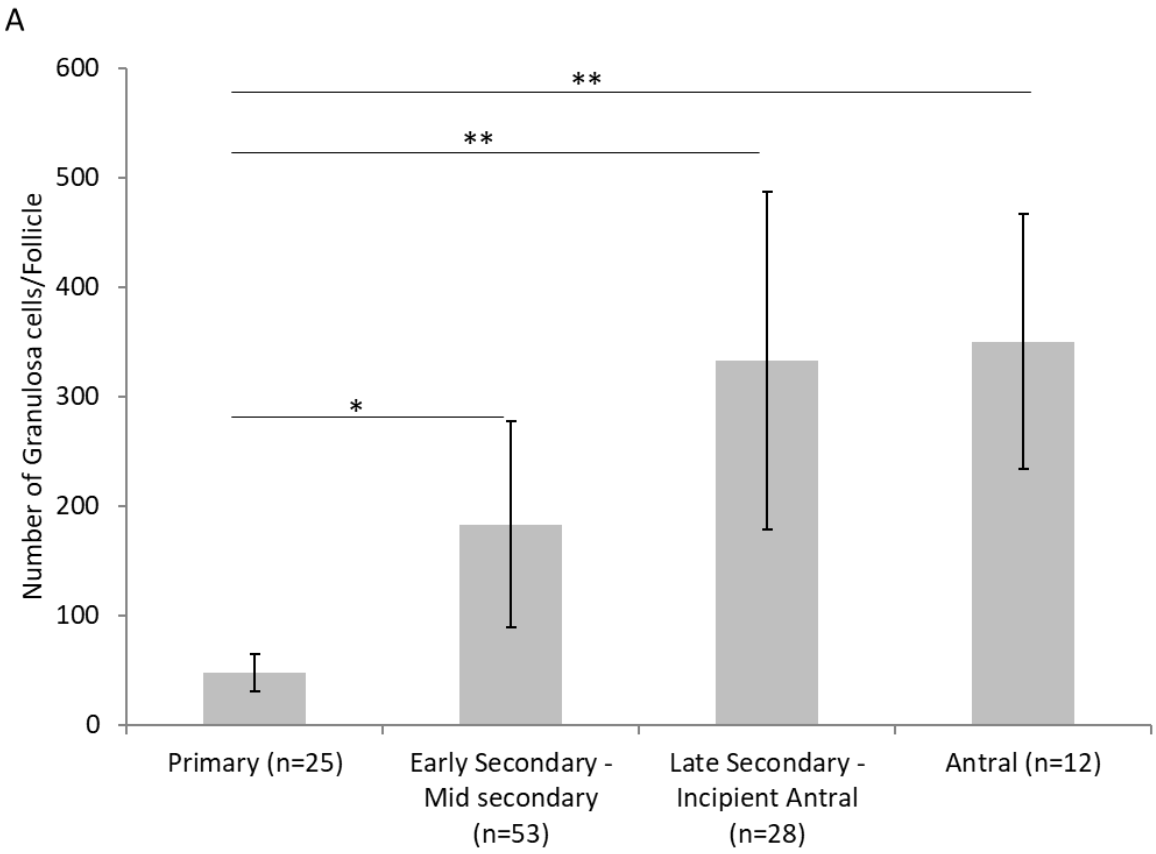
Follicles isolated from three mice were stained with Calcein AM (Live) and Ethidium Homodimer-1 (Dead stain). Images of ten clearly focused GCs from each of six morphologically intact follicles from each of the primary (72±4 µm), secondary (139±5 µm) and antral (240±23 µm) size cohorts were measured using a circular tool in ImageJ. Mean SD of n=60 nuclei and granulosa cell cytoplasmic areas from each size cohort presented. Data were analysed by 1-way ANOVA and Tukey's Multiple Comparison Test. Insert shows a fluorescence photomicrograph of red EthD-1 stained granulosa cell nuclei in a secondary follicle with diameter 176. 22 µm.

3.3.4.3 Number of DAPI-Stained GCs Per Follicle

The numbers of GCs in primary follicles were significantly lower than in early ($p<0.05$) and late ($p<0.01$) secondary follicles and antral ($p<0.01$) follicles (Figure 3. 10A). Image analysis of DAPI-stained follicles yielded granulosa cell numbers that increased as the size of the follicle increased from primary to late secondary ($R^2=0.61$, Figure 3. 10 B).

The total numbers of GC per primary, secondary and antral follicle were within the range of consensus values (Table 3. 5) estimated by histological sectioning (a) and Griffin's equation (b), but DAPI stained GC in canted primary follicles were higher than GC numbers estimated using follicle and oocyte diameters (c).

A total of 30 deidentified DAPI-stained primary, secondary and antral follicles were given to three trained examiners to measure the diameters, assess morphology, and estimate the number of DAPI stained areas per follicle. In 70% of cases different examiners allocated the same follicle morphological grade. The coefficients of variation of DAPI-positive GC number estimation ranged from 1.4 to 12.4 % and follicle diameter measurement ranged from 1.6 to 12.1% (Table 3. 7).



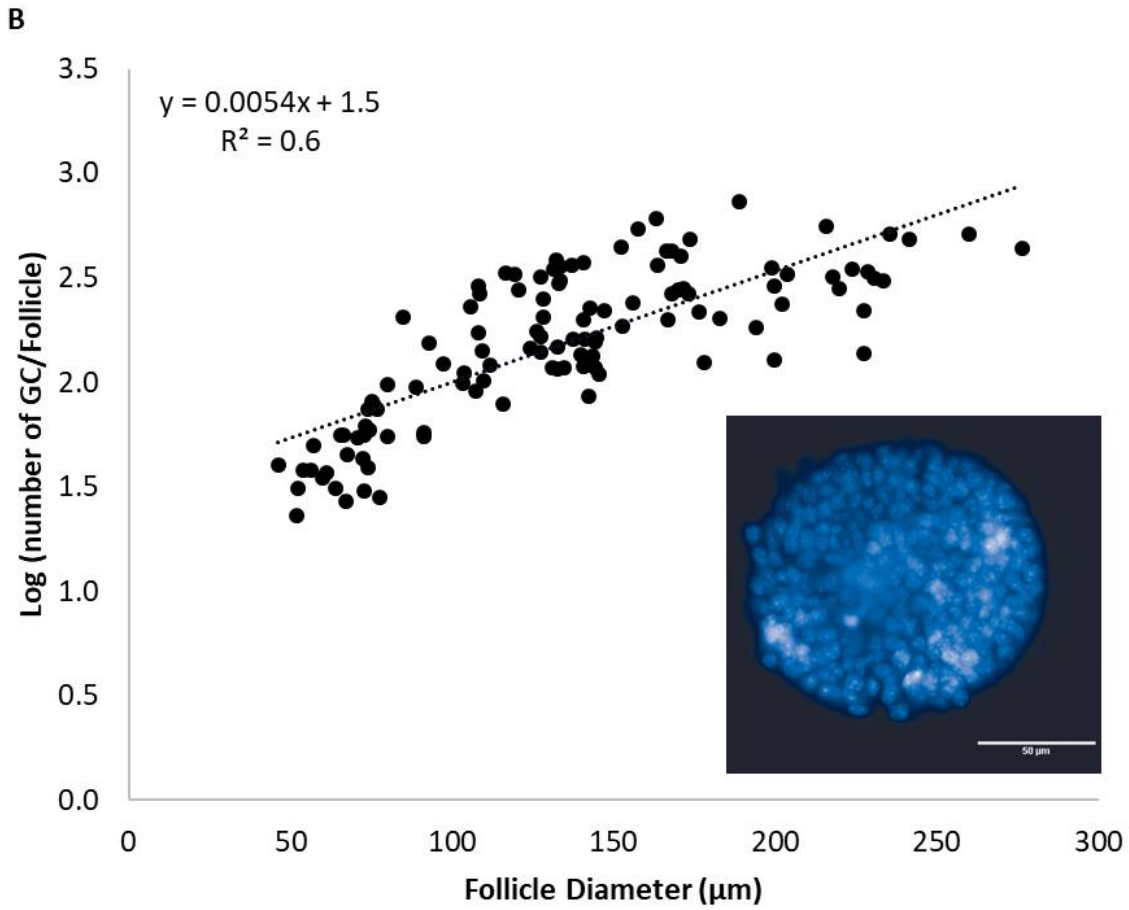


Figure 3. 10: Number of DAPI-stained GCs per follicle.

*Primary (66±9µm), early to mid-secondary (122 ± 19 µm), late secondary to incipient antral (176 ± 18 µm), and antral (235 ± 17 µm) follicles (total of 118 follicles) were collected from the enzyme disaggregated ovaries of five mice (n=5) and stained with DAPI. Image J software was used to objectively quantify the number of discrete DAPI-stained areas in each follicle. (A) Mean±SD GCs per follicle. Data were analysed by 1-way ANOVA and Tukey's Multiple Comparison Test. Significant difference from primary follicles *<0.05, **p<0.01. (B) Linear Regression analysis of log number of GC against diameter of follicles. Insert shows a DAPI-stained antral follicle with diameter 220.72 µm.*

Table 3. 7: Interpersonal variation in estimating GC numbers and diameters of follicles

Photomicrographs of 10 DAPI-stained morphologically intact follicles from four size cohorts of follicles were deidentified before being assessed by co-authors who were supplied with the standard operating procedure for image analysis to determine follicle diameters and the number of GCs in each follicle. Mean±SD of three assessor's estimations and coefficients of variation between those three estimations shown.

	Primary		**Early-Mid Secondary**		**Late Secondary-Incipient Antral**		**Antral**	
	GC Number	**Diameter (µm)**	**GC Number**	**Diameter (µm)**	**GC Number**	**Diameter (µm)**	**GC Number**	**Diameter (µm)**
Mean±SD	41±1	65±2	116±5	123±10	174±22	178±22	301±13	231±4
% Coefficient of variation	1	3	4	8	12	12	4	2

3.3.4.4 Number of Calcein AM and Ethidium Homodimer-1 (Live-Dead) Stained GCs per Follicle.

Image analysis of Live-Dead stained follicles yielded granulosa cell numbers that increased as the size of the follicle increased from primary to late secondary (R^2=0.63, Figure 3. 11B). The total numbers of GCs in primary follicles were significantly lower than in early secondary ($p<0.05$) or late secondary and antral follicles ($p<0.01$). Although all the follicles had good morphology and were graded as M1 or M2 quality (Lierman et al. 2015; Young et al. 2017), approximately 57±28% of the GCs in the follicles were not viable. The proportions of viable GCs were 38±25%, 41±28%, 45±29% and 61±28% in the primary (71±8 µm), early (125±15 µm), late (197±19 µm) secondary and antral (248±33 µm) follicles respectively.

Estimations of live-dead stained GC numbers were in the middle of the consensus ranges estimated by method (a) and (b) (Table 3. 5) but were at the lower end of the estimations from follicle and oocyte diameters (Table 3. 5, method c).

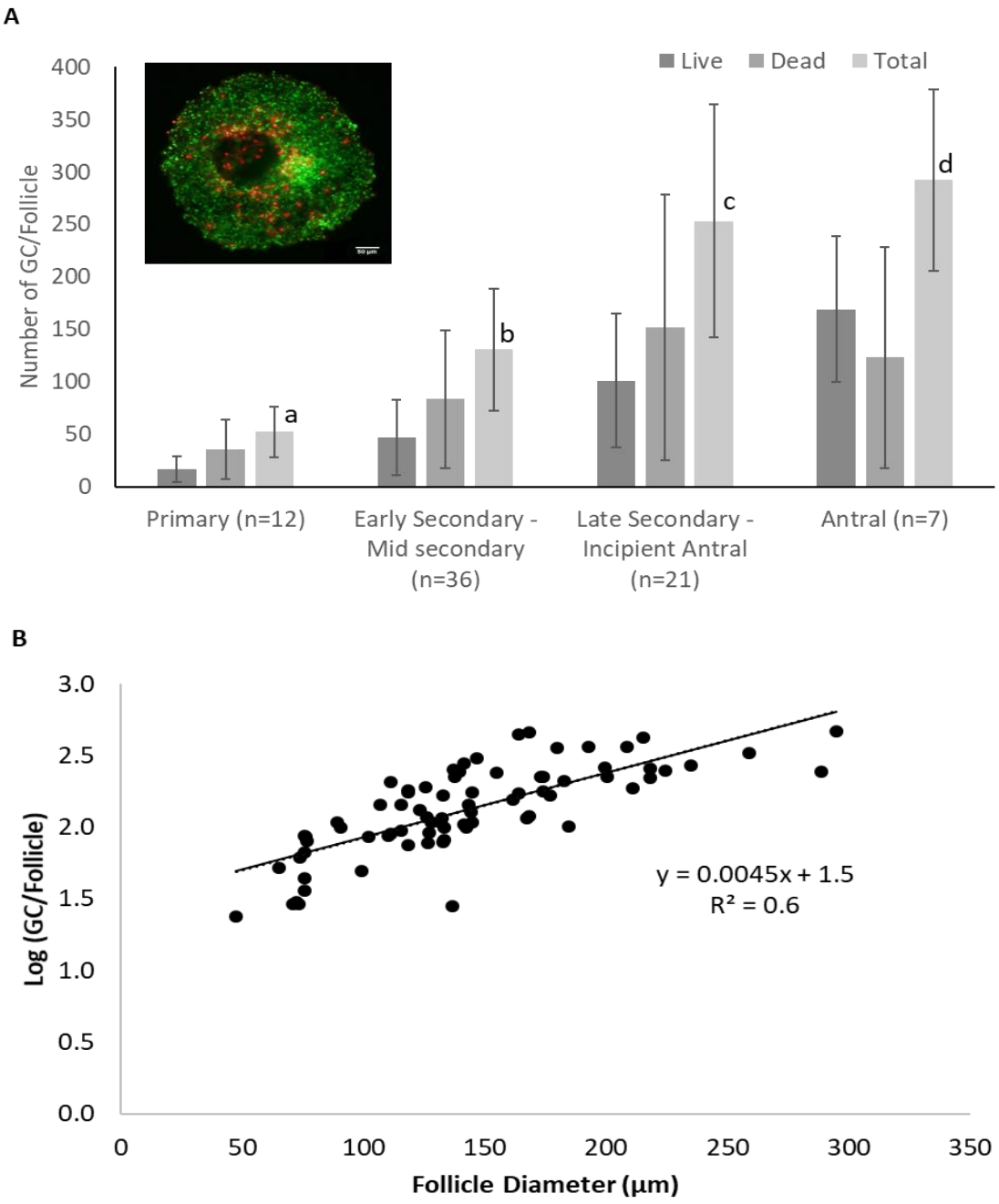
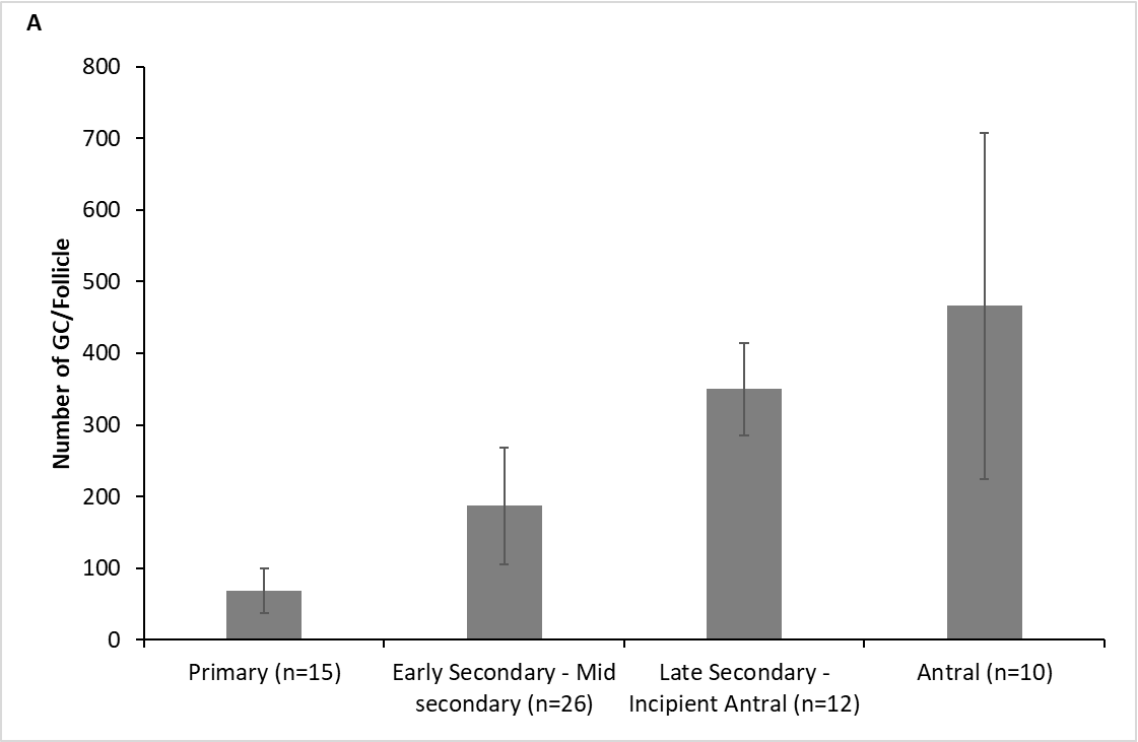


Figure 3. 11: Number of CalceinAM and Ethidium Homodimer-1 stained GCs per follicle.

Primary (71 ± 8 µm), early to mid-secondary (125 ± 15 µm), late secondary to incipient antral (179 ± 19 µm), and antral (248 ± 7 µm) follicles (total of 76 follicles) were collected from the enzyme disaggregated ovaries of three mice (n=3) and stained with Calcein AM (Live, green) and Ethidium Homodimer 1 (Dead, red). Image J software was used to objectively quantify the number of Live-Dead stained areas in each follicle (A) Mean±SD of live and dead GCs per follicle. Total numbers of GCs per follicle in each size cohort were analysed by 1-way ANOVA and Tukey's Multiple Comparison Test. a v b, b v d, $p<0.05$, and a v c & d, $p<0.01$. Insert shows fluorescence photomicrograph of a Live-Dead stained antral follicle with diameter 444.25 µm. (B) Linear Regression analysis of log number of GC against diameter of follicles.

3.3.4.5 Number of Ethidium Homodimer-1 Stained 'Dead' GCs per Follicle

Image analysis of follicles exposed to 70% EtOH and stained with Ethidium Homodimer 1 yielded granulosa cell numbers that increased as the size of the follicle increased from primary to late secondary (R^2=0.60, Figure 3. 12B). The numbers of GCs in primary follicles were significantly lower than in early ($p<0.05$), late ($p<0.01$) secondary and antral follicles ($p<0.01$). The numbers of GC were at the higher end of the estimations from consensus values [*Table 3. 5*, method (a) and (b)] but were lower than that estimated from using follicle and oocyte diameters.



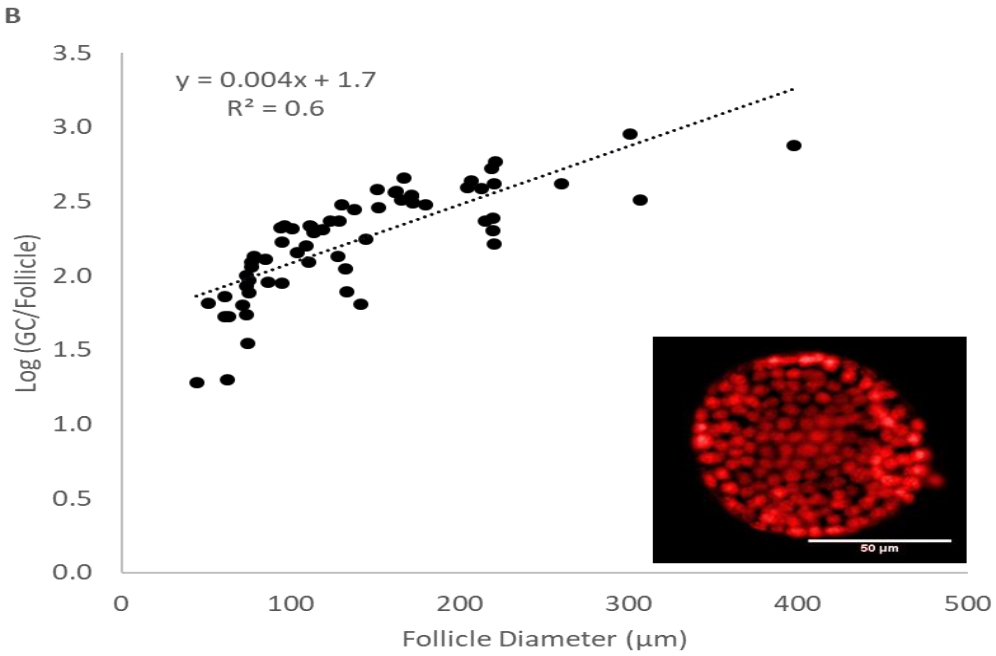


Figure 3. 12: Number of Ethidium Homodimer-1 stained GCs per follicle.

*Primary (68±15 µm), early to mid-secondary (117±23 µm), late secondary to incipient antral (181±23 µm), and antral (255±60 µm) follicles (total n=63) were killed with 70% ethanol before staining with Ethidium Homodimer 1. Image J software was used to objectively quantify the number of EthD1-stained nuclei in each follicle. Data were analysed by 1-way ANOVA and Tukey's Multiple Comparison Test. (A) Mean±SD GCs in follicles shown. Significant difference to primary follicles for GC per follicles indicated, *p<0.05 and **p<0.01 (B) Linear Regression analysis of log number of GC against diameter of follicles.*

3.3.4.6 Comparison of Granulosa Cell per Follicle Values Obtained from Different Methods

The total numbers (i.e. live plus dead) of GCs per µm diameter of follicle obtained after objective, semi-automated image analysis of isolated follicles stained with DAPI or Live-Dead stain were similar to each other, but approximately half the consensus values obtained by counting GCs in H&E stained histological sections, or by calculations using formulae, or follicle and cell volumes (Table 3. 5 and Figure 3. 13). The GC per follicle values obtained by counting Trypan Blue stained cells from disaggregated follicles however were similar to the consensus values (Figure 3. 13). There was no statistical difference between the GC per follicle values estimated using the three different methods in this study; Trypan Blue, DAPI nor Live-Dead stain.

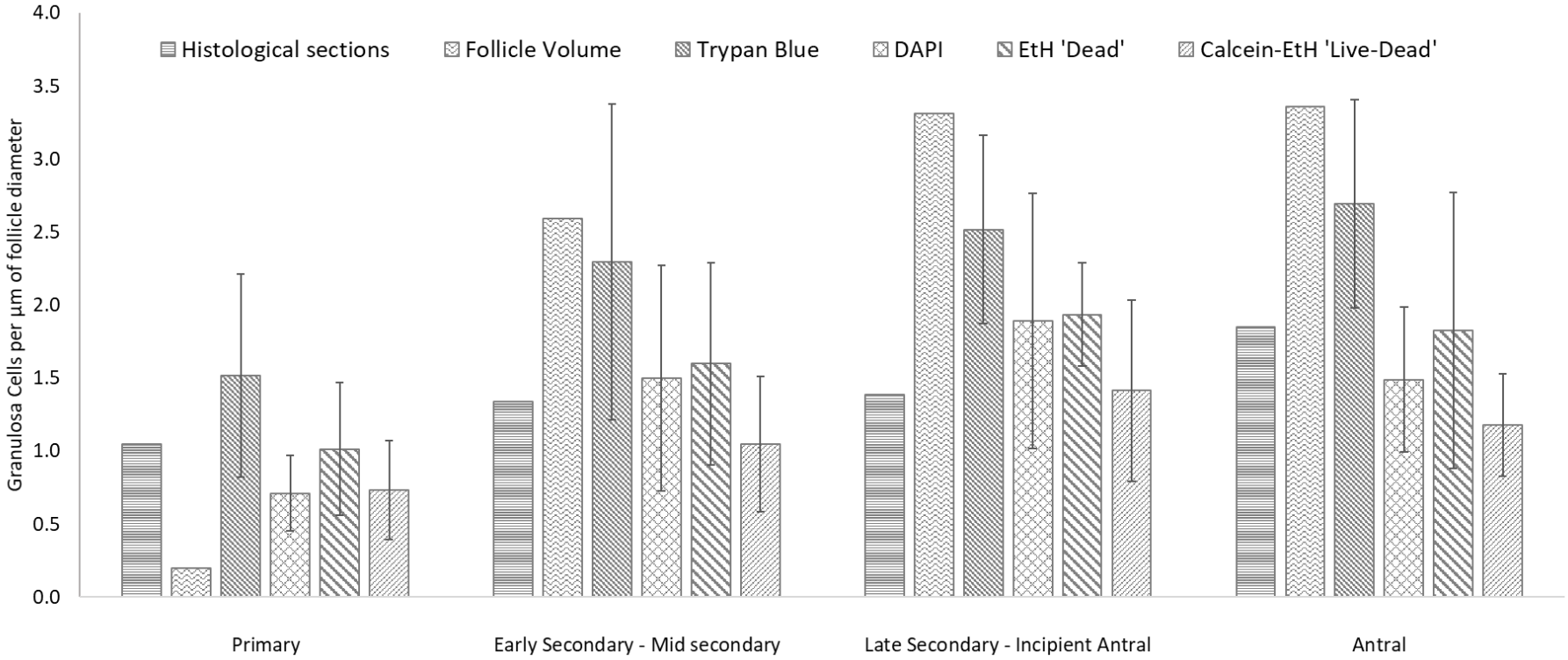


Figure 3. 13: Comparison of granulosa cell per follicle values.

Total granulosa cell numbers a) in follicles in fixed ovarian sections b) calculated from follicle volumes, c) determined by disaggregating follicles then counting the granulosa cell numbers in a Trypan Blue Exclusion assay, d) in follicles stained with DAPI e) in follicles treated with 70% EtOH and stained with Ethidium Homodimer 1, f) determined by using image analysis to quantitate the number of Live-dead stained cells in an intact follicle. Mean±SD GCs per µm diameter of follicle shown for each size cohort. Number at the base of each column is the number of follicles used to derive the data point.

3.3.5 Discussion

At the commencement of this study, there was no definitive consensus value for the number of GCs in a follicle, although many researchers (Bagger et al. 1993; Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Pedersen & Peters 1968) had estimated the numbers of GCs in fixed histological sections of mouse ovaries. Values derived from histological sections cannot be applied to experiments using whole, intact follicles, because the distribution of GCs in follicles is polarized (van Wezel, Ingrid L & Rodgers, Raymond J 1996). This is the first study to quantify GC in isolated murine follicles which also compares three different methods (Table 3. 5).

It was assumed that all GC in freshly-isolated follicles would be viable, because when histological sections of human or marmoset ovaries were 3' end labelled, there appeared to be no apoptotic cells in non-atretic follicles (Lierman et al. 2015; Young et al. 1997). TUNEL labelling does not identify necrotic cells, and therefore the number of dead GC in situ may have been underestimated, nevertheless the finding that only half the GC in freshly-isolated follicles were viable was surprising. It was initially thought that enzyme disaggregation of the ovary may have reduced GC viability within the follicle, but when ovaries were disaggregated mechanically without enzymes there was no increase in GC viability. Two different methods were used in the present study to assess GC viability (Trypan Blue and Live-Dead stain) and both demonstrated that the proportion of viable cells increased from 40% in small primary follicles to 60% in the larger secondary and antral follicles. In addition to this the Live-Dead staining suggested that the majority of dead GC were located in the peripheral layers of the follicles. It is possible that follicle size or volume affected the proportion of viable GC, and this observation supports the proposal that the process of isolating follicles from the ovary may have reduced GC viability. Lierman et al. (2015) also concluded that the separation of follicles from the extracellular matrix of the ovary caused GC death, because there was no follicular apoptosis in histological sections of ovaries, but 27% of isolated follicles were labelled at 3'-OH end of fragmented DNA of apoptotic cells after TUNEL assay This was not surprising because vascularization of the outer theca layer of secondary follicles would appear more susceptible to damage during the isolation process to separate the follicles from the ovary. Also, it is possible that the higher proportion of dead GC in primary follicles reflects the poorly formed basal lamina – not as robust as in larger follicles.

Others have applied live-dead stain to mouse (Kreeger et al. 2006), ovine (Lunardi et al. 2012) (Merdassi et al. 2011), cow (Aerts et al. 2008) and human (Campos et al. 2011) ovarian follicles. In these studies, green-stained areas corresponding to 'live' cells were presented as a percent of the

whole follicle area, rather than being counted as individual cells as in the present study. In those in situ studies, more than 80% of each follicle was viable, irrespective of the species (Aerts et al. 2008; Campos et al. 2011; Kreeger et al. 2006; Lunardi et al. 2012; Lundy et al. 1999; Merdassi et al. 2011). However, it is possible that some of the 'live' staining was non-specific, and this elevated the estimation of viable GC numbers. When the number of individually 'live'-stained GC were compared to the numbers of viable GC that excluded Trypan Blue, the numbers were similar to each other. In another study in which GCs from mouse large antral follicles were isolated by puncturing and assessed by Trypan Blue staining, 60% of GCs were dead (Li et al. 2016). When mechanically isolated ovine whole preantral follicles were cultured *in vitro*, on the second day the follicular cell viability was found to be 62.5% (Lunardi et al. 2012). These two studies support our own finding that approximately half the GC in freshly isolated follicles are viable.

Six different methods were used to estimate number of GC per follicle. All the staining procedures estimated lower GC per follicle numbers than the estimations from histological sections, estimations from follicle and oocyte diameters or the Trypan Blue assay on GC from disaggregated follicles.

Although the DAPI staining combined with objective image analysis estimated lower GC per follicle numbers than disaggregated follicles combined with Trypan Blue staining, the difference between GC quantification values was not statistically significant, nor was there any statistical difference between these and the Live-Dead staining method. The DAPI staining protocol is inexpensive and straightforward and the objective image analysis protocol highly reliable and reproducible; the inter-assay coefficient of variation for quantification of GC numbers in secondary follicles was only 4% and means that the method can be applied to assess the effects of test reagents on GC proliferation *in vitro*.

Trypan Blue assay is considered to be the best way of estimating the granulosa cell numbers because it directly estimated live and dead cells disaggregated from follicles. Moreover, unlike the staining approach it does not depend on the penetration of dye to the inner layers of GCs. Hence, there is no risk of underestimation due to low penetration of dye. In addition, it is a direct estimation of GC in single cell suspension and so there is no underestimation resulting from overlapping of stained GCs. Also, the granulosa cell numbers estimated from Trypan Blue linearly increase with increasing diameters (R^2=0.97). Distribution of GCs as a function of diameter represented the pattern of best fit that indicated that there was no systematic or gross error (Karp et al. 2010). On the other hand, regression analysis from GC numbers estimated from DAPI stained (R^2=0.61), live dead stained

(R^2=0.57) and positive dead stained (R^2=0.60) as function of follicle diameter represented poor fit with regression line. Therefore, regression equation obtained from TB estimations can be used to estimate the total number of GCs present in a follicle with known diameter because in 97% cases, it can predict accurately the follicular cell numbers as a variation of follicular diameter.

In conclusion, the direct estimation of GC in a disaggregated follicle overcomes the limitations of other estimation methods of follicular cell number and viability including, histological cross section, 2D staining and follicular volume. The total and viable GCs per follicle can therefore be used as a baseline estimation to compare with that after exposure to chemotherapy.

4. CHAPTER FOUR: PRELIMINARY STUDIES ON FOLLICLES *IN VITRO*

4.1. Introduction

Although follicle culture is an *in vitro* technique commonly used for facilitating the development, growth, and maturation of oocytes (Dewailly et al. 2016; Diclemente et al. 1994; Durlinger et al. 2001; Eppig & Schroeder 1989; Lebbe & Woodruff 2013; Spears et al. 1994), the application of *in vitro* culture of ovarian follicles to study the toxic effects of new drugs and existing chemotherapies on reproductive function has been limited. With the development and discovery of new molecules of pharmaceutical importance, the need for developing *in vitro* follicle culture systems has emerged to characterise aspects of the reproductive toxicity (Cortvrindt & Smitz 2002). In addition to this the international Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals and the Organization for Economic Co-operation recommend that any pharmaceutical molecule intended for human use should be tested for reproductive toxicity. The *in vitro* culture of murine follicles is an easy, economic and safe way to investigate the toxicity of therapeutically used pharmaceuticals, including chemotherapies, and can contribute to the development of new treatment guidelines with reduced reproductive toxicity (Cortvrindt & Smitz 2002).

The aim of this project is to examine the role of chemotherapies in ovarian failure. Follicles produce different hormones at different stages of follicular growth and development. Pharmaceuticals and chemotherapeutics may exert detrimental effects at different stages of follicle development and thus affect hormone production (Channing et al. 1980; Jamin & Hazard 1981; Reed & Carr 2015) and can lead to infertility. Breast cancer is the most commonly diagnosed cancer among women, among the diagnosed patients about 12% are of premenopausal age (below 45 years) (Rugo & Rosen 2011) and approximately 90% survive after chemotherapy (Ferlay et al. 2015; Morgan et al. 2012). Commonly used chemotherapeutics for treating breast cancer include Doxorubicin (Adriamycin) with Cyclophosphamide (AC) (Dees et al. 2000; Ganz et al. 2017; Jones et al. 2006; Singh et al. 2017; Swenson et al. 2003). Chemotherapeutics kill rapidly proliferating cancer cells. During folliculogenesis, follicles grow from primary to antral follicles through the proliferation of GCs. Hence it is likely that chemotherapeutics will kill the proliferating GCs of growing follicles; however, the effects of these chemotherapeutics on ovarian follicles have not been examined *in vitro*.

Previous studies suggested that culture of murine secondary follicles in a 2D culture system allowed the growth of follicles to antral stage and supported GC proliferation, maturation of oocyte, and steroidogenic functions (Cortvrindt & Smitz 2002; Eppig & O'Brien 1996; Eppig & Schroeder 1989; O'Brien, Pendola & Eppig 2003; Spears et al. 1994; Spears, De Bruin & Gosden 1996; Telfer et al. 2008; Wang et al. 2011). Here two related preliminary studies involving short term 48h or 72h 2D *in vitro* culture of murine secondary follicles are presented to examine follicle growth, granulosa cell proliferation, follicle integrity and hormone synthesis. The IC25s (of doxorubicin, cyclophosphamide and gamma tocopherol) estimated in the previous study (chapter 2) were used in this study.

4. 2 Methods

4.2.1 Isolation of Murine Secondary Follicles

Mice that were surplus to the breeding colony requirements of the College of Medicine and Public Health Animal Facility were allocated to routine cull. Female Balb/C or C57/BL cull mice of mean age 8-20 weeks were used in this experiment. On each occasion one mouse was killed by cervical dislocation and the ovaries collected. Ovaries with oviduct and adipose tissue were carried to the laboratory in alpha MEM (Sigma) with 10% FCS (Bovogen Biologicals Pty Ltd, VIC, Australia), 1% ITS (Gibco, New York, USA) and 1% penicillin and streptomycin (Sigma) (complete media) at 37°C. Ovaries were dissected free of oviducts, adipose tissue and extraneous tissue before mechanical disaggregation using 22-gauge syringe needles until all individual follicles were isolated. On separate occasion (for exposing to chemotherapeutics), follicles were dissected out after exposing ovaries to 2 mg/mL collagenase IV in Dulbecco's Modified Eagle's Medium (DMEM)/F12 for 30 minutes at 37°C. Only the secondary follicles with diameters from 79 to 145 µm were transferred to a well of a 96-well plate containing complete media to produce a pool of secondary follicles from both ovaries.

4.2.2 *In Vitro* Culture of Murine Follicles

On each occasion, 10 isolated intact murine secondary follicles with spherical morphology (intact basal lamina with no loss of GC) were cultured in a group of 5 follicles per well in 200µL complete media with 25IU/mL follicle stimulating hormone (FSH) (Merck Serono Australia Pty Ltd., NSW) under humidified conditions at 37°C with 5% CO_2 for 72 hours. On the commencement of culture, follicles were photographed using an inverted microscope and thereafter every 24 hours until the end of culture. Every 24h, 150µL conditioned culture media were collected and replaced with same volume of fresh culture media with FSH. Collected conditioned media were stored in eppendorfs at -20°C for hormone assay.

4.2.3 Follicle Exposure to Chemotherapeutics *In Vitro*

Only the secondary follicles with intact morphology were collected in a well of a 96-well plate containing complete media (DMEM/F12 with 10% FCS + 1% ITS). Groups of 4 follicles were transferred to wells of a 96-well round bottom plate and exposed to 100 µL of the test reagents (*Table 4. 1*). Doxorubicin (Dox) (Sigma, Sydney, Australia) and 4-hydroperoxycyclophosphamide (a prodrug of active metabolite of cyclophosphamide, 4HPCYP) (ThermoFisher Scientific, Victoria, Australia) were each added to sterile water to make stock solutions of 100 µM and 1000 µM respectively. These were stored at 4°C and 20°C respectively (Hoffman et al. 1979; Ulukaya et al. 2008). Immediately before use they were diluted further to the desired concentrations (see below and Table 4. 1) in complete media. Gamma-tocopherol (gTоc) (Sigma, Sydney, Australia) was dissolved in DMSO to prepare a stock solution of 1000µM, and stored at 4°C. Immediately before use it was diluted further to the desired concentrations (see below and Table 4. 1) in complete media, such that the concentration of DMSO that cells were exposed to was 0.3% DMSO. There was no significant difference in cell viability, or estrogen production by cells exposed to 0.3% DMSO when compared to complete cell culture medium alone.

Table 4. 1: Test reagents

Test reagent	**Concentration**
Control (DMEM/F12+910%FCS+1%ITS)	0
Doxorubicin + Cyclophosphamide (AC)	1.21 µM + 21.23 µM
Gamma Tocopherol (gToc)	35.1 µM
Doxorubicin + Cyclophosphamide + gToc (AC+gToc)	1.21 µM + 21.23 µM+35.1 µM

Follicles in treatments were incubated for 48 hours at 37°C with 5% CO_2. Follicles were photographed under inverted microscope at the commencement of culture and after 48 hours.

4.2.4 Follicle Disaggregation

At the end of 72h (*in vitro* 2D culture) and 48h (chemotherapeutics exposed to follicles) culture period, 150µL and 100 µL of conditioned media respectively were collected from each well. 10µL of Collagenase IV 12mg/mL (Worthington, New Jersey, USA) was added to the remaining 50µL of media containing 5 or 4 follicles to produce an effective concentration of 2mg/mL. Follicles with collagenase IV were subjected to incubation at 37°C with 5%CO_2 for 30 minutes with gentle shaking

for one minute every 15 minutes. After that, 50µL 0.05% hyaluronidase was added and further incubated for 30 minutes. All the contents of each well after incubation were transferred to 1.5mL centrifuge tubes and the volume was made up to 500µL with PBS. Isolated GCs were retrieved after centrifugation at 2040g for 5 minutes, supernatants were discarded, and cells were re-suspended in 20µL compete media.

4.2.5 Follicular Cell Viability Assessment

The GCs harvested from each group of 5 or 4 follicles were suspended in 20µL of complete media and 20µL trypan blue were added to each of these single cell suspensions of GCs. Two aliquots of 10µL were assessed by counting all the live and dead cells in 9 large squares of haemocytometer. Mean±SD of viable and dead GCs per follicle are reported. Experiment was repeated on four separate occasions (n=4).

4. 2.6 Hormone Produced by Follicles *In Vitro*

4.2.6.1 Estradiol Enzyme Immunoassay (EIA)

The conditioned media were examined in a competitive Estradiol (E2) EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA). The manufacturer reports a detection range from 6.6 to 4000 pg/mL, and an intra-assay coefficient of variation (CoV) from 7.8% to 18.8%. For this study, the estradiol standard was diluted in the alpha MEM complete cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. All supernatants from four separate experiments were examined in one EIA, in which the standard curve R^2 value was 0.99.

4.2.6.2 Progesterone Enzyme Immunoassay (EIA)

The conditioned media were examined in a competitive progesterone EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA). The manufacturer reports a detection limit of 10pg/mL, an intra-assay coefficient of variation (CoV) of 7.5%, and an inter-assay CoV of 2.9%. For this study, the progesterone standard was diluted in the alpha MEM complete culture medium supplemented to give concentrations that ranged from 7.18 to 1000 pg/mL. All supernatants from four separate experiments were examined in one EIA, in which the standard curve R^2 value was 0.99.

4.2.6.3 Anti-Mullerian Hormone (AMH) Enzyme Immunoassay (EIA)

Conditioned media were examined in a two-immunological step sandwich type EIA (Immunotech, Marseille Cedex, France). The manufacturer reports a detection limit of 1pg/mL, an intra-assay CoV

of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standards were diluted in complete medium to give concentrations that ranged from 0 to 150 pM. All supernatants from four separate experiments were examined in one EIA, in which the standard curve R^2 value was 0.99.

4.3 Results

4.3.1 Follicle Morphology and Growth

Although follicles cultured in 2D culture system maintained intact structures with clearly visible oocytes surrounded by GCs within basement membrane (Figure 4. 2), follicles did not grow over the 72h culture period ($p>0.05$) (Figure 4. 1). Addition of FSH did not stimulate GC proliferation or growth of follicles. At the commencement of culture, morphologically follicles had intact basal membrane (Figure 4. 3 A, C, E, and G) with multiple layers of GCs surrounding the oocyte. After 48h *in vitro* either with chemotherapy or gamma tocopherol there was a loss of spherical structure and GCs were dispersed across the surface of the well (Figure 4. 3 B, D, F and H). Exposure to doxorubicin and cyclophosphamide in combination caused dispersal of the cumulus oocyte complex (COC) (Figure 4. 3 D) in contrast to the compact appearance of the COC in follicles cultured under control conditions in DMEM/F12 (Figure 4. 3 B). Follicles exposed to Gamma tocopherol had a similar morphological appearance to the control follicles cultured in DMEM/F12, but possibly with less compact COCs and more dispersed GC (Figure 4. 3 F).

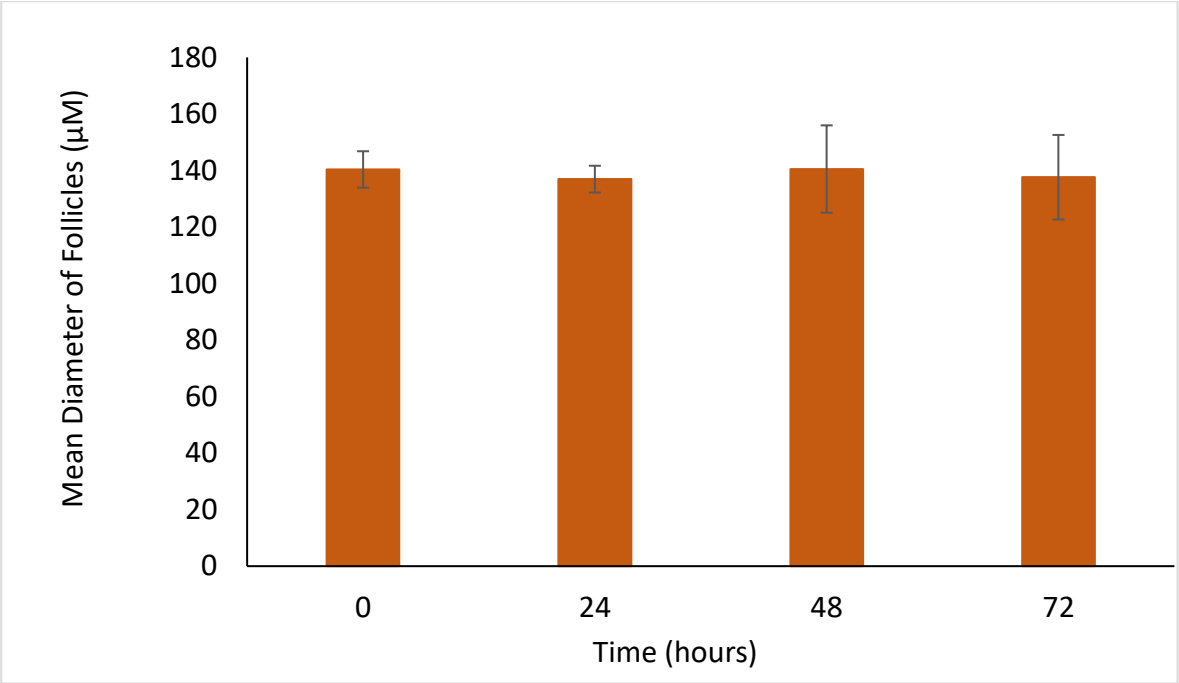


Figure 4. 1: Ovarian follicle growth in vitro.

Mechanically isolated murine secondary follicles were allocated in groups of 5 to two wells of a 96-well round bottom plate and cultured for 72 hours at 37°C with 5% CO_2. Follicles were photographed at commencement of culture and thereafter every 24-hour using an inverted microscope. Photomicrographs were used to determine diameters of follicles by drawing three straight lines through the oocyte using Image J. Experiment was repeated on four separate occasions (n=4), Mean±SD shown for each time point. Data were analysed using a one-way ANOVA with Tukey's multiple comparison test.

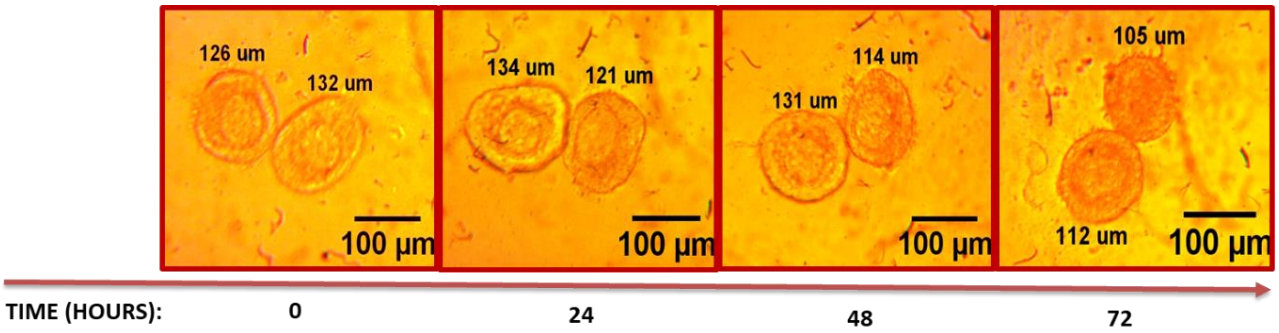


Figure 4. 2: Follicle morphology during in vitro culture.

Mechanically isolated murine secondary follicles were cultured in groups of 5 follicles in duplicate wells of a 96-well round-bottom plate for 72 hours at 37°C with 5% CO_2. Follicles were imaged individually at 25X magnification under inverted microscope on commencement of culture and thereafter 24h interval.

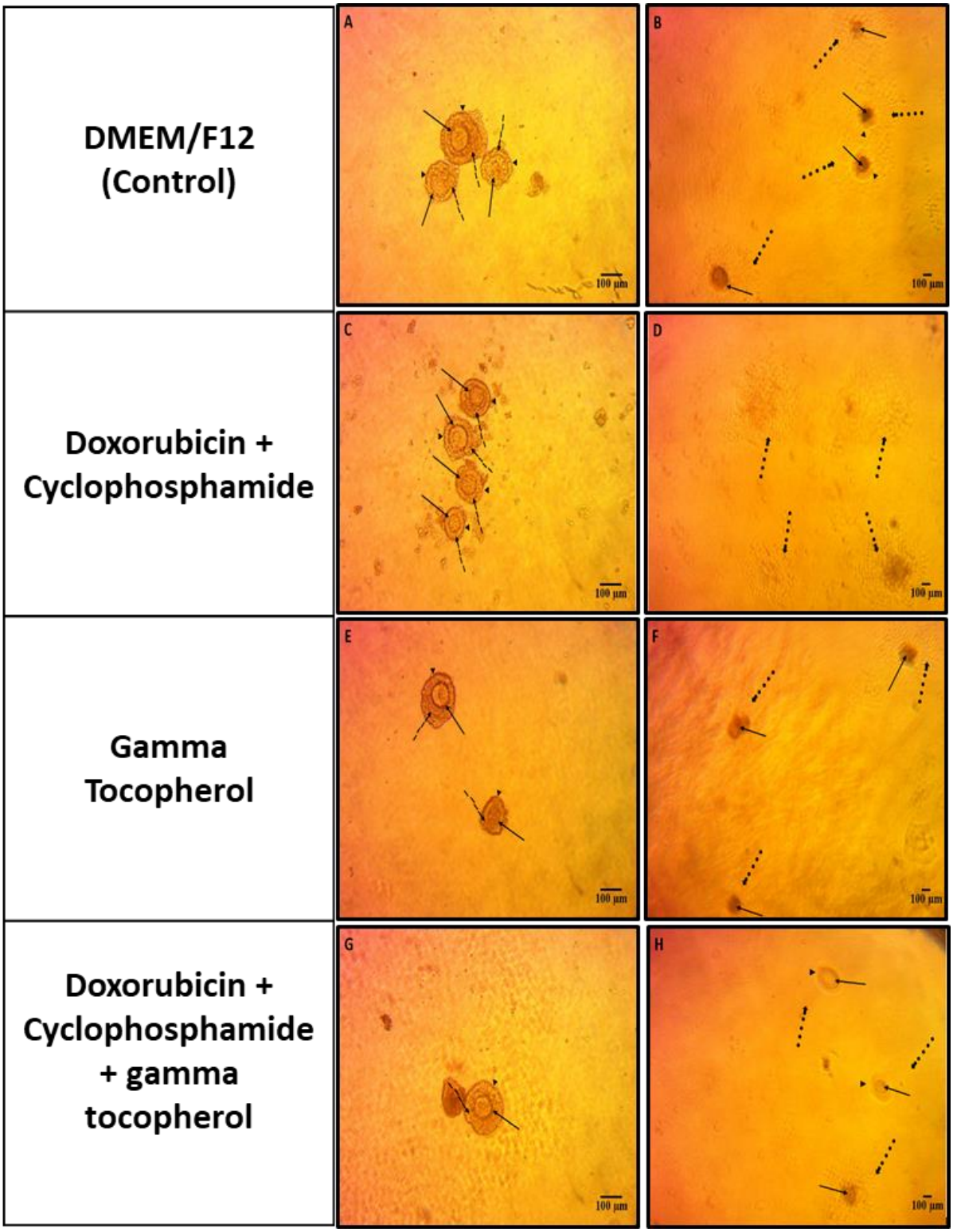


Figure 4. 3: Follicle morphology before and after 48h culture.

Groups of 4 follicles were exposed to DMEM/F12, doxorubicin+cyclophosphamide, gamma tocopherol or doxorubicin+cyclophosphamide+gamma tocopherol for 48 hours in wells of 96-well plate under humidified condition at 37°C with 5% CO_2. Follicles were photographed at commencement (A, C, E and G) at 10X magnification and at the end of culture period (B, D, F and H) at 4X magnification. Oocyte indicated by solid arrow (→), basement membrane (▽) and dispersed GCs (…>).

4.3.2 Follicle Cell Viability

There were 1087 GCs per secondary follicle after 72-hour *in vitro* culture of which 63% were viable (Figure 4. 4). Addition of gToc to doxorubicin and cyclophosphamide resulted the number of viable granulosa cell numbers per μm^2 of follicle, and the percent of viable cells to be similar to control and to the gToc treated group of follicles after 48 hours (Figure 4. 5). However, doxorubicin and cyclophosphamide reduced the numbers and percentage of viable GCs per μm^2 of follicle (Figure 4. 5).

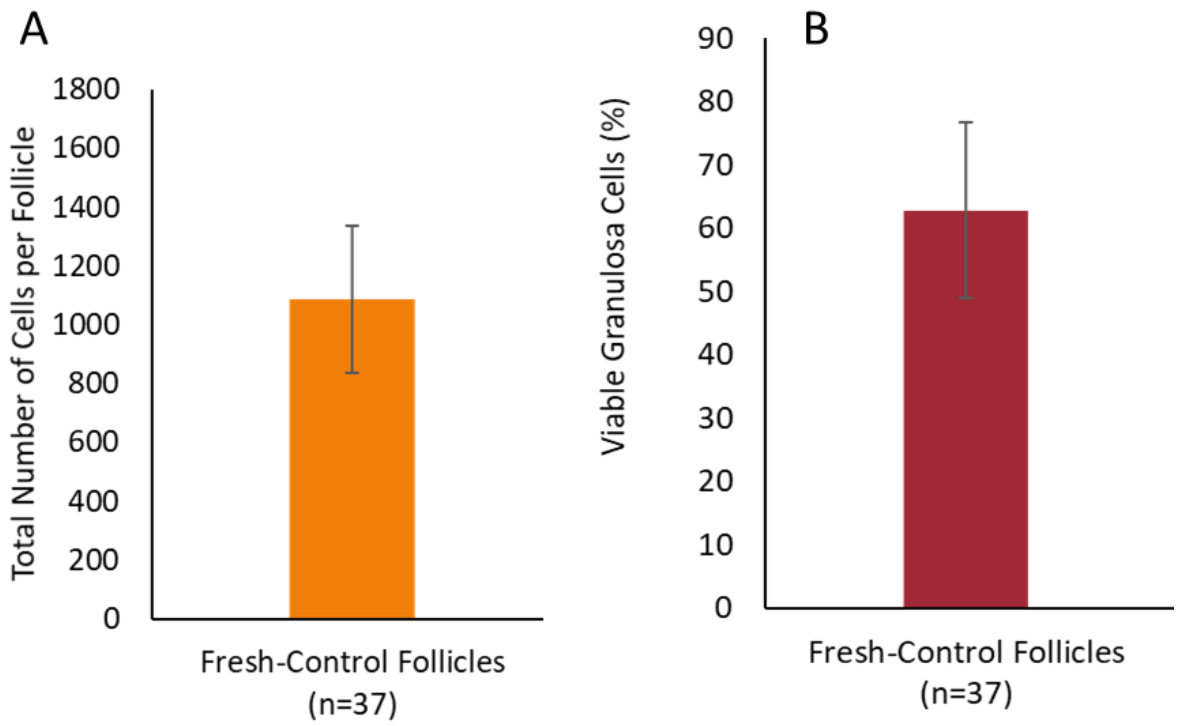


Figure 4. 4: Follicular cell viability in vitro.

Mechanically isolated murine secondary follicles were cultured in groups of 5 follicles in duplicate wells of a 96-well round-bottom plate for 72 hours at 37°C with 5% CO_2. At the end of culture period, follicles were disaggregated using collagenase IV and hyaluronidase to produce single granulosa cell suspension to be assessed by trypan blue assay. Mean±SD of total GCs (A) and viability (% of viable GC) (B) four experimental replicates (n=4) shown.

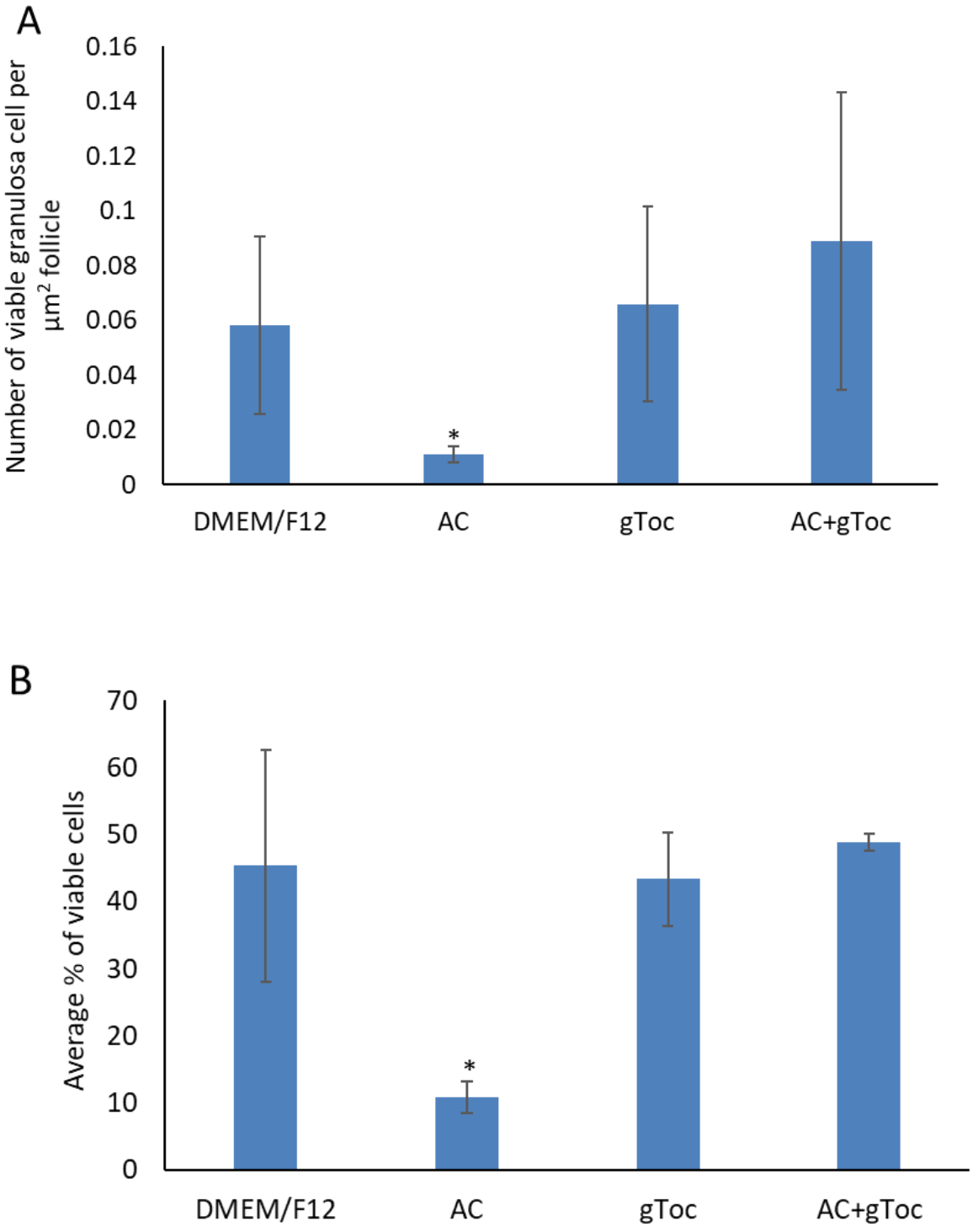


Figure 4. 5: Follicular cell viability.

*4 follicles in duplicate wells were treated with DMEM/F12, doxorubicin + cyclophosphamide (AC), gamma tocopherol (gToc) or doxorubicin + cyclophosphamide + gamma tocopherol (AC+gToc) for 48 hours. After culture period, follicles were disaggregated to isolate GCs and live and dead GCs were assessed by trypan blue assay. Mean±SD of viable GCs per µm² of follicle (A) and percent of viable GCs per treatment presented (B). Data analysed by one-way ANOVA with Tukey's multiple comparison test, significant difference, * p<0.05 from control (DMEM/F12).*

4.3.3 Hormone Production

Follicles produced estrogen, and progesterone but not AMH. The synthesis of estrogen and progesterone was not significantly different in each 24h interval (Figure 4. 6)

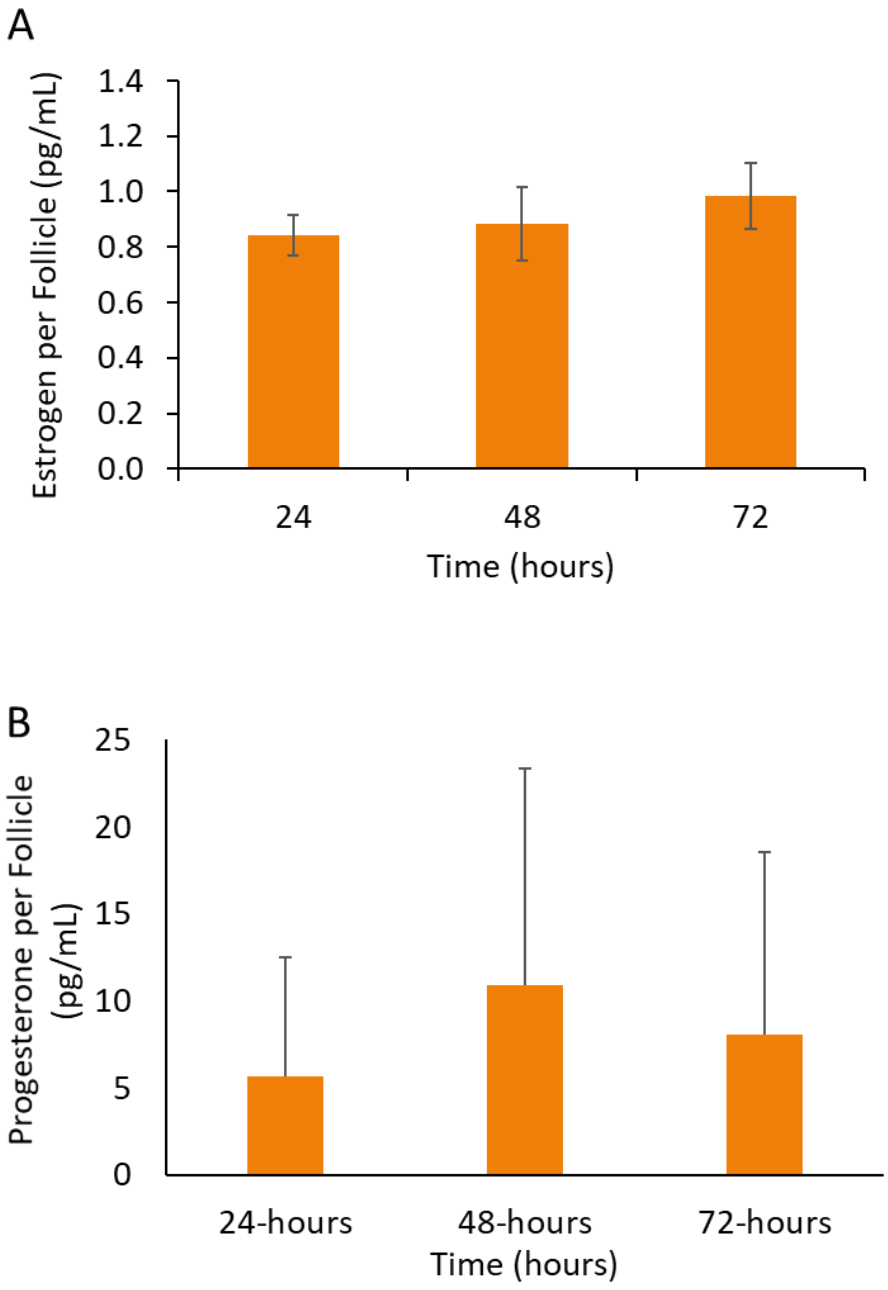


Figure 4. 6: Hormone synthesized by murine follicles in vitro.

Groups of 5 mechanically isolated murine secondary follicles were cultured for 72 hours. Conditioned media were collected every 24-hour interval and subjected to estrogen, progesterone and AMH ELISA. Hormone synthesized per well were adjusted for follicle number. Experiment was repeated on three separate occasions (n=3), mean±SD of hormone produced shown, (A) estrogen synthesized per µm diameter of follicle and (b) progesterone synthesized per µm diameter of follicles. Data were subjected to one-way ANOVA with Tukey's multiple comparison test.

4.4 Discussion

The 2D culture system did not support follicle growth. This finding was supported by Eppig and Schroeder (1989) and Eppig and O'Brien (1996). This preliminary study to examine the effects of chemotherapeutics on follicles suggests that the IC25s of chemotherapeutics against MCF-7 cells were more cytotoxic to follicles than breast cancer cells (killed more than 25% of follicular GCs). Doxorubicin and cyclophosphamide at MCF-7 IC25s killed about 90% of non-cancerous GCs but when in combination with gToc only caused the death of 49% of GC which suggests that addition of gToc prevented the death of about 40% of the GCs. However, about half of the control GCs were dead. One factor may have been suboptimal culture conditions that used high atmospheric oxygen levels (20%). This high level of oxygen might have contributed to the low GC viability in control follicles. Previously follicle viability was higher in 5% oxygen than in 20% (Gook et al. 2013; Hartshorne 1997). Similar to the study conducted by Telfer et al. (2008) this preliminary study suggests that hormone synthesis is reduced in a 2D culture system. This may be because loss of spherical structure in a 2D culture system causes disruption to autocrine and paracrine functions through increasing stress in gap junctions and that this contributes to impairment of hormone synthesis (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989; Telfer et al. 2008). These preliminary studies indicate a need to develop a robust and reliable 3D follicle culture system to examine the effects of chemotherapeutics on follicle cell viability and hormone synthesis *in vitro*. However, these initial results were promising with respect to the potential for gToc to reduce granulosa cell death in follicles exposed to chemotherapeutics.

5. CHAPTER FIVE: DEVELOPMENT OF A 3D FOLLICLE CULTURE SYSTEM

5.1 Overview

Ovaries contain follicles which are three dimensional (3D) spheres made up of a central oocyte (egg) surrounded by layers of GCs, and additional layers of theca cells in the larger, more mature follicles. The main function of the granulosa and theca cells is to support oocyte maturation so that it can be fertilized by spermatozoa after ovulation *in vivo* (Macchiarelli et al. 1992). Ovarian follicles are cultured *in vitro* for many reasons including *in vitro* maturation to produce fertilizable oocytes, and to allow investigation of the fertility-suppressing effects of chemotherapeutics on granulosa cell viability and hormone production (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Desai et al. 2010; Heidari et al. 2012; Mohammadi-Rousheh et al. 2006; Moon et al. 1978; Picton, HM et al. 2008; Telfer, Evelyn E & Zelinski, Mary B 2013; Zhang et al. 2011). Two-dimensional (2D) culture systems have been used to grow follicles but these 2D culture systems cause collapse of the 3D spherical structure with consequent impairment of follicle and oocyte maturation (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989). In contrast, culturing ovarian follicles in 3D matrices supports the growth of follicles in all directions, maintains inter-cellular gap junctions and maintains enclosure of the oocyte within the granulosa cell layers (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Desai et al. 2010). In addition, culturing follicles in a 2D system *in vitro,* in a preliminary study (chapter 4) made it clear that a more complex culture system that modelled the *in vivo* situation was required. Therefore, the purpose of this study is to compare two 3D matrices (established versus novel) with a 2D follicle culture system (negative control).

5.2 Three-dimensional *In Vitro* Culture of Murine Secondary Follicles in a Defined Synthetic Matrix

5.2.1 Introduction

Two dimensional (2D) culture systems allow cells to grow in a monolayer on a rigid and flat substrate (Edmondson et al. 2014). 2D follicle culture systems have been evaluated by examining oocyte germinal vesicle break-down (GVB) (Eppig & Schroeder 1989; O'Brien, Pendola & Eppig 2003) and meiotic arrest (Eppig & Schroeder 1989), both indicators of oocyte maturation and fertilisability, as well as follicle granulosa cell viability (Heidari et al. 2012), follicular growth to antral stage (Heidari et al. 2012; Spears, De Bruin & Gosden 1996), follicular integrity (Buccione et al. 1987; Eppig 1977, 1979), and the production of steroid hormones; estrogen (E2) and progesterone (P4) (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Heidari et al. 2012). However, the application of 2D follicle culture systems to the *in vitro* maturation of follicles and oocytes has been criticized because they do not maintain follicular integrity throughout the culture period, and GCs tend to grow out through the follicle basal lamina within four days of culture (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996). Moreover, 2D culture systems do not model the *in vivo* 3D environment in which follicles are enclosed by the extracellular matrix of the ovary (Edmondson et al. 2014). Follicular integrity is essential for follicle and oocyte maturation because GCs communicate constantly with each other and the oocyte through gap junctions (Buccione et al. 1987; Desai et al. 2010; Eppig 1977, 1979). When follicles are cultured in 2D systems, stress on the intercellular gap junctions causes a significant reduction in oocyte growth, extrusion of oocytes, loss of spherical structure (Cortvrindt, R, Smitz, J & Van Steirteghem, A 1996; Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Desai et al. 2010; Eppig & O'Brien 1996; Eppig & Schroeder 1989) and flattening of the follicles (Gomes et al. 1999). Although follicles cultured in 2D systems grow and can exhibit 2.1 to 5.3-fold increases in diameter (Eppig & O'Brien 1996; Eppig & Schroeder 1989), only 62% of the oocytes undergo GVB (O'Brien, Pendola & Eppig 2003) and the oocyte diameters tend to be significantly smaller after 10 days in culture than those of freshly isolated oocytes from follicles of comparable age (Eppig & O'Brien 1996; Eppig & Schroeder 1989). It was argued that the enzymatic disaggregation of ovarian tissue to collect follicles damaged the basal laminae of the isolated follicles and that this compromised follicular integrity (Desai et al. 2010) contributed to the detachment of GCs from each other and the oocyte (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996) and impaired maturation. However, when follicles were collected mechanically from the ovaries without enzymes, cultured in a 2D system for 6 days and the viability assessed using Live-Dead stain, the follicular viability was

only 8% (Gook et al. 2013). In some studies, follicles were said to be viable if the follicle retained a 3D spherical structure and GCs enclosed the oocyte (Heidari et al. 2012), but in situ studies indicated that about 20% to 40% of GCs in a follicle can be non-viable, irrespective of species (Aerts et al. 2008; Campos et al. 2011; Kreeger et al. 2006; Li et al. 2016; Lunardi et al. 2012; Lundy et al. 1999; Merdassi et al. 2011) and in isolated morphologically intact, spherical follicles 10% of GCs were not viable when measured by staining the whole follicle with Live-Dead stain (Santos et al. 2006) or by Trypan Blue staining (Santos et al. 2008). Together, these studies indicate that 2D culture systems are not optimal for follicular and oocyte maturation.

Three dimensional (3D) *in vitro* culture matrices have been developed to overcome the limitations of 2D culture systems. *In vivo*, the distinct cellular compartments of ovarian follicles; theca cells, basal laminae, GCs and the cumulus oocyte complex (COC) (Desai et al. 2010), are embedded in an extracellular matrix consisting of collagen, fibrinogen and hyaluronic acid (Gomes et al. 1999; Irving-Rodgers et al. 2010; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; van Wezel, Ingrid L & Rodgers, Raymond J 1996). The follicles are in turn embedded in the extracellular matrix of the ovary which consists of collagen, proteoglycans, and tenascin C (Salani et al. 2007; Salvetti et al. 2003). There are two broad types of 3D matrices; alginate and Matrigel. Alginate matrices are derived from algae and comprise β-d-mannuronic acid and α-l-guluronic acid that forms gel by ionic cross-linking of the guluronic residue. Higher concentrations of alginate (0.25%) however reduced the growth and maturation of follicles and oocytes (Parrish et al. 2011; Xu et al. 2011; Xu et al. 2006) but incorporation of components of extra cellular matrix such as collagen I and IV, fibronectin and laminin into alginate increased steroid hormone (P4 and E2) production by follicles, probably because it promoted the adhesion of GCs to each other and the oocyte (Kreeger et al. 2006; Kreeger et al. 2005; Kreeger, Woodruff & Shea 2003). Fibrin-alginate based matrix is responsive to follicle growth because proteases present in the matrix are activated by follicular cells during growth (Shikanov et al. 2011). Matrigel is an extract from tumor cells and consists of collagen IV, fibronectin, laminin, entactin, sulfate proteoglycans, heparins and growth factors (Oktem & Oktay 2007a). Liquid Matrigel forms a gel at or above 20°C and hence when cultured at 37°C, supports follicular growth and integrity (Oktem & Oktay 2007a). Matrigel however, does not allow expansion or growth of follicles over time (Desai et al. 2010). Another problem associated with alginate and Matrigel 3D matrices is the need to use enzymes to retrieve follicles when the culture is finished (Desai et al. 2010; Kreeger et al. 2006; Shikanov et al. 2011).

Three-dimensional culture systems must allow gas exchange and diffusion of nutrients, and this permeation is affected by the porosity of the matrix. 3D culture matrices must be not toxic to the growing cells and ideally should also allow sustainable growth for the duration of the culture period (Desai et al 2010) by supporting the growth of follicles in all directions and by holding the cellular and non-cellular compartments close together within a spherical structure (Gomes et al. 1999). Another factor affecting *in vitro* follicle culture is the co-culture of groups of follicles together, because this has been reported to promote oocyte development and follicular growth irrespective of other aspects of the culture system (Gomes et al. 1999; Spears, De Bruin & Gosden 1996) possibly because follicles can communicate with each other, as in the *in vivo* situation.

Human follicles are collected from various patient cohorts, primarily to culture and produce fertilizable oocytes that can be submitted to established clinical IVF protocols which will enable infertile couples to have children. Regulatory agencies do not permit human cells that will be implanted or transferred into patients to be exposed to animal-derived products (Lee, Cuddihy & Kotov 2008). Many advances have been made to optimize *in vitro* culture systems for follicles, but at the time of writing no method has been unequivocally proven to be effective for consistent follicular development *in vitro*. The animal-derived Matrigel culture system holds promise but needs modification to allow non-destructive isolation of the follicle from the gel, and further data are required to determine if the fibrin-alginate interpenetrating network (FA-IPN) follicle culture system supports correct morphological development.

Healthy premenopausal human ovarian tissue is scarce, and few human follicles are available for research and development. Therefore, the objective of this study was to evaluate SFX-1, a novel 3D culture matrix, in terms of its ability to support murine 3D follicular growth, and to determine if the new culture matrix is worth further study using human follicles in the future. SFX-1 is a thermosensitive synthetic matrix that is fully defined and has no components derived from animals, hence it has potential for clinical application for ovarian follicles, and also for the culture of other 3D mammalian structures *in vitro*. The SFX-1 matrix is in solution status below 33°C and gels above this temperature. This property suggested it might be easier to encapsulate and retrieve follicles from SFX-1 than from Matrigel. On the other hand, SFX-1 does not contain proteins, growth factors or structural components whereas Matrigel does, and this led to the hypothesis that follicles cultured in DMEM/F12 with 10% FCS in a 2D system would flatten, lose their spherical structure and fail to produce hormones, whereas follicles cultured in SFX-1 with DMEM/F12 and 10% FCS would retain their spherical structure and synthesize hormones, and follicles cultured in Matrigel with

DMEM/F12 and 10% FCS would have the best morphology and highest hormone production. Since this is the first study to examine follicle culture in SFX-1, it was important to determine if the new matrix was immediately and acutely cytotoxic, therefore the study aims were to quantify murine follicle cell viability, follicular growth, morphological integrity (maintenance of 3D spherical structure), and hormone production in a short 48-hour culture *in vitro*.

5.2.2 Materials and Methods

All reagents were from Sigma Aldrich (Australia) unless otherwise stated.

5.2.2.1 Synthesis of SFX-1

The SFX-1 matrix was synthesized by free radical emulsion polymerization as reported previously (Cui et al. 2016). Before the polymerization, the monomer N-Isopropylacrylamide (NIPAM) was recrystallized in n-hexane and dried in vacuum at room temperature, and acrylic acid (AA) was purified by vacuum distillation. For the SFX-1 synthesis, 9.9 mmol NIPAM, 0.1 mmol AA, 0.2 mmol N, N´-methylenebis (acrylamide) and 0.12 mmol of sodium dodecyl sulphate were added in 97 mL of water. The solution was bubbled with nitrogen for 30 min. 3.0 mL of potassium persulphate aqueous solution (0.1 mmol) was then injected into the degassing solution to initiate the polymerization at 70°C. After polymerization for 5 hours with continuous supply of nitrogen and stirring, the temperature was reduced to the ambient temperature. The as-prepared products were purified by membrane dialysis with a cut-off molecular weight of 12-14 kDa against Milli-Q water for one week with a daily water change.

5.2.2.2 Preparation of Reagents

Dulbecco's Modified Eagle's Medium with Hams F12 was supplemented with 1% insulin (5ug/mL), transferrin (5ug/mL) and selenium (5ng/mL, 'ITS', Gibco, New York, USA), and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) and hereafter referred to as DMEM/F12. For use in all three-culture systems heat inactivated foetal calf serum (FCS, DKSH, Melbourne, AUS) was added to DMEM/F12 to 10% v/v.

SFX-1 (50mg/mL) was diluted to 30 mg/mL with 40% v/v DMEM/F12 and 10% FCS on the day before each experiment. Matrigel (8.61 mg/mL) was diluted 1:1 with DMEM/F12 and 10% FCS immediately before encapsulating follicles. Live-Dead combined stain containing 10 µM Calcein AM and 20 µM Ethidium Homodimer-1 (Invitrogen, Paisley, UK) was prepared in 1X phosphate buffered saline (PBS). Collagenase IV (12 mg/mL, 295 units/mg, Worthington, New Jersey, USA) was prepared in DMEM/F12 and hyaluronidase (0.025%, 1228 units/mg, Lakewood, NJ) was prepared in 1X PBS.

5.2.2.3 Mouse Ovary Collection

Mature adult female mice (C57 BL, 10.7±1 weeks, n=8) were killed by cervical dislocation and the isolated ovaries placed in 37°C DMEM/F12 and transported to the laboratory at 37°C. These mice were excess to the needs of the breeding colony and allocated to routine cull. They were euthanized by College of Medicine animal facility staff, and the use of their ovaries for this research project was approved by the Animal Welfare Committee at Flinders University, Adelaide, South Australia.

5.2.2.4 Follicle Isolation from Ovaries

Whole ovaries were dissected free of oviducts, adipose tissue, and extraneous tissue, cut in half, and disaggregated using 0.5 mL collagenase IV (2mg/mL in DMEM/F12) for 30 minutes at 37°C with 1 min agitation after every 10 minutes, then mechanically disaggregated with 22-gauge syringe needles for 5 minutes. All released follicles were collected and transferred to a 96-well plate containing DMEM/F12 with 10% FCS.

5.2.2.5 Follicle Size Classification

Follicle diameters were initially estimated using a scale graticule in the lens eye piece of a dissecting microscope at 3X magnification. Follicles were separated into pools of primordial, primary, early secondary, late secondary or antral follicles according to the size ranges described by Griffin, Jeanine et al. (2006) and Young and McNeilly (2010). The follicles were collected from two mice on four separate occasions (n=4 experimental replicates). All the secondary follicles from two mice were combined into one pool of follicles and examined using a dissection microscope to identify follicles with excellent morphology (see below for scoring system). These were then randomly distributed to culture in SFX-1, Matrigel or DMEM/F12, such that at the beginning of culture, all follicles had high M1 or M2 scores for morphology and spherical integrity.

5.2.2.6 Follicle Culture

20 µL of Matrigel or SFX-1 or DMEM/F12 with 10% FCS were added to a 96 well plate and incubated at 37°C in a humidified incubator for 5 minutes. Groups of three secondary follicles were added to each well and overlain with 30 µL of Matrigel or SFX-1 or DMEM/F12. These were incubated at 37°C for another 5-10 minutes, to ensure that follicles were encapsulated within each matrix. The solid hydrogels (each well containing three follicles) were then overlain with 100 µL DMEM/F12 with 10% FCS, and the follicles cultured for 48h at 37°C with 5% CO_2. The SFX-1 gel contained 40% and the Matrigel contained 50% DMEM/F12 with 10% FCS, whereas the follicles in the 2D culture system were surrounded by 100% DMEM/F12 with FCS. Each of the three culture systems (Matrigel, SFX-1

and DMEM/F12) were examined in 6 replicate wells, so a total of 18 follicles were used to examine each culture condition within each of 4 experimental replicates. Follicles were photographed using an inverted microscope immediately after encapsulation and before culture. After 48 hours at 37°C, media were collected, and the follicles were left at room temperature for ten minutes to allow the matrices to cool and become liquid gel. This allowed the follicles to be retrieved without enzymes and washed with PBS. Nine follicles from 3 replicate wells were subjected to Live-Dead staining whereas follicles from the other three replicate wells were disaggregated (see below) and the isolated GCs assessed using a Trypan Blue exclusion assay.

5.2.2.7 Live-Dead Staining

Follicles (n=27) in each replicate experiment (n=4) were washed with PBS then stained with 150 µL Live-Dead combined stain at 37°C for 45 minutes. The follicles were washed with PBS before fixation in 150 µL of 4% paraformaldehyde (PFA) for 40 minutes in the dark at room temperature. The Live-Dead stained whole follicles were placed onto a poly-L-lysine coated glass microscope slide with 5 µL buffered glycerol and a cover slip.

Images of Live-Dead stained whole follicles were captured using a fluorescence microscope (Bright field BX50, Olympus) at 20x magnification using Micro-Manager (v1.4.13) image capture software. The fluorescence microphotographs were analysed using Image J (1.49v), and the diameters of each follicle were determined by drawing a straight line across the follicle three times then calculating the average.

After subtracting non-specific background fluorescence, the grey scale image was used to identify all Live-stained, or Dead-stained areas. Overlapping stained areas were subdivided by applying an Image J plugin program 'Watershed'. A 'Particle analyser' function in Image J was then used to count the number of separate stained areas. These were outlined to check against the original image, and the estimated numbers were recorded for each follicle.

5.2.2.8 Follicle Disaggregation and Trypan Blue Exclusion Assay

Follicles (n=27) in each replicate experiment (n=4) were disaggregated in collagenase IV for 30 minutes before treatment in hyaluronidase for another 30 minutes. The GCs harvested from each group of 3 disaggregated follicles were resuspended in 20 µL DMEM/F12, then 20 µL Trypan Blue was added to these cell suspensions. 10 µL was loaded onto a haemocytometer, and the live and dead cells in 9 large squares were counted. The mean±SD numbers of viable or dead GCs were

expressed per follicle, and the numbers of viable cells were expressed as percentage of the total number of GCs from one follicle.

5.2.2.9 Follicle Growth and Morphology

After 48h culture, only the diameters of the Live-Dead stained follicles were measured because follicles from the other three technical replicates were disaggregated and the GCs were assessed using Trypan Blue. The morphology of each Live-Dead stained follicle was classified as being intact (M1, the follicle appeared completely enclosed by the basal lamina), or largely intact but of lower morphological integrity (M2, the basal lamina appeared irregular or broken in places, but without any loss or displacement of GC), or slightly (M3) or completely disrupted (M4) (Dolmans et al. 2006; Young et al. 2017).

5.2.2.10 Progesterone Measurement

In each experiment and for each culture condition (SFX-1 or Matrigel or DMEM/F12), conditioned media were collected from two replicate wells, each containing three follicles, after 48 hours of culture. The conditioned medium from each culture well was examined in one well of a competitive enzyme-linked immunoassay (EIA, Cayman Chemical ELISA, Ann Arbor, MI, USA), and duplicate wells were used to measure progesterone production. A mouse monoclonal anti-rabbit IgG, and an acetylcholinesterase progesterone tracer were used for the progesterone EIA. The manufacturer reported a detection limit of 10pg/mL, an intra-assay coefficient of variation (CoV) of 7.5%, and an inter-assay CoV of 2.9% for this assay. For this study, the progesterone standard was diluted in DMEM/F12 with 10% FCS to give concentrations that ranged from 7.18 to 1000 pg/mL.

5.2.2.11 Estradiol Measurement

In each experiment, and for each culture condition, conditioned media were collected from two replicate wells (each containing 3 follicles) after 48 hours of culture and examined in a competitive EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that used a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. The manufacturer reported a detection limit of 15 pg/mL, and an intra-assay coefficient of variation (CoV) of 7.8 to 18.8% for this assay. For this study, the estradiol standard was diluted in the DMEM/F12 with 10% FCS to give concentrations that ranged from 6.6 to 4000 pg/mL.

5.2.2.12 Anti-Mullerian Hormone (AMH) Enzyme Immunoassay

As described above, conditioned media from two replicate wells (each containing 3 follicles) were collected after 48 hours of culture. Conditioned media samples were examined in a two-step

sandwich type EIA (Immunotech, Marseille Cedex, France) that used an anti-AMH monoclonal antibody for capturing AMH, and biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH in the wells. The manufacturer reported a detection limit of 1 pg/mL, an intra-assay coefficient of variation (CoV) of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standards were diluted in DMEM/F12 with 10% FCS to give concentrations that ranged from 0 to 150 pM.

5.2.3 Statistical Analysis

The mean±SD values of four experimental replicate (n=4) were determined for each culture condition and experimental output (follicle diameter, granulosa cell viability, P4, E2 or AMH concentration). Follicle diameters were subjected to one-way ANOVA with Tukey's Multiple Comparison test, whereas the numbers of live and dead GCs were subjected to a two-way ANOVA with Bonferroni post-test. Statistical analyses were carried out using GraphPad Prism, and statistical significance was assigned at $p<0.05$.

5.2.4 Results

5.2.4.1 Follicle Growth

The initial diameter of the subset of secondary follicles that were allocated to Live-Dead staining was the same as the entire cohort of secondary follicles (Table 5.1), and there was no difference between the follicles that were randomly allocated to each of the three culture conditions (SFX-1, Matrigel or DMEM/F12) at the beginning of the experiment (Table 5.1)). There was a significant increase ($p<0.05$) in follicle diameter in all three matrices after 48-hour culture, but no difference between the three culture conditions; the SFX-1 matrix supported follicle growth to the same extent as Matrigel.

Table 5. 1: Follicle growth after 48h in vitro.

*Murine secondary follicles (n=216) were cultured in groups of three for 48h in SFX-1, Matrigel or DMEM/F12. Half the follicles were assessed using Live-Dead stain. Photomicrographs of follicles were taken before and after culture, and the images used to determine diameter. The mean±SD diameter of follicles in 4 replicate experiments shown (n=4), and the data assessed by one-way ANOVA with Tukey's Multiple Comparison test. *p<0.05.*

Culture system	Initial Follicle Diameter (µm)	Initial Diameter (µm) of Follicles allocated to Live-Dead staining	Final Diameter (µm) of Live-Dead stained follicles	Percent increase (%)
SFX-1 (3D)	144±20	153±28	201±38 *	43±30
Matrigel (3D)	152±8	152±5	213±42 *	40±29
DMEM/F12 (2D)	148±6	145±6	205±22 *	41±18

5.2.4.2 Follicle Morphology

Green Live-staining was localised to the cytoplasm of GCs, although in some follicles the staining was more punctate (Figure 5.1A) than in others (Figure 5.1 B). Diffusive Live-staining was seen in the vicinity of the oocyte. Red Dead-staining was localised to the nuclei of GCs (Figure 5.1 C) and to the large nucleus of the oocyte in highly disrupted follicles with low M4 morphology scores (Figure 5.1D). Fewer than half the follicles were retrieved from the wells (SFX 44%, Matrigel 41%, DMEM/F12 25% retrieved) because in many cases the GCs attached to the floor or wall of the culture wells and formed a monolayer, and the oocytes were not enclosed by GCs (Figure 5.1E).

All the follicles (n=36 in each matrix) had an intact spherical morphology which was scored as being M1 or M2 at the commencement of culture. The highest numbers of follicles were retrieved (and Live-Dead stained) from SFX-1 after 48h culture (n=16 follicles), a similar number (n=15) were retrieved after culture in Matrigel, but only nine follicles were retrieved after 48h culture in the 2D DMEM/F12 system. The follicles that could not be retrieved from the culture wells did not have an intact spherical structure and were more disrupted than the follicles given M4 scores (Figure 5.1 F), hence the highest number of follicles with poor, non-spherical morphology resulted from culture in DMEM/F12.

Matrigel maintained an intact spherical M1 or M2-graded morphology in 47% of the follicles that could be retrieved (Figure 5. 2), the 2D DMEM/F12 culture system did not support the maintenance of any follicles with M1 scores, although 44% of the low number of follicles that were retrieved had M2 scores, and in SFX-1 only 12.5% of retrieved follicles maintained M1 or M2 morphological scores. There were however significantly higher numbers of follicles with M3 scores ($p<0.01$) in SFX-1 than those with M1 or M2 scores.

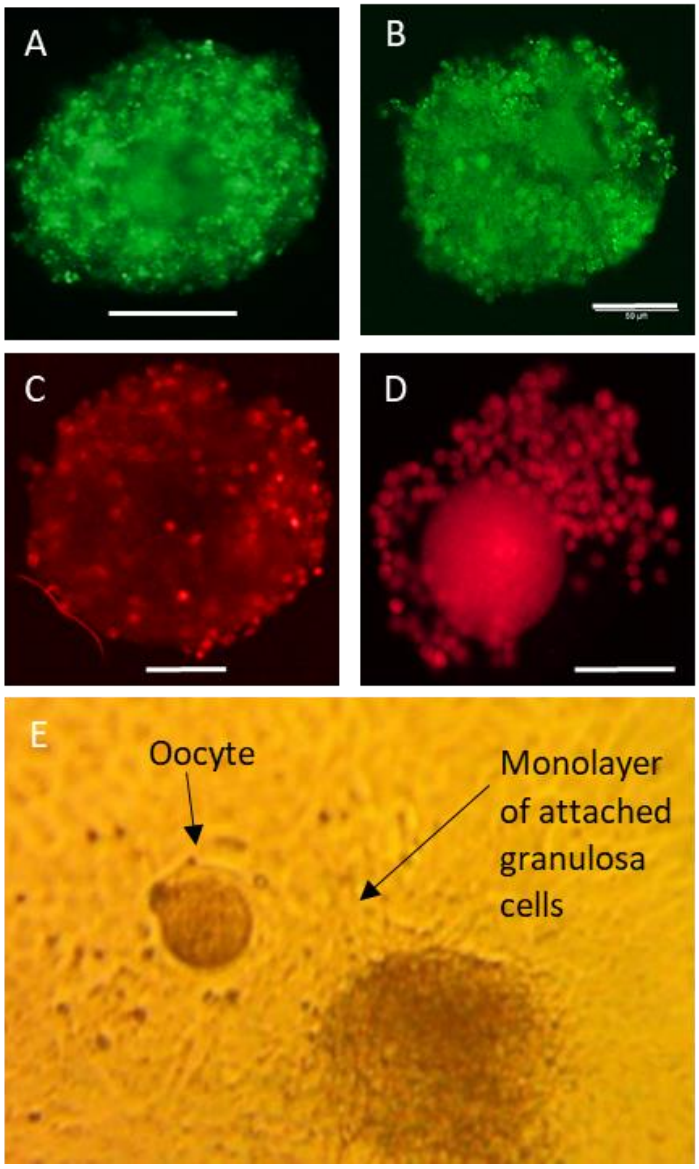


Figure 5. 1: Effect of culture on follicle viability and morphology.

Intact spherical murine secondary follicles with high morphological quality scores of M1 and M2 were cultured for 48h in 3D or 2D matrices, retrieved and Live-Dead stained. A) An M2 Live-stained follicle cultured in Matrigel. B) M3 Live-stained follicle cultured in SFX-1. C) M3 Dead-stained follicle cultured in SFX-1. D) Disrupted M4 Dead-stained follicle cultured in DMEM/F12. E) Disrupted follicle with extruded oocyte in a culture well that could not be retrieved nor Live-Dead stained.

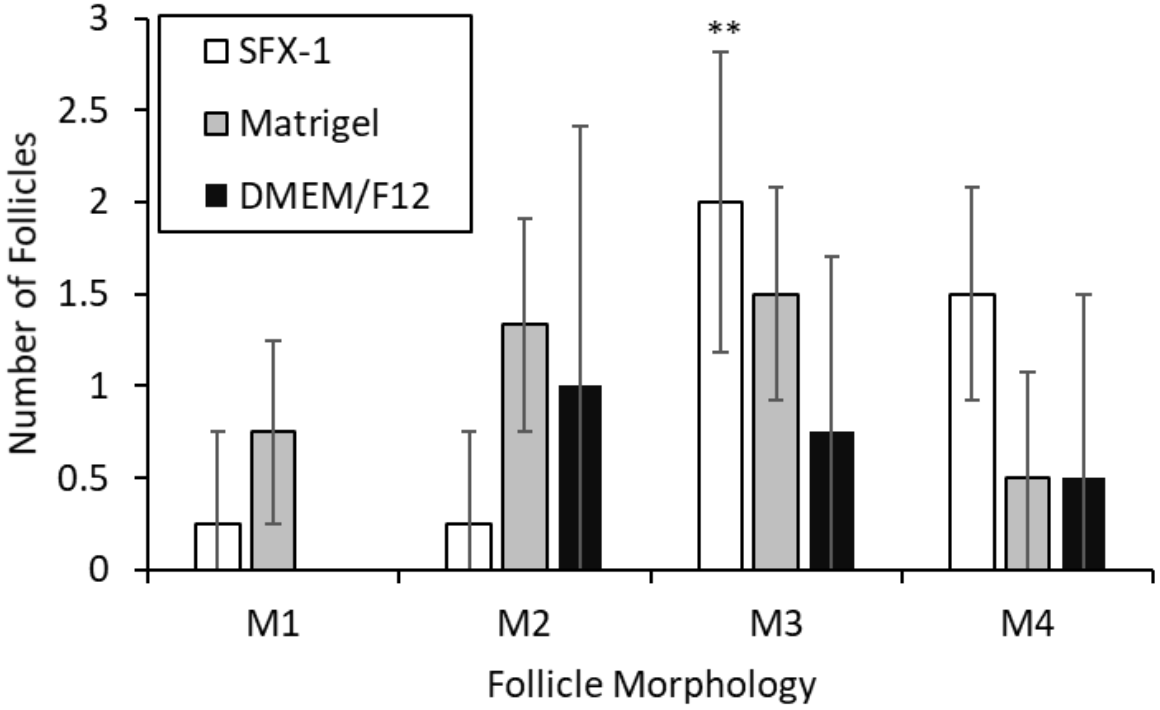


Figure 5. 2: Effect of culture on follicle morphology.

*Intact spherical murine secondary follicles with high morphological quality scores of M1 and M2 were cultured for 48h in 3D SFX-1 (n=36) or Matrigel (n=36), or in 2D DMEM/F12 (n=36). The follicles were retrieved, Live-Dead stained and given scores for spherical integrity and morphological quality. M1 and M2 no loss of GCs, M3 <10% loss of GCs, M4 highly disrupted with >10% loss of GCs. The experiment was repeated on four separate occasions (n=4, 9 follicles per matrix per experiment) and Mean ± StDev number of follicles in each morphological category shown. SFX-1 data subjected to 1-Way ANOVA with Tukey post-test, ** p<0.01 compared to M1 & M2.*

5.2.4.3 Follicular Cell Viability

Although follicles were washed free of Matrigel, it was not possible to produce a single cell suspension that could be assessed in a Trypan Blue Exclusion assay. The numbers of cells in follicles grown in 3D SFX-1 were not significantly higher than in follicles grown in 2D DMEM/F12 cell culture medium (Figure 5. 3 A) when assessed by Trypan Blue, and the percentage of viable cells was similar; 64±8 and 69±9 in SFX-1 and DMEM/F12 respectively. Intact Live-Dead stained follicles were assessed using an image analysis protocol, which generated data suggesting that these follicles contained more GCs (average 486±49, Figure 5. 3 B) than the disaggregated follicles assessed by Trypan Blue (average 279±108, Figure 5. 3 A). The percentages of viable cells in intact Live-Dead stained follicles were similar after culture in the three matrices; 8±10% in SFX-1, 20±6% in Matrigel and 14±13% in DMEM/F12 (Figure 5. 3 B).

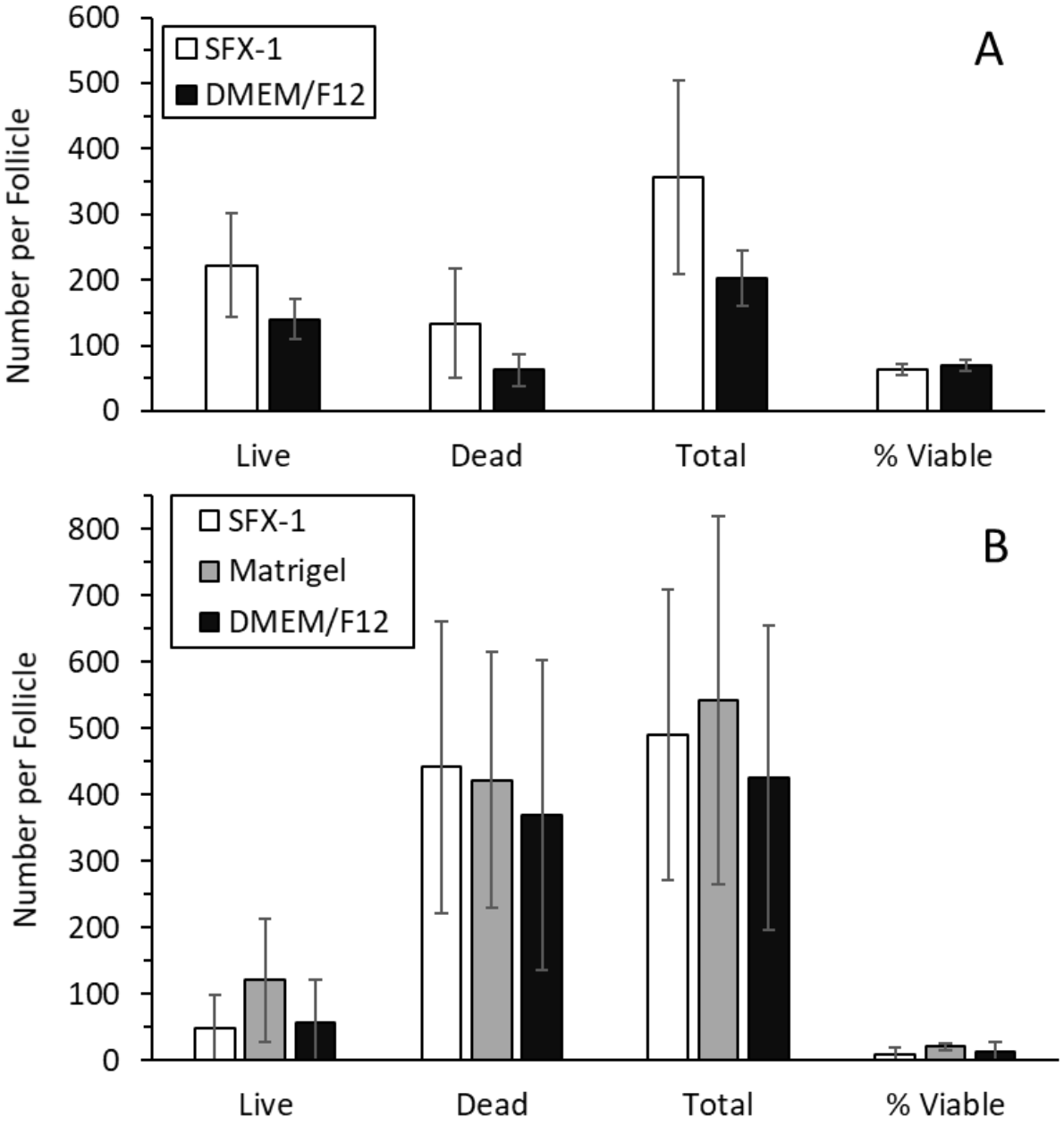


Figure 5. 3: Effect of 48h follicle culture on cell viability.

Groups of three murine secondary follicles per well were cultured in SFX-1, Matrigel or in DMEM/F12 medium. After 48h in vitro, follicles were A) disaggregated to produce a single cell suspension which was assessed using a Trypan Blue Exclusion assay to identify live and dead cells or B) Live-Dead stained and Image Analysis used to quantify the number of areas of staining. Live cells were expressed as a percentage of total cells per follicle. The experiment was repeated on four separate occasions (n=4) and Mean ± StDev number per follicle shown.

5.2.4.4 Follicle Hormone Production

After a 48h culture period, follicles cultured in SFX-1, Matrigel and DMEM did not produce any detectable AMH (Table 5. 2). Follicles cultured in SFX-1 and Matrigel produced progesterone, and only follicles that were cultured in Matrigel produced estrogen.

Table 5. 2: Hormone produced by follicles after 48 hours culture.

Groups of three follicles were cultured in SFX-1, Matrigel or DMEM/F12 for 48h. Conditioned media were collected, and anti-mullerian hormone (AMH), estradiol 17β (E2) and progesterone (P4) produced by groups of 3 follicles were measured in duplicate by enzyme immuno-assay. The mean±SD pg/mL (n=4) shown. ND: not detectable.

Hormone	SFX-1	Matrigel	DMEM/F12
AMH	ND	ND	ND
E2	ND	17.8±9	ND
P4	478±285	506±210	ND

5.2.5 Discussion

Mouse ovarian follicles were cultured in a novel, fully defined 3D synthetic gel system for the first time and compared with follicles grown in Matrigel or DMEM/F12 for a short 48h period. Although the only nutrients in the SFX-1 3D gel system derived from the medium (DMEM/F12 with 10% FCS and 1% ITS), the diameters and morphological integrity of follicles grown in SFX-1 were not significantly lower than those grown in Matrigel and were clearly superior to those grown in DMEM/F12 with 10% FCS. All three culture systems supported short-term growth as indicated by increases in follicle diameters, but the morphological integrity scores were highest in Matrigel, as expected. This was probably because Matrigel is a biologically active matrix that contains collagen, laminin and sulfate proteoglycans, (Rodgers et al. 1998) as well as other growth factors such as EGF, FGF, IGF-1, PDGF and TGF-β (Oktem & Oktay 2007a), and these facilitated adhesion of GCs to each other and the oocyte (Kreeger et al. 2006). Nevertheless, it was clear that 3D structural support in the presence of minimal nutrients, as characterized by the SFX-1 culture system, was sufficient to support follicle growth and maintain a spherical structure. In addition to this, the highest numbers of follicles were retrieved from SFX-1. Although more than half of the encapsulated follicles in the 3D systems could not be retrieved, a problem noted by others who used 3D matrices containing collagen (Desai et al. 2010; Telfer 1996; Torrance, Telfer & Gosden 1989), in future studies components of the ovarian ECM (Gomes et al. 1999; Irving-Rodgers et al. 2010; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; van Wezel, Ingrid L & Rodgers, Raymond J 1996) may be

incorporated into SFX-1 in a gradual process designed to identify the combination that maximizes follicle growth and retrieval.

The total numbers of GCs estimated by Live-Dead staining were higher than those estimated by Trypan Blue. This discrepancy was probably a consequence of the heterogeneous Live-staining localization which confounded our automated Image Analysis protocol; many areas of Live-staining were smaller than the area of a granulosa cell, and hence the total number of GCs in Live-stained follicles appeared higher than that obtained using the Trypan Blue exclusion assay. Nevertheless, both protocols yielded values similar to those in published reports; 100-400 GCs per secondary follicle (Griffin, Jeanine et al. 2006; Lintern-Moore & Moore 1979; Pedersen & Peters 1968).

There were concerns that the enzyme disaggregation of follicles, and the centrifugation force applied to retrieve the disaggregated GCs, might have reduced viability before assessment in the Trypan Blue exclusion assay (Li et al. 2016; Lunardi et al. 2012; Orimoto et al. 2008; Rodgers et al. 1998; Stojkovic et al. 2001), but the proportions of viable GCs were lower when quantified using Live-Dead stain (~15%) than when using Trypan Blue (~65%). The Live-Dead stain identification of viable cells is based on activity of cytoplasmic esterase enzyme and conversion of Calcein AM to fluorescent Calcein. However, *in vitro* follicle culture at atmospheric oxygen levels and 5% CO_2 is associated with increased production of Reactive Oxygen Species which in turn reduce the activity of cytoplasmic esterase (Devine, Perreault & Luderer 2012; Filatov, Khramova & Semenova 2015; Gook et al. 2013; Xian et al. 2013). Trypan Blue measures cell membrane integrity while Live-Dead stain measures enzyme activity, and the different mechanisms of action may account for the differences in viable cell quantification found in this study. Irrespective of the method used to quantify viable cells, fewer than half of the cells appeared to be viable in each follicle after 48h *in vitro*, whereas in situ studies reported that 80% of GCs were viable (Aerts et al. 2008; Campos et al. 2011; Kreeger et al. 2006; Lundy et al. 1999; Merdassi et al. 2011). It was surprising that Matrigel did not maintain significantly higher viable cell numbers than either of the other two culture systems. Altogether our results suggest that the isolation of follicles from ovarian tissue, followed by a 48h culture at atmospheric oxygen levels and 5% CO_2 in any system, reduced the viability of follicular GCs. In future, modified SFX-1 will be used in conjunction with other factors that promote follicle growth *in vitro*, such as the partial pressures of oxygen and CO_2 (Gook et al. 2013; Heise et al. 2009; Silva et al. 2010; Xu et al. 2011)

Hormones produced by GCs are an important indicator of follicle viability and maturation. Matrigel supported the production of both estrogen and progesterone but none of the culture systems supported AMH production, even though it has been reported that secondary follicles express the gene for AMH (Baarends et al. 1995; Pask et al. 2004; Webber et al. 2003; Weenen et al. 2004a). It is possible than the relatively low levels of granulosa cell viability, and impaired cell-cell communication (Desai et al. 2010), may have prevented AMH synthesis and secretion.

Our hypothesis was supported; Matrigel supported the growth of high quality morphologically intact follicles that produced steroid hormones, but the retrieval of follicles from Matrigel was difficult. Follicles cultured in DMEM/F12 with 10% FCS in a 2D system lost their spherical structure and failed to produce hormones, in contrast to follicles also cultured in DMEM/F12 and 10% FCS but with the addition of SFX-1, which retained their spherical structure and synthesized the steroid hormone progesterone. Importantly, SFX-1 was not acutely cytotoxic to follicular cells during 48h *in vitro*.

We conclude that SFX-1 is a promising synthetic 3D culture matrix that was not cytotoxic and supported follicle growth, in the absence of any animal-derived structural components or growth factors, apart from those in 10% FCS. This study supports further development of SFX-1 for clinical application to the *in vitro* maturation of follicles, and we recommend future studies in which defined extracellular components and recombinant growth factors are added to SFX-1 for the extended culture of follicles. Although SFX-1 is a promising 3D culture matrix, it did not support follicle integrity. Also, follicular granulosa cell viability was low in Matrigel and SFX but Matrigel had higher number of total GC and higher viability. Most importantly, Matrigel supported the synthesis of both estrogen and progesterone. Therefore, for the effect of chemotherapeutics on follicle *in vitro* study, we decided to use Matrigel for culturing follicle.

6. CHAPTER SIX: EFFECT OF CHEMOTHERAPEUTICS AND GAMMA TOCOPHEROL ON MURINE OVARIAN FOLLICLES CULTURED IN MATRIGEL

Some replication of content (introduction and background) follows because thesis section 2.6 has been submitted as a manuscript for publication. The datasets presented in section 2.6 are distinct and different from previous datasets.

6.1 Introduction

Breast cancer is the most commonly diagnosed cancer in women with 1.7 million diagnoses globally (DeSantis et al. 2016; Tao et al. 2015). In the United States of America, 246,660 new cases of breast cancer were diagnosed in women in 2016 (Siegel, Miller & Jemal 2016) and according to the National Breast Cancer Foundation (NBCF 2018), one in 8 women in Australia are diagnosed with breast cancer. There were 17,586 breast cancer patients in Australia in 2017 (AIHW 2017), of whom 37% were 15 to 54 years of age and 5% were younger than 40 years of age (AIHW 2017). The five year survival rate for breast cancer is now 80% to 90% (Ferlay et al. 2015; Morgan et al. 2012).

Breast cancer patients are treated with doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 (Ganz et al. 2017; Guo et al. 2011; Nahleh et al. 2016; Shulman et al. 2014; Singh et al. 2017; Swenson et al. 2003). After infusion of both chemotherapeutics plasma concentrations of doxorubicin were 1.8±0.4µM during the first cycle of chemotherapy (Swenson et al. 2003). Cyclophosphamide however is metabolized to 4-hydroxycyclophosphamide (4OHCYC) *in vivo*, and this hepatic activation is required for chemotherapeutic efficacy (De Jonge, Milly E et al. 2005; Struck et al. 1987; Teicher, B et al. 1996; Yuksel et al. 2015). A synthetic compound, 4-hydroperoxycyclophosphamide (4HPCYP), undergoes spontaneous hydrolysis to 4-hydroxycyclophosphamide (4OHCYC) in aqueous solutions such as cell culture media. These two molecules cause similar levels of DNA cross-linking and cytotoxicity at equimolar concentrations (Ozer et al. 1982), and hence 4-hydroperoxycyclophosphamide or its metabolite 4OHCYC have been used to examine the effects of cyclophosphamide *in vitro* (Dees et al. 2000; Ozer et al. 1982) and *in vivo* (Teicher, B et al. 1996; Yuksel et al. 2015). Struck et al. (1987) administered doses of cyclophosphamide (600mg/m^2) to different oncology patients and recorded serum concentrations of 4OHCYC that ranged from 0.02 to 0.18 µg/mL in the 24h period after administration. The serum concentrations of 4OHCYC were highest 2-4h after the IV administration of cyclophosphamide (Struck 1987), approximately 0.063

µg/mL (0.2 µM). Hence, when doxorubicin and cyclophosphamide are co-administered, the *in vivo* serum concentrations are likely to be 1.8µM doxorubicin and 0.2µM 4OHCYC, a ratio of 9:1.

The co-administration of doxorubicin and cyclophosphamide to breast cancer patients is associated with a number of acute side-effects (Legha et al. 1982; Morgan et al. 1997; Wallace 2003; Yeh & Bickford 2009). Doxorubicin inhibits topoisomerase II activity and causes DNA double-strand breaks (Kalyanaraman, Perez-Reyes & Mason 1980; Swift et al. 2006; Tewey et al. 1984; Thorn et al. 2011; Xiao et al. 2017). Other mechanisms of action of doxorubicin include the generation of reactive oxygen species (ROS) with subsequent lipid peroxidation (Batist et al. 2001; Gewirtz 1999; Kasapović et al. 2010), and DNA intercalation that prevents DNA replication and synthesis (Legha et al. 1982; Rogalska et al. 2011). Doxorubicin has also been associated with cardiotoxicity through the production of ROS such as hydrogen peroxide and superoxide. These stimulated the production of nitric oxide synthase in myocytes and endothelial cells and caused apoptosis of cardiomyocytes. This led to cardiomyopathy and the production of hydroxyl and perferryl ions, which altered mitochondrial respiration and enzyme functions in cardiac cells (Goodman & Hochstein 1977; Kalyanaraman et al. 2002; Minotti, Cairo & Monti 1999; Singal & Panagia 1984).

Cyclophosphamide is an alkylating agent and it's active metabolites including 4OHCYC act in three ways; cross linking of DNA though attachment to guanine bases, prevention of RNA transcription and DNA synthesis and mutations due to nucleotide mispairing (Arnold, Bourseaux & Brock 1958; Bagley, Bostick & DeVita 1973; Boddy & Yule 2000; De Jonge, Milly E et al. 2005; Emadi, Jones & Brodsky 2009; Peigne & Decanter 2014; Siddik 2002; Sladek 1972; Struck et al. 1987; Teicher, B et al. 1996; Yuksel et al. 2015; Yule et al. 1995). The metabolite, acrolein, is responsible for producing ROS. In addition, oxidative metabolism of cyclophosphamide produces ROS; such as the hydroxyl radical, hydrogen peroxide, nitric oxide (NO) and superoxide anion. Together these ROS depress cellular antioxidant defence systems and cause lipid peroxidation, protein oxidation and DNA strand breaks (Boddy & Yule 2000; Korkmaz, Topal & Oter 2007; Selvakumar et al. 2005; Sevko et al. 2013; Yule et al. 1995). The active metabolite 4OHCYC is distributed throughout the body as well as to cancer cells (Siddik 2002).

Undesirable side-effects of chemotherapy include infertility and premature menopause (Ben-Aharon et al. 2010a; Desmeules & Devine 2006; Jurisicova et al. 2006; Meirow et al. 1999; Morgan et al. 2012; Oktem & Oktay 2007c; Perez et al. 1997; Petrillo et al. 2011; Roti et al. 2012; Yucebilgin et al. 2004; Yuksel et al. 2015). In general chemotherapeutics reduce fertility to varying degrees

depending on the age of the patient, the chemotherapeutics, and the number of administered cycles (Blumenfeld 2012; Morgan et al. 2012). Girls are born with a fixed number of small primordial follicles (Anderson & Wallace 2011; Morgan et al. 2012). After puberty, some primordial follicles are activated at regular intervals, after which the GCs proliferate and the follicles grow and support meiotic maturation of the oocyte (Knight & Glister 2006). Proliferating GCs in secondary and early antral follicles secrete anti-Mullerian hormone (AMH), which prevents activation of primordial follicles. It is thought that chemotherapy-induced granulosa cell death reduces AMH production, which results in the activation of more primordial follicles, which in turn are destroyed in subsequent rounds of chemotherapy (Anderson & Cameron 2011; Morgan et al. 2012). A number of clinical studies have described decreases in serum AMH concentrations and amenorrhea one year post-chemotherapy (Anderson & Cameron 2011; Peigne & Decanter 2014), but the direct effect of chemotherapeutics on follicular GCs is an emerging field of research (Morgan et al. 2012).

Depending on the patient's age, treatment with doxorubicin alone led to amenorrhea in 20 to 80% of patients (Ben-Aharon et al. 2010a; Knobf 2006; Lee et al. 2006). Doxorubicin caused oocyte apoptosis, reductions in the size of ovaries (Ben-Aharon et al. 2010b; Grzanka, Domaniewski & Grzanka 2005; Perez et al. 1997; Roti et al. 2012), reductions in primordial follicle reserves (Familiari et al. 1993; Marcello et al. 1990) and estrogen synthesis *in vitro* (Xiao et al. 2017). Hence doxorubicin is associated with amenorrhea, infertility and cardiotoxicity. Similar to doxorubicin, cyclophosphamide also caused premature ovarian failure subsequent to the depletion of ovarian follicle reserves (Ataya et al. 1990; Detti et al. 2013; Himelstein-Braw, Peters & Faber 1978; Oktay & Oktem 2007), apoptosis of GCs (Desmeules & Devine 2005; Meirow 2000; Oktem & Oktay 2007c; Perez et al. 1997; Ramahi-Ataya et al. 1988), fibrosis in the ovarian cortex (Meirow et al. 2007), damage to oocytes (Ataya et al. 1990; Desmeules & Devine 2005), and reduction of progesterone (Ramahi-Ataya et al. 1988), estrogen (Oktem & Oktay 2007c)and AMH synthesis (Detti et al. 2013). Kalich-Philosoph et al. (2013) also reported that cyclophosphamide upregulated the PI3K pathway which subsequently initiated recruitment of dormant follicles and led to burnout of ovarian follicles. When both doxorubicin and cyclophosphamide were used in combination to treat cancers of various types, there were significant reductions in primordial follicle counts per square mm of ovarian sections, and corresponding reductions in estrogen production (Oktem & Oktay 2007c). Depletion of ovarian follicle reserves leads to the development of infertility (Bines 1995; Martin et al. 2005; Nabholtz 2002; Partridge et al. 2004; Schmidt et al. 2005; Stone et al. 2000; Tham et al. 2007). However, the assessment of the impact of chemotherapy on ovarian function is mainly based on

the indirect parameters such as amenorrhea and hormone levels (Ben-Aharon et al. 2010a; Knobf 2006; Lee et al. 2006) and not on direct observations of ovarian follicles.

Vitamin E refers to eight different molecules synthesized by plants; alpha, beta, gamma and delta tocopherol, and the four tocotrienol forms of these molecules (Brigelius-Flohe et al. 2002; Dietrich et al. 2006; Jiang et al. 2004; Lu, J et al. 2010; Smolarek, AK & Suh, N 2011). They are lipophilic antioxidants which can reduce ROS-induced lipid peroxidation (Brigelius-Flohe et al. 2002; Smolarek, AK & Suh, N 2011). Among all the tocopherols, gamma tocopherol (gToc) is the dominant form in the human diet (Baumeister et al. 2009; Bieri & Evarts 1974; Lu, J et al. 2010). Gamma and delta tocopherols reduced the growth of human large-cell lung carcinoma cells *in vivo* and *in vitro*, whereas alpha tocopherol had no effect on cell viability (Lu, J et al. 2010).

In vitro studies have indicated that gToc inhibited neoplastic transformation and cancer cell proliferation (Betti et al. 2006; Cooney et al. 1993; Gysin, Azzi & Visarius 2002), and had anti-tumor activity in animal models of colon and prostate cancer (Campbell et al. 2003; Smolarek, AK & Suh, N 2011). Gamma tocopherol delayed the formation of breast cancer tumors in rodent models (Smolarek, AK & Suh, N 2011) and inhibited the proliferation of human breast and prostate cancer cells *in vitro* (Gysin, Azzi & Visarius 2002; Lee et al. 2009; Smolarek, AK & Suh, N 2011). Another study supported the anticancer activity of gToc because it showed that it increased the levels of pro-apoptotic proteins and inhibited expression of anti-apoptotic proteins *in vivo* (Smolarek, AK & Suh, N 2011). However, it is not known if gToc has any effect on normal or primary-derived cells *in vitro*. In addition to cytotoxic effects, gToc also appears to have protective antioxidant effects; it decreased lipid hydroperoxides in colon cancer (Gao et al. 2002), and also had cardioprotective effects (Baumeister et al. 2009).

The idea to add antioxidant gamma tocopherol to the combined doxorubicin and cyclophosphamide chemotherapeutic regimen arose from the realization that, its anticancer effect might augment doxorubicin and cyclophosphamide while its antioxidant effects might ameliorate adverse side-effects caused by ROS. It is recognized that ROS might be integral part of the chemotherapeutic effect, and that *in vitro* studies are required to examine the interaction between cytotoxic and antioxidant activities. Another reason for examining the effect of doxorubicin and cyclophosphamide with gToc on the ovary is that steroidogenesis in the ovary is associated cytochrome P450 generated ROS. *In vivo* this is counteracted by intrinsic superoxide dismutase and catalase, but in situations in which oxidative stress is increased by treatment doxorubicin and

cyclophosphamide, we propose that the increased generation of ROS may be neutralized by gToc and so maintain hormone production by the ovarian cells (Hanukoglu 2006; Rapoport, Hanukoglu & Sklan 1994; Rapoport, Sklan & Hanukoglu 1995). Hence, if the undesirable side-effects of cyclophosphamide and doxorubicin can be reduced by co-administration of gToc, there may be scope for the development of modified chemotherapeutic regimen with fewer adverse effects on the ovary.

We therefore hypothesise that the exposure of normal primary-derived, non-cancerous murine ovarian follicles to cyclophosphamide and doxorubicin singly and in combination will cause granulosa cell death and ROS generation and will reduce the number of viable cells in a time-dependent manner. Chemotherapeutic-induced ROS will additionally inhibit hormone production. Gamma tocopherol alone will cause a decrease in the number of proliferating follicle cells (because it kills proliferating cells), but the antioxidant properties of gToc will support steroid hormone production. The addition of gToc to the two chemotherapeutic agents will inhibit ROS generation and support hormone synthesis whilst augmenting the anti-proliferative activities of doxorubicin and cyclophosphamide. The aim of this study is to characterize the balance or tipping point between hormone production and the death of proliferating cells in ovarian follicles.

6.2 Materials and Methods

All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

6.2.1 Preparation of Culture Medium and 3D Follicle Culture Matrix

Alpha Modified Eagle's Medium (αMEM) was supplemented with 1% insulin (5ug/mL), transferrin (5ug/mL) and selenium (5ng/mL, 'ITS', Gibco, New York, USA), 0.5% glutamine (200mM), 1µM testosterone (University of California, Davis) and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) and is referred to as complete medium. Ovary disaggregation medium was prepared by supplementing αMEM with 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) and 0.6% HEPES. Matrigel (8.61 mg/mL) was used as a three-dimensional (3D) follicle culture system that was diluted 1:1 with 2X concentration of test reagents (see below and Table 6. 1) in complete media immediately before encapsulating follicles.

6.2.2 Preparation of Test Reagents

Doxorubicin (Dox) (Sigma, Sydney, Australia) and 4-hydroperoxycyclophosphamide (active metabolite of cyclophosphamide, 4HPCYP) (ThermoFisher Scientific, Victoria, Australia) were each added to sterile water to make stock solutions of 100 μM and 1000 μM respectively. These were stored at 4°C and -20°C respectively (Hoffman et al. 1979; Ulukaya et al. 2008). Immediately before use they were diluted further to the desired concentrations (see below and Table 6. 1) in complete media with 100 mIU Follicle Stimulating Hormone (FSH) (Merck Serono Australia Pty Ltd., NSW). Gamma-tocopherol (gToc) (Sigma, Sydney, Australia) was dissolved in DMSO to prepare a stock solution of 1000μM, and stored at 4°C. Immediately before use it was diluted further to the desired concentrations (see below and Table 6. 1) in complete media with 100 mIU FSH, such that the concentration of DMSO that cells were exposed to was 0.3% DMSO. There was no significant difference in cell viability, or estrogen production by cells exposed to 0.3% DMSO when compared to complete cell culture medium alone.

6.2.3 Determination of Inhibitory Concentrations of Chemotherapeutics and gToc

The human epithelial breast adenocarcinoma cell line (MCF-7, ATCC® HTB-22™) was obtained from the America Type Culture Collection (ATCC) and maintained in RPMI media, supplemented with 10% foetal calf serum (FCS) and 1% v/v of 10,000 units/mL penicillin and 10mg/mL streptomycin. Media were replaced every 2-3 days and cells were harvested with 0.1% trypsin EDTA solution and sub-cultured when 80% confluent. Cell culture flasks containing cells in exponential growth phase were used for all experiments. A 96 well plate was seeded with 20,000 cells per well and incubated for 24 hours to allow cell adherence condition at 37°C and 5% CO_2. Cells were exposed to incremental concentrations of test reagents for 24h and cell viability was examined in a crystal violet assay. Inhibitory concentrations corresponding to the values that killed 25% (IC25) and 50% (IC50) of the MCF-7 cells were calculated using a non-linear regression analysis generated by GraphPad Prism (Version 5.00, San Diego, California, USA). The experiment was repeated on 3 separate occasions. Mean IC25 and IC50 values are shown (Table 6. 1). The selection of doses for examination in this study were therefore based on the IC25 values obtained from MCF7 breast cancer cells (Table 6. 1).

Table 6. 1: Inhibitory concentrations against MCF-7 breast cancer cells.

MCF-7 cells were allowed 24h for adherence at a density of 20,000 per well in a 96 well plate before exposing to test reagents at incremental doses for 24 hours at 37°C with 5% CO_2. After treatment, cell viability was examined in a crystal violet assay. Inhibitory concentrations corresponding to the values that killed 25% (IC25) and 50% (IC50) of the MCF-7 cells were calculated using a non-linear regression analysis. Mean±SD of 3 (n=3) experimental replicates presented.

Test Reagents	IC 25 (µM)	IC 50 (µM)
Doxorubicin (Dox)	1.21	3.63
4 Hydroperoxy-Cyclophosphamide (4HPCYC)	21.23	63.69
Gamma Tocopherol (gToc)	35.1	105.3

6.2.4 Mouse Ovary Collection

Mature adult female mice (BalbC, 12±2 weeks, n=12) were killed by cervical dislocation and the isolated ovaries placed in 37°C disaggregation medium and transported to the laboratory at 37°C. Mice used for this study were excess to the needs of the breeding colony and were therefore allocated for routine cull. The use of ovaries from cull mice for this study was approved and registered by the Animal Welfare Committee at Flinders University, Adelaide, South Australia.

6.2.5 Follicle Isolation from Ovaries

Ovaries were dissected free of oviducts, adipose tissue and extraneous tissue, and mechanically disaggregated using 22-gauge syringe needles until all individual follicles were isolated. The follicles were collected and transferred to a 4-well GPS plate (LifeGlobal, Victoria, Australia) containing complete media.

6.2.6 Follicle Size Classification

The diameter of isolated follicles was initially measured at 3X magnification by using a scale graticule in the eye piece of a dissection microscope. Only secondary sized follicles (Griffin, Jeanine et al. 2006; Young & McNeilly 2010) that were morphologically intact (M1/M2) (Dolmans et al. 2006; Young et al. 2017) were used for this study. The follicles were collected from three mice (n=3) on each occasion and follicle collection was performed on four separate occasions (n=4).

6.2.7 *In Vitro* exposure to chemotherapeutics

For each experimental replicate, morphologically intact (M1 or M2) secondary follicles (n=48) were cultured in a 3D culture system using Matrigel in two 4-well GPS plates, i.e. 6 follicles in each well. Since the culture medium did not contain any FCS, testosterone was supplied as the substrate for CYP450 aromatase, to support E2 synthesis (Boon & Simpson 2012; Pruitt 2016; Strauss 2014). Follicles *in vivo* are constantly stimulated to grow by the gonadotrophin follicle stimulating hormone (FSH), which was therefore supplied to stimulate granulosa cell proliferation and follicle growth

(Abel et al. 2000; Babu et al. 2000; Kreeger et al. 2005; Kumar et al. 1997) and also to stimulate expression of CYP450 aromatase (Pruitt 2016; Strauss 2014; Strauss, Modi & McAllister 2014). The chemotherapeutics were soluble in water, but gToc required preparation in the solvent DMSO, therefore the vehicle control of 0.3% DMSO was added to the experiment (Table 6. 2).

Table 6. 2: Single or combination test reagents

Test reagents	GPS plate well number							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Testosterone (T) (1µM)	√	√	√	√	√	√	√	√
FSH 100 mIU		√	√	√	√	√	√	√
DMSO 0.03%			√			√		√
Doxorubicin (Dox) (1.21 µM)				√			√	√
4 Hydroperoxy-Cyclophosphamide (4HPCYC) (21.23 µM)					√		√	√
Gamma Tocopherol (gToc) (35.1 µM)						√		√

50µL of Matrigel was added to each of the 4 large well (300µL) and 20µL was added to each of the 4 small wells (100µL) in each GPS plate. Groups of six secondary follicles were loaded to each large well, but no follicle was added to the small wells. The follicles were thus encapsulated in Matrigel and were incubated for 5 minutes at 37°C with 5% O_2 and 6% CO_2 in nitrogen to solidify the liquid matrix into a gel. The matrix containing six follicles in each well was then overlain with 200µL warmed (37°C) test reagent in medium (Table 6. 2), and the follicles were photographed using an inverted microscope and a warming stage (37°C). These images were later used to calculate the initial diameter of the follicles and to confirm the morphology of the follicles. The follicles were cultured for a total of 6 days, but at 2-day intervals 150µL of conditioned media were collected and replaced with 150µL of fresh media containing the different test reagents and the follicles were photographed again. This experiment was repeated on 4 separate occasions (n=4).

6.2.8 Follicle Cell Viability Assay

After 6 days of culture, media were collected, and the follicles were left at room temperature for ten minutes to allow the Matrigel to form a liquid gel, which facilitated retrieval of the follicles. The groups of 6 follicles were disaggregated in 2 mg/mL collagenase IV (295 units/mg, Worthington,

New Jersey, USA) for 30 minutes before further disaggregation using 0.05% hyaluronidase (1228 units/mg, Lakewood, NJ) for another 30 minutes. This formed a single cell suspension of GCs suitable for assessment using a Trypan Blue exclusion assay (Asaduzzaman, Gonzalez & Young 2018).

6.2.9 Assessment of Follicle Morphology and Growth

Follicle integrity and growth (diameter) was determined using the photomicrographs obtained at the start of culture and every 2 days. The diameters of the follicles were measured using Image J (1.49v, NIH, USA) to draw straight line through the centre of the follicle three times at different angles, from the outside edges of the follicles. The average of these three determinations was taken as the diameter of the follicle. The morphology of each follicle was classified as intact, M1 (regular spherical shape with intact basal lamina), M2 (irregular shape of follicle with no loss of GC), M3 (damaged basal lamina with loss of <5% GCs) and M4 (completely disrupted basal lamina with loss of >5% GC) (Dolmans et al. 2006; Young et al. 2017).

6.2.10 Estradiol Enzyme Immunoassay (EIA)

Conditioned media from wells that contained follicles, as well as media from small wells that contained Matrigel and test reagents but no follicles, were examined in a competitive Estradiol immunosorbent assay (EIA) (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti rabbit IgG, and an acetylcholinesterase estradiol tracer. The manufacturer reports a detection limit of 15pg/mL, an intra-assay coefficient of variation (CoV) of 7.8 to 18.8%. For this study, the estradiol standard was diluted in the complete media to give concentrations that ranged from 6.6 to 4000 pg/mL. A separate standard plot was constructed for each experimental replicate (n=4) and the lowest R^2 value was 0.99. The background contributed by the Matrigel with or without test reagents was subtracted from the E2 produced by the follicles. Neither 0.3% DMSO nor the test reagents interfered with the ELISA.

6.2.11 AMH Enzyme Immunoassay

Conditioned media were examined in a two-immunological step sandwich type EIA (Immunotech, Marseille Cedex, France) that uses an anti-AMH monoclonal antibody for capturing AMH, and biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH in the wells. The manufacturer reports a detection limit of 0.52 ng/mL (3.74 pM), an intra-assay coefficient of variation (CoV) of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standard was diluted in complete media to give concentrations that ranged from 0 to 166.67 pM. A separate standard plot was constructed for each experimental replicate (n=4) and the lowest R^2

value was 0.99. Neither control nor treated follicles produced detectable levels of AMH. Also, neither Matrigel nor test reagents interfered with the AMH ELISA. Purified AMH used to construct standard curves for the ELISA was added to culture media at different concentrations. AMH was measured accurately in the ELISA; there appeared no degradation of AMH in the culture media. Also, the conditioned medium from the culture matrix without follicle were examined in the same ELISA and reported no interference.

6.3 Statistical Analysis

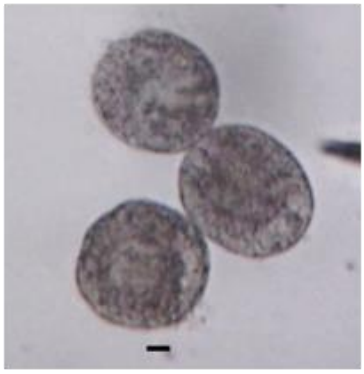
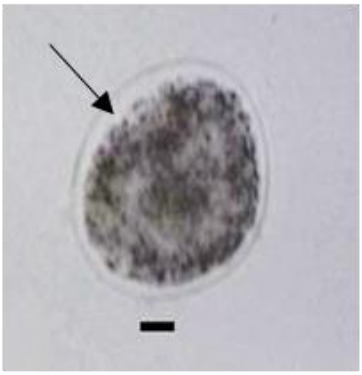
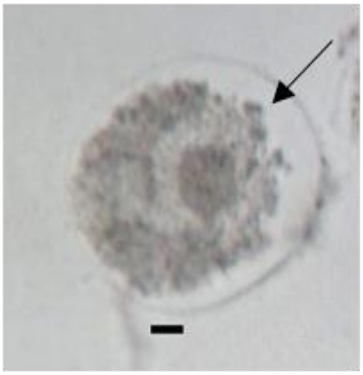
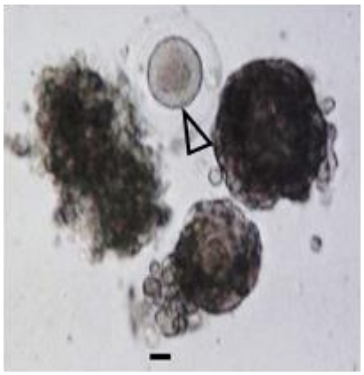
The mean and standard deviation was calculated for each experimental parameter, and statistical analysis was performed using GraphPad Prism software. Trypan blue data were subjected to two-way ANOVA with Tukey's multiple comparison test and the effects on follicle morphology, growth and hormone production were subjected to two-way ANOVA with Bonferroni post-hoc test. Significance was assigned at $p<0.05$.

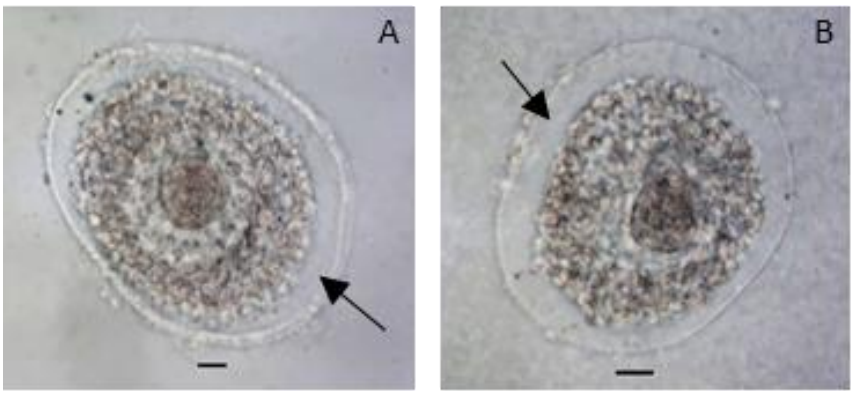
6.4 Results

6.4.1 Follicle morphology

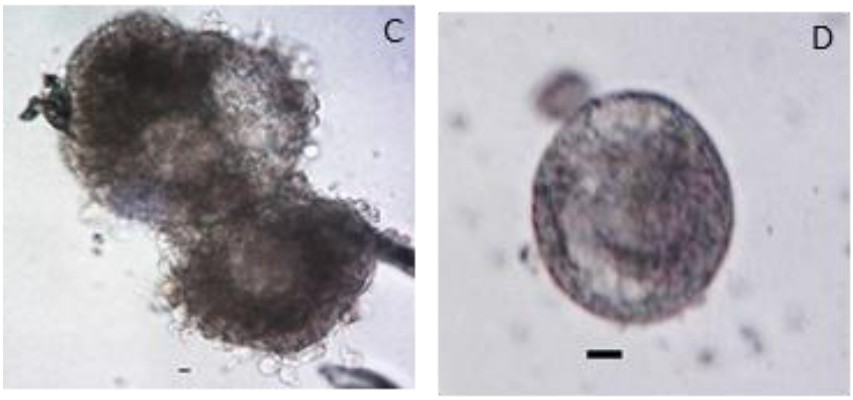
Immediately after isolation, follicles were assessed using a dissection microscope, and only follicles which appeared to have a regular spherical morphology with an intact basal lamina (i.e. M1 or M2) were transferred to the GPS plates and encapsulated in Matrigel. Different reagents (Table 6. 2) were added to each encapsulated group of 6 follicles and photomicrographs were taken of individual follicles on day 0 before commencement of culture. It was unlikely that any of the test reagents would have affected the morphology in the 15-30 minutes between encapsulation and photography. All the day 0 images were therefore of comparable, 'control' follicles. Ideally, there would have been 6 M1 or M2 follicles in each Matrigel plug, but review of the micrographs taken on day 0 allowed a more accurate assessment than the initial "live" scoring. In the whole experiment, which used 6 follicles in each of 8 different culture conditions and was repeated on 4 separate occasions (a total of 192 follicles) there were (5±0.5) intact good quality M1 or M2 follicles in each Matrigel plug at time 0 at the commencement of culture. Testosterone was supplied as a substrate for E2 synthesis. FSH stimulates granulosa cell proliferation and follicle growth, and also stimulates expression of CYP450arom, the enzyme crucial for E2 synthesis. It was anticipated that the combination of FSH and testosterone would provide the optimal control conditions for follicle growth and morphology. It was therefore surprising to find that testosterone alone, and the combination of testosterone with FSH, did not maintain high quality follicle morphology (Figure 6.

1 and Figure 6. 2). There appeared to be a cell-free margin between the outermost layer of mural GC and the basal lamina. Many follicles exposed to testosterone, and to FSH with testosterone, appeared to have a regular, unbroken outer layer of GC (Figure 6. *1* A) although sometimes this was disrupted (Figure 6.1B) but not as much nor as frequently as in follicles exposed to doxorubicin (Figure 6. *1* E) or 4OH-cyclophosphamide (Figure 6. *1* F). It was also surprising that the addition of 0.3% DMSO to testosterone and FSH, and also the combination of 0.3% DMSO, testosterone and FSH with gToc, maintained the highest numbers of spherical good quality morphologically intact follicles (Figure 6. *1* C & D and Figure 6. 2) throughout the 6-day culture period. Doxorubicin and 4OHCYC each had significantly fewer intact M1 or M2 follicles on day 2 ($p<0.001$, Figure 6. 2). Addition of gToc did not prevent this structural damage to follicles although there were more M1 and M2 follicles remaining after 6-days exposure to doxorubicin with 4OHCYC and gToc (1.5±1.7) than there were after 2-days exposure to doxorubicin (0.5±0.6, Figure 6. 2) and cyclophosphamide (0, Figure 6. 2) or to 4OHCYC alone.

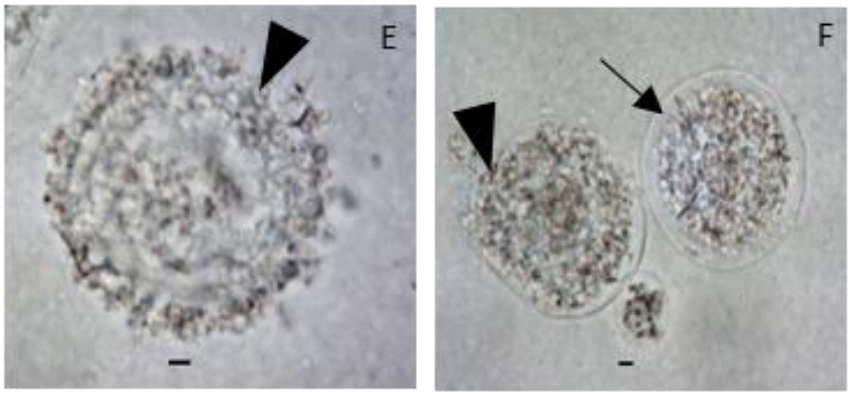
Morphological class	Representative image
M1/M2	
M3	
M4	
M3 and M4 with extruded oocyte	



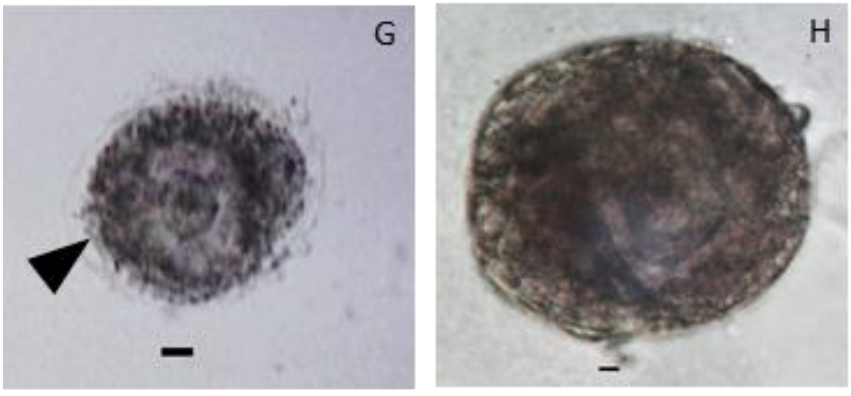
Testosterone | Testosterone+FSH



DMSO | Gamma Tocopherol



Doxorubicin | 4OH-cyclophosphamide

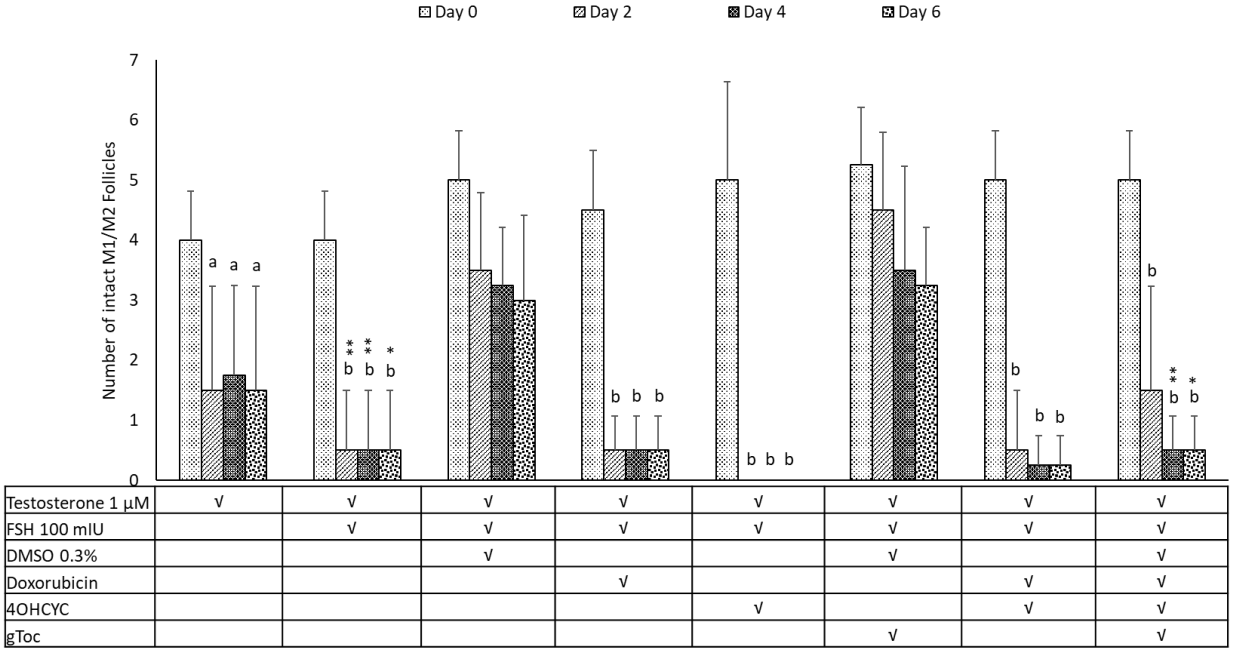


Doxorubicin+4OH-cyclo-phosphamide | Doxorubicin+4OH-cyclo-phosphamide+gToc

Figure 6. 1: Follicle morphology

Groups of 6 follicles were encapsulated and photographed through Matrigel at the commencement of culture (Day 0) and every 2 days for 6 days using an inverted microscope. Follicles were scored as M1-intact basal lamina with regular spherical morphology, M2-intact basal lamina but irregular non-spherical shape, M3-disrupted basal lamina with 5-10% loss of GCs sometimes also appearing retracted from basal lamina, M4 disrupted basal lamina with >10% loss of GC and sometimes an extruded oocyte (open arrow head).

Follicles were exposed to A. testosterone (T), B. T + follicle stimulating hormone (FSH), C. T + FSH + 0.3% DMSO, D. T + FSH + 0. 3% DMSO + gamma tocopherol (gToc), E. T + FSH + Doxorubicin (DOX), F. T + FSH + 4hydroxycyclophosphamide (CYC), G. T + FSH + DOX + CYC, H. T + FSH + DOX + CYC + 0.3% DMOS + gToc. Follicles A – H after 6 days in vitro. Long arrow-GC retracted inside basal lamina. Arrow head-disrupted basal lamina. Scale bars 20 µm.



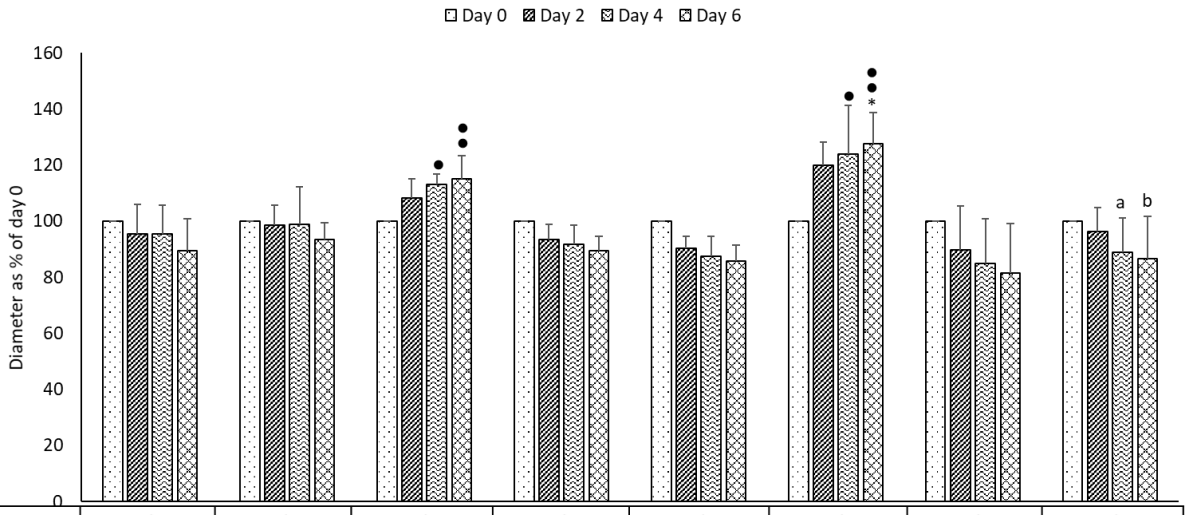
Testosterone 1 µM	√	√	√	√	√	√	√	√
FSH 100 mIU		√	√	√	√	√	√	√
DMSO 0.3%			√			√		√
Doxorubicin				√			√	√
4OHCYC					√		√	√
gToc						√		√

Figure 6. 2: Effect of chemotherapeutics on follicle morphology

*Groups of 6 morphologically intact (M1/M2) murine follicles (n=48) were cultured in Matrigel with test reagents for 6 days. Follicles were photographed at commencement of culture (day 0) and every 2 days thereafter. Follicles were scored as M1-intact basal lamina with regular spherical morphology, M2-intact basal lamina but irregular non-spherical shape, M3-disrupted basal lamina with 5-10% loss of GCs sometimes also appearing retracted from basal lamina, M4 disrupted basal lamina with >10% loss of GC and sometimes an extruded oocyte. Follicles with M1 and M2 scores were considered to be intact with good, spherical morphology. The experiment was repeated on four separate occasions (n=4). Data were analysed using a two-way ANOVA with Bonferroni post-test. Mean±SD shown. Significant difference from day 0, (a) $p<0.05$ and (b) $p<0.001$. Significant difference from T+FSH+DMSO control at the same exposure * $p<0.05$, ** $p<0.001$.*

6.4.2 Effect of Chemotherapeutics on Follicular Growth

The average initial diameter of all follicles (n=192 on day 0) was 122±18µm. The addition of 0.3% DMSO with testosterone and FSH caused significantly more follicle growth than testosterone and FSH alone, from 122±1µm on day 0 to 140±18µm on day-6 ($p<0.01$, Figure 6. 3), and the addition of gToc to 0.3% DMSO (with testosterone and FSH) also caused significantly more follicle growth after 6-day exposure than FSH with testosterone alone from 112±12µm on day 0 to 143±21 on day 6 ($p<0.001$). Although doxorubicin with cyclophosphamide affected the morphology of follicles (Figure 6. *1* and Figure 6. 2) they did not significantly reduce follicle growth during a 6-day exposure in this model (Figure 6. 3). The combination of gToc, doxorubicin and cyclophosphamide caused a significant reduction in follicle growth compared to the 0.3% DMSO and gToc control. After 4 days exposure follicles were 138±18µm in diameter, whereas follicles exposed to doxorubicin and 4OHCYC with gToc for 2 days were 109±14µm in diameter. The chemotherapeutics appeared to prevent the follicle growth promoting effects of 0.3% DMSO and gToc, but gToc neither increased nor prevented the toxic effects of doxorubicin with 4OHCYC.



Testosterone 1 µM	√	√	√	√	√	√	√	√
FSH 100 mIU		√	√	√	√	√	√	√
DMSO 0.3%			√			√		√
Doxorubicin				√			√	√
4OHCYC					√		√	√
gToc						√		√

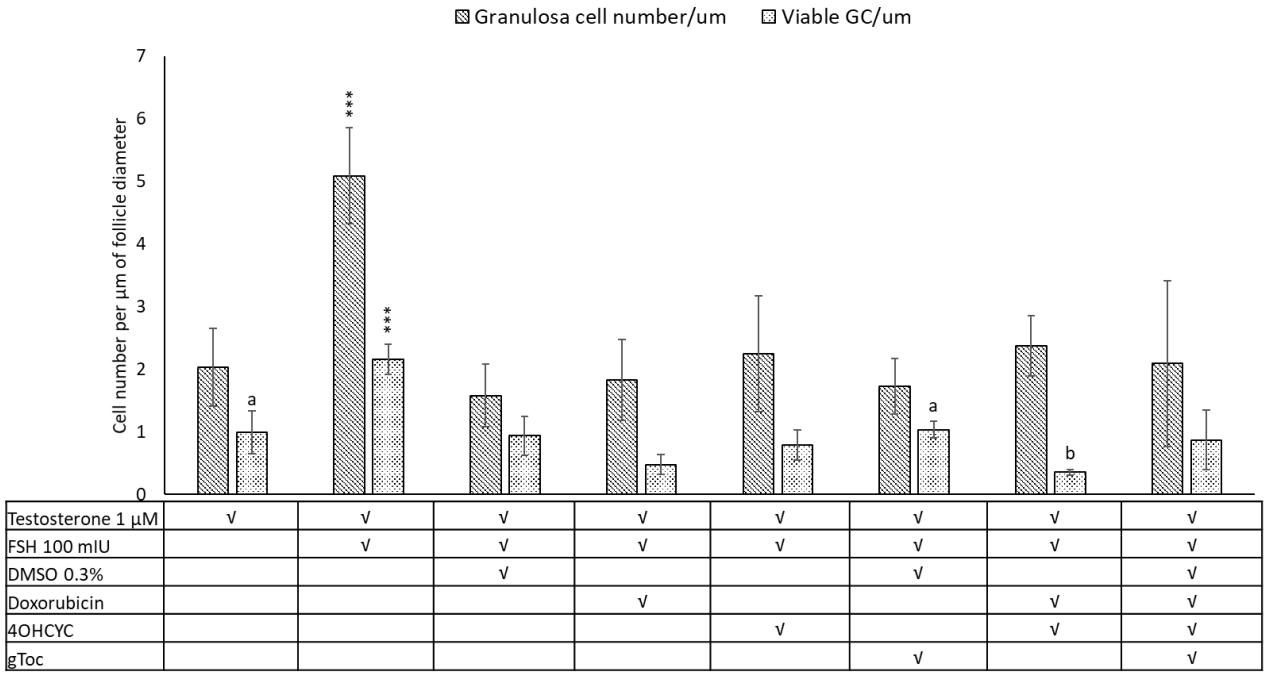
Figure 6. 3: Effect of chemotherapeutics on follicle growth

Groups of 6 follicles were photographed through the Matrigel at the commencement of culture (Day 0) and every 2 days for 6 days using an inverted microscope. Diameters of individual follicles in each photomicrograph were measured and the mean±SD of all 6 follicles in the same well determined. The diameters on day 0 were set as 100%, and diameters of the same follicles on subsequent days expressed as a percentage of the commencing diameters. The experiment was repeated on four separate occasions (n=4), and the mean±SD percent change shown. The diameters (µm, not

*percentage) were analysed using two-way ANOVA with Bonferroni post-test. Significant difference, * p<0.05, in comparison to day 0 for the same test reagents. Significant difference from DMSO control (with FSH+T) at the same exposure; (a) p<0.05, (b) p<0.01 and from testosterone with FSH at the same exposure; (•) p<0.05, (••) p<0.01 and (•••) p<0.001.*

6.4.3 Effect of Chemotherapeutics on Follicle Cell Viability

FSH stimulated a significant increase in the total number of cells ($p<0.001$) and the number of viable cells ($p<0.001$) in each follicle but the addition of 0.3% DMSO, chemotherapeutics and gToc prevented the stimulatory effect of FSH on GC proliferation (Figure 6. 4). After 6-day of culture in testosterone with FSH, the follicles had a diameter of 103±15µm and 518±54 GCs of which 224 (43%) were viable. Although the total number of cells was lower (228±93 per follicle) the percentage of viable cells was highest in follicles cultured T+FSH+ 0.3% DMSO (60±9%) and T+FSH+ 0.3% DMSO with gToc (57±14%) which did not appear to be cytotoxic since viable cell numbers were similar to 0.3% DMSO control. The combination of doxorubicin and 4OH-cyclophosphamide caused a significant reduction ($p<0.05$) in the number of viable cells compared to gToc. And the addition of gToc to the combination of doxorubicin and 4OH-cyclophosphamide increased cell viability and there was no significant difference between follicles exposed to gToc with doxorubicin and 4OH-cyclophosphamide, and the gToc control. The percentage of viable cells after 6 days exposure to gToc with doxorubicin and 4OH-cyclophosphamide was 44±7% as compared to 16±5% in follicles exposed to doxorubicin with 4OH-cyclophosphamide.



Testosterone 1 µM	√	√	√	√	√	√	√	√
FSH 100 mIU		√	√	√	√	√	√	√
DMSO 0.3%			√			√		√
Doxorubicin				√			√	√
4OHCYC					√		√	√
gToc						√		√

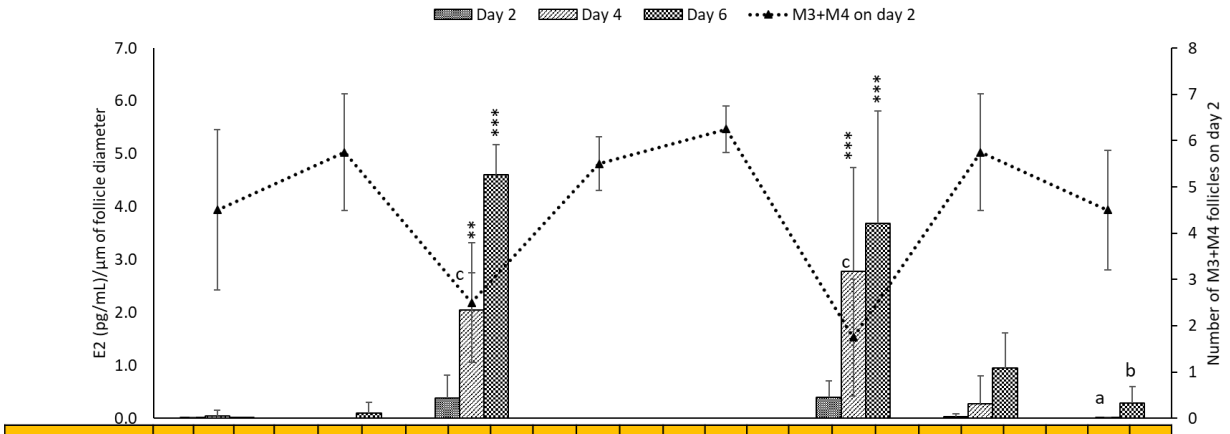
Figure 6. 4: Effect of chemotherapeutics on granulosa cells (GC)

*Morphologically intact (M1/M2) follicles (n=48) from mice (n=3) were cultured in Matrigel with test reagents for 6 days, then retrieved and disaggregated to form a single cell suspension suitable for assessment by trypan blue assay. The total numbers and viable cell numbers from each group of 6 follicles were divided by the average diameter of the same 6 follicles immediately before disaggregation, to give the number of cells per µm of diameter. The experiment was repeated on four separate occasions (n=4). Data was analysed using one-way ANOVA with Tukey's multiple comparison test. Mean±SD shown. Significant difference, a v b p<0.05 or *** p<0.001 compared to all other total and viable cell numbers.*

6.4.4 Hormone Production

Although FSH stimulated cell proliferation, it did not stimulate E2 production in this model (Figure 6. 5), whereas the combination of testosterone and FSH with 0.3% DMSO or gToc stimulated increasing synthesis of E2 in each 2-day period (Figure 6. 5). Similar to morphological damage, follicles exposed to doxorubicin and 4OH-cyclophosphamide alone did not produce any E2, although follicles exposed to the combined chemotherapeutics produced E2 between days 2 and 4 (0.3±0.5 pg/mL) and produced more E2 between days 4 and 6 (1±0.7) ($p>0.05$). Addition of gToc to the doxorubicin and 4OH-cyclophosphamide combination caused a significant reduction in E2 synthesis in comparison to the DMSO and gToc control, but E2 was not significantly lower than in follicles exposed to doxorubicin with 4OH-cyclophosphamide alone. The chemotherapeutics appeared to prevent the E2 synthesis enhancing effect of 0.3% DMSO and gToc. Follicle morphology and integrity

appeared to affect the estrogen synthesis because E2 synthesis was highest in groups of follicles with the highest numbers of good quality M1 and M2 follicles and low numbers of poor quality M3 and M4 follicles (Figure 6. 5). None of the follicles synthesised detectable levels of AMH.



No of intact M1/M2	1.5	1.75	1.5	0.5	0.75	0.5	3.5	3.5	3	0.5	0.5	0.5	0	0	0	4.5	3.5	3.25	0.5	0.25	0.25	1.5	0.5	0.5
Testosterone 1 µM		√			√			√			√			√			√			√			√		
FSH 100 mIU					√			√			√			√			√			√			√		
DMSO 0.3%								√									√						√	
Doxorubicine											√									√			√	
4OHCYC														√						√			√	
gToc																	√						√	

Figure 6. 5: Effect of chemotherapeutics on hormone production by follicles

*Mouse secondary follicles (n=48) were encapsulated in Matrigel in groups of 6 and cultured for 6 days. Every 2 days, 150µL of conditioned media were collected and replaced. Conditioned media were examined in an Estradiol ELISA. The concentration of E2 (pg/mL) per µm diameter of follicles is shown. Experiment was repeated on four separate occasions (n=4). Data was analysed using two-way ANOVA with Bonferroni post-test. Significant difference from day 2, ** p<0.01 and *** P<0.001. Significant difference, (a) p<0.01, (b) p<0.001 at same exposure from 0.3% DMSO (with FSH and testosterone) and (c) significantly lower in comparison to T+FSH, T+FSH+Dox, T+FSH+Cyc and T+FSH+Dox+Cyc, p < 0.05 at least.*

6.5 Discussion

This is the first study to examine the effects of chemotherapeutics that are commonly used to treat breast cancer, on murine secondary follicles *in vitro*. Gamma tocopherol was added to the combination treatment of doxorubicin and 4OH-cyclophosphamide with the objective of reducing undesirable side-effects on the ovaries.

Although the highest numbers of cells were retrieved from follicles cultured in testosterone with FSH, and these follicles also had the highest absolute numbers of viable cells (but not the highest percentages of viable cells) the high cell numbers did not give rise to large follicle diameters, nor to intact follicle morphology, nor to estrogen production. The percentage of viable cells, and the maintenance of intact M1 and M2 morphology however, were related to follicle growth and E2 synthesis (Boon & Simpson 2012; Erickson 1993; Williams & Erickson 2012). Matrigel was constant throughout the experiment and it was surprising that neither testosterone alone nor in combination with FSH maintained follicle integrity. Although testosterone is an important precursor for estrogen synthesis in GCs (Boon & Simpson 2012; Miller & Auchus 2011; Pruitt 2016; Smyth et al. 1993; Strauss, Modi & McAllister 2014), it is also a prooxidant and can induce oxidative stress (Aydilek, Aksakal & Karakılçık 2004; Mooradian 1993). Hence, it is possible that the lower morphological grade of follicles cultured in testosterone with FSH was a consequence of ROS induced apoptosis and the associated loss of dead cells (theca and GCs) from the follicles (Devine, Perreault & Luderer 2012). In the normal *in vivo* situation glutathione, an intrinsic antioxidant, is present in GCs and oocytes but when the ROS production exceeds the REDOX capacity of the cell, increased oxidative stress leads to apoptosis and damage (Anderson & Luo 1998; Devine, Perreault & Luderer 2012; Shan, Aw & Jones 1990). Therefore, it was concluded that follicle damage was caused by testosterone but not by the FSH. In addition to testosterone, chemotherapeutics (cyclophosphamide and doxorubicin) have been reported to produce ROS with the capacity to deplete antioxidant enzyme systems in growing follicle which in turn can cause granulosa cell apoptosis (Batist et al. 2001; Devine, Perreault & Luderer 2012; Hanukoglu 2006; Rapoport, Hanukoglu & Sklan 1994; Rapoport, Sklan & Hanukoglu 1995). Gamma tocopherol or DMSO may have prevented follicle damage because they are both potent antioxidants (Brigelius-Flohé et al. 2002; Hanukoglu 2006; Rapoport, Hanukoglu & Sklan 1994; Rapoport, Sklan & Hanukoglu 1995; Smolarek, AK & Suh, N 2011). DMSO has neutralised hydroxyl ions immediately (within 1.2-3.5 nano second) after release (Lee & De Mora 1999; Scaduto Jr 1995; Sunda et al. 2002). It is possible that, gToc did not maintain the follicle integrity when added to doxorubicin and 4OH-cyclophosphamide

because the chemotherapeutics act in multiple ways to cause granulosa cell apoptosis in a follicle (Batist et al. 2001; Dees et al. 2000; Gook et al. 2013; Jones et al. 2006; Legha et al. 1982; Morgan et al. 1997; Morgan et al. 2012; Ozer et al. 1982) but gToc could only prevent the GC apoptosis caused by ROS.

Gamma tocopherol kills proliferating cancer cells (Betti et al. 2006; Campbell et al. 2003; Cooney et al. 1993; Gysin, Azzi & Visarius 2002; Smolarek, AK & Suh, N 2011). As far as we know, this is the first study to show that gToc does not kill primary derived, normal proliferating cells. the different activity of gToc to malignant and normal proliferating cells holds promise for the development of more effective chemotherapeutic regimen.

A previous study by Gook et al. (2013) indicated that low levels of oxygen supported follicular growth and follicle cell viability and we confirm their findings in this study. The 3D follicle culture model supported follicle growth under control conditions, and allowed us to conclude that exposure to doxorubicin and 4OH-cyclophosphamide may have disrupted the autocrine and paracrine functions (Cortvrindt, R, Smitz, J & Van Steirteghem, AC 1996; Richings et al. 2006; Wang et al. 2011; Xiao et al. 2015) essential for GC proliferation and follicle growth.

The antioxidants 0.3% DMSO and gToc maintained the morphological integrity, follicle growth and viability of follicles. However, the growth of follicles on day 6 was higher in the gToc exposed follicles in comparison to follicles exposed to 0.3% DMSO compared to day 0, suggesting that gToc has an additional effect to 0.3% DMSO in follicle growth. Follicle integrity, viability of GCs in turn supported steroidogenesis. The production of estrogen begins in the theca interna when cholesterol is converted to androstenedione. Androstenedione and testosterone are transferred to GC and converted into estrogen (Boon & Simpson 2012; Miller & Auchus 2011; Pruitt 2016; Smyth et al. 1993; Strauss 2014; Strauss, Modi & McAllister 2014). Disruption of the basal membrane and loss of theca cells may have reduced steroidogenesis and E2 production (Williams & Erickson 2012). Hence, follicles damaged by exposure to chemotherapeutics sufficient to produce visibly obvious low grade M3 or M4 morphology may also have had impaired steroidogenesis (Erickson et al. 1985; Kamada et al. 1997; Telfer et al. 2008; Williams & Erickson 2012). In addition, increased ROS production by doxorubicin and 4OH-cyclophosphamide may have interrupted hormone production by the follicles because of the fact that the activity of CYP450 enzyme system that is responsible for steroidogenesis is reduced by ROS (Diemer et al. 2003; Hanukoglu 2006; Rodgers et al. 1995; Sugino 2005; Young et al. 1994). *In vivo*, AMH is produced by the growing secondary follicle (Garrel et al. 2016; Hanna et

al. 2006; La Marca et al. 2005; Pask et al. 2004; Weenen et al. 2004a), until they reach small antral stage (Baarends et al. 1995; Hanna et al. 2006; Orisaka et al. 2009; Pask et al. 2004; Weenen et al. 2004a; Zec et al. 2011). Also, a previous study indicated that 10,000 GCs retrieved from COC during IVF produced 18.1ng AMH every 2 days and this equates to 1.81pg AMH per granulosa cell (Kollmann et al. 2015). The average diameter of follicles at the commencement of culture was 122±18µm indicating follicles were early to mid-secondary stage. In this study, 6 secondary follicles could have over 1,700 GCs of which 40% were dead in the control group, leaving over 1000 viable GCs, hence it was expected that the GCs in follicle cultured in control conditions would produce AMH at detectable levels (minimum detection level was 0.52 ng/mL) but the early secondary follicles in this study did not. Apparently, AMH synthesis by murine follicles cannot be predicted by IVF-derived human GCs. these data were in agreement with a found that primary derived murine GCs did not produce any AMH even at 20,000 cells after 24h (Asaduzzaman, Gonzalez & Young 2018) though another study reported that 10,000 primary-derived human GCs produced 0.025 to 1.7 ng/mL (Pellatt et al. 2007). From this it was concluded that murine and human primary-derived GCs produce AMH at different levels. In this study, AMH was not degraded over time (Kollmann et al. 2015) and neither the matrix components nor culture media interfered with the AMH ELISA suggesting that follicles do not produce AMH *in vitro* to a detectable level. It has been reported that a 16 week old mouse having 153 growing follicles produced 28.27ng/mL AMH *in vivo* equivalent to 1.08ng/mL by 6 secondary murine follicles considering all GCs are viable (Kevenaar et al. 2006), but with 60% viable GCs in controlled follicle, AMH production might only have been 0.54 ng/mL which is the minimum detection level of the AMH kit. Hence, in this study, it was concluded that no AMH was detected after exposure to test reagents.

This study found that gToc retained follicular integrity, growth and viability of GCs, but that it could not prevent the toxic effects of doxorubicin and cyclophosphamide. Therefore, further *in vitro* and *in vivo* studies are required with higher doses of gToc to evaluate its potential to protect ovarian follicles from the toxic effects caused by exposure to chemotherapeutics.

7. CHAPTER SEVEN: DISCUSSION

Chemotherapy induced premature ovarian failure is a serious consequence for female breast cancer survivors. Ovarian failure is diagnosed by serum AMH levels, the reduced number of dormant primordial follicles or by antral follicle counts, but the underlying cause of these indirect markers of ovarian insufficiency are not evaluated. These indirect effects however are thought to be a result of the direct cytotoxic effects of chemotherapeutics on proliferating GCs of ovarian follicles. GCs play an important role in follicle development through autocrine and paracrine regulations of oocyte maturation and through their contribution to hormone synthesis (Boon & Simpson 2012; Knight & Glister 2006; Miller & Auchus 2011; Orisaka et al. 2009; Strauss 2014; Tajima et al. 2006). This project describes the direct ovo-toxic effects of commonly used breast cancer chemotherapeutics (doxorubicin and cyclophosphamide: AC) on murine ovarian secondary follicles. This project used 3D follicle morphology, growth, follicle cell numbers, cell viability and hormone measurement (estrogen, progesterone and AMH) for optimizing follicle culture systems, and for estimating the ovo-toxicity of chemotherapeutics. An experiment using human breast cancer cells (MCF-7) and ovarian carcinoma-derived granulosa like cells (KGN) demonstrated that the chemotherapeutics used for breast cancer are also cytotoxic to ovarian carcinoma cells. Clinically relevant doses of cyclophosphamide however were not cytotoxic to ovarian carcinoma cells, and this led to determination of the 24h IC25 values of these chemotherapeutics against the breast cancer MCF-7 cell line.

A preliminary study using murine secondary follicles suggested that the 2D culture systems do not support the maintenance of follicle morphology, growth and AMH synthesis. Therefore, two 3D culture matrices; SFX-1 and Matrigel, were evaluated, and this study led to the conclusion that Matrigel was better for supporting follicle morphology, granulosa cell viability and hormone synthesis, and hence Matrigel was used in a subsequent study to examine the effects of commonly used breast cancer chemotherapeutics; doxorubicin and cyclophosphamide, on follicle growth *in vitro*.

To assess the cytotoxic effect of chemotherapeutics, follicle cell viability was measured by direct assessment using trypan blue assay and live-dead stain. It was unexpected that approximately half the GCs were dead in freshly isolated secondary follicles and that there was no difference in follicle cell viability between enzymatically or mechanically isolated follicles. Similarly, follicle cell viability was not affected by the culture systems and cell viability in follicles cultured for 48h was similar to

cell viability in freshly isolated follicles. Previous studies reported 80% viable GCs after assessment using live-dead staining of whole follicles (Aerts et al. 2008; Campos et al. 2011; Lunardi et al. 2012; Merdassi et al. 2011), but whole follicle staining gives an indirect and subjective estimation of viable cells because follicles are spherical and GCs overlap when stained follicles are mounted on microscope slides as was proven in the present study in which indirect viable granulosa cell counts were approximately half of that measured by direct trypan blue counts. In contrast to previous reports we suggest that since trypan blue allows direct estimation of granulosa cell viability in a single cell suspension, this is more accurate and reliable than live-dead stain of whole follicles, and that those studies may have overestimated viable cell numbers in whole follicles. It is acknowledged that the loss of follicle cell viability in the trypan blue direct assay may be attributed to the disruption of the gap junctions between GCs (Desai et al. 2010) during follicle disaggregation, but the follicle isolation method and the follicle culture system did not affect the follicle cell viability. In addition to this, in the present study live-dead staining of whole follicles was optimised, and this also found lower numbers of viable cells in isolated follicles than were reported previously.

In an early study using KGN cells, tocopherols were prepared in 0.8% DMSO in complete media. Hence, 0.8% DMSO in complete media was used as a vehicle control. KGN cell viability was not affected by 24h exposure, but there was reduced viability after 72h continuous exposure to 0.8% DMSO. When ovarian cancer KGN cells were exposed to tocopherols in 0.8% DMSO, gToc neither promoted cell proliferation nor killed KGN cells. gToc was not toxic to KGN cells up to 100µM concentration. In a later study using murine follicles however, 0.3% DMSO with testosterone and FSH, or with testosterone, FSH and gToc, maintained the intact follicle morphology over a 6-day culture in a 3D culture system with 60% follicle cell viability, similar to the cell viability of freshly isolated follicles. Hence it was concluded that low concentrations of DMSO (0.3%) supported follicle cell viability over 6 days *in vitro,* and the effects of 0.3% DMSO were thought to be because follicles are sensitive to reactive oxygen species and DMSO is a potent antioxidant that was effective in neutralizing ROS in this culture system (Lee & De Mora 1999; Scaduto Jr 1995; Sunda et al. 2002). It is possible that at higher concentrations (0.8%) DMSO can be cytotoxic to ovarian cancer cells after longer 72h continuous exposures, but not after shorter 24h exposures. The difference in toxicity of the DMSO vehicle control in the two studies was therefore attributed to differences in concentration.

Alpha tocopherol was not cytotoxic to breast cancer cells (MCF7) nor to ovarian carcinoma cells (KGN), but gamma tocopherol was more toxic to breast cancer cells than ovarian carcinoma cells

(KGN). This was confirmed in a later experiment using murine secondary follicles in a 2D culture system. Despite the loss of the spherical structure of the follicles, follicles exposed to gamma tocopherol had a similar number of total and viable GCs per secondary follicle as follicles cultured under control conditions. gToc was not cytotoxic to primary derived follicle cells. In the optimized 3D follicle culture system, gamma tocopherol again showed the same growth promoting properties; the morphological integrity of follicles was retained, and growth, total number of follicle cells, viability and hormone synthesis were maintained. However, no definitive conclusions could be drawn because these effects were not significantly higher than the vehicle control containing 0.3% DMSO, which is also a potent antioxidant and was at such a low concentration that it did not cause cytotoxicity (Lee & De Mora 1999; Scaduto Jr 1995; Sunda et al. 2002). Part of the protective effect found in gamma tocopherol treated follicles was probably contributed by DMSO, which leads to the conclusion that the effects of higher concentration of gToc (in the same 0.3% DMSO) on murine follicles should be examined, to determine if gToc does provide benefits for follicle growth and physiological functions, and also to find the concentration that does cause damage, since this will define the maximum doses that might potentially be administered *in vitro*.

Follicles are unlike breast cancer or ovarian carcinoma cells, in that they remain embedded in the ovary, they are spherical with an oocyte in the centre and this 3D structure maintains endocrine and paracrine functions through gap junctions and other mechanisms dependent on the 3D structure. Previous studies reported that 2D culture systems do not support follicle or oocyte growth, maturation or hormone production, due to loss of the spherical structure (Eppig & O'Brien 1996; Eppig & Schroeder 1989; Spears, De Bruin & Gosden 1996; Telfer et al. 2008). Results from this project confirms these findings because follicles cultured in 2D culture systems did not grow and produced less hormone, and GCs dispersed and attached to the floor of the wells showing loss of spherical structure and extrusion of oocytes. In contrast the 3D culture matrix, Matrigel, supported spherical morphology of follicle and hormone synthesis. Culturing isolated follicles in Matrigel modelled the *in vivo* conditions in which follicles remain embedded in the ovarian ECM. Components of ECM support the follicle growth *in vivo*, and collagen in Matrigel provides a fibrous structure with elasticity (Gomes et al. 1999; Irving-Rodgers et al. 2010; Rodgers et al. 1998; Rodgers, Irving-Rodgers & Russell 2003; van Wezel, I. L. & Rodgers, R. J. 1996; Woodruff & Shea 2007). Follicle integrity is a crucial requirement for follicle development, growth, oocyte maturation and hormone synthesis because different compartments of ovarian follicles (GCs, oocyte and theca cells) communicate with

each other through gap junctions (Boon & Simpson 2012; Desai et al. 2010; Knight & Glister 2006; Pruitt 2016; Smyth et al. 1993; Strauss 2014; Voutilainen et al. 1986; Zec et al. 2011).

Hormone synthesized by follicles was used as an assessment parameter because its synthesis reflects follicle morphology and cell viability. Under control conditions, Matrigel maintained high quality M1 or M2 morphology during a 48h culture period and therefore also supported the synthesis of both estrogen and progesterone, in contrast to the 2D culture system which did not maintain follicle integrity (M1/M2 morphology) or hormone production. This was also supported by the study conducted by Telfer et al. (2008) and in this study, estrogen production by a single secondary follicle was 0.84 pg/mL in a 2D culture system and 5.93 pg/mL in a 3D matrix (Matrigel). These observations give rise to the promise that when the 3D follicle morphology is damaged, steroidogenesis is interrupted, possibly because, in an intact follicle, GCs communicate with theca cells and oocyte (Knight & Glister 2006), and steroidogenesis requires the transfer of pregnenolone from GC to theca cells where it is converted to androstenedione and testosterone which are transferred back to the GC before production of estrogen (Boon & Simpson 2012; Miller & Auchus 2011; Pruitt 2016; Smyth et al. 1993; Strauss 2014; Voutilainen et al. 1986; Whitelaw et al. 1992). Hence damage to follicle morphology represented by M1 or M2 to M3 or M4 grades may be associated with disrupted steroidogenesis because of interrupted gap junctions (Erickson et al. 1985; Telfer et al. 2008; Williams & Erickson 2012). The association of morphological damage with hormone synthesis is supported by the finding that follicles exposed to doxorubicin and 4OH-cyclophosphamide alone did not produce any E_2 and had low numbers of morphologically intact follicles on day 2 but higher numbers of M3 or M4 follicles. Therefore, it is suggested that the chemotherapeutics used for treating breast cancer cause morphological damage of follicles which has immediate effects on hormone synthesis that are independent of granulosa cell death.

The active metabolite of cyclophosphamide, 4-hydroxycyclophosphamide, at its clinically relevant dose did not kill KGN cells. In later study, 4OHCYC was applied at a concentration (0.5µM) that still maintained the therapeutic dosage ratio of 10:1 to doxorubicin. However, this higher concentration of 4OHCYC was not also cytotoxic to KGN cells. A subsequent study determined that the IC25 of 4OHCYC against the MCF-7 breast cancer cells was 21.23 µM which was much higher than that of its plasma concentration *in vivo* (0.2 µM) (Struck et al. 1987). When secondary follicles were exposed to this MCF-7 24h IC25 values for 48h the 4OHCYC caused loss of spherical structure of follicle and dispersion of GCs to the floor of the wells. Similar results were observed when follicles were exposed

to 4OHCYC in a 3D culture system over 6-day period; there were no morphologically intact M1 or M2 follicles by day 2 of culture.

Doxorubicin was cytotoxic to KGN cells after a 24h exposure to a therapeutically relevant dose. In a later study, the IC25 value of doxorubicin, 1.21 µM, caused morphological damage, prevented follicle growth, caused the death of 74% of follicle cells, and prevented hormone production. It seems likely that the morphological damage may have caused subsequent follicle cell death, and reduced growth and hormone synthesis (Boon & Simpson 2012; Desai et al. 2010; Knight & Glister 2006; Pruitt 2016; Smyth et al. 1993; Strauss 2014; Voutilainen et al. 1986; Zec et al. 2011).

The combinations of doxorubicin and 4OHCYC (AC) at a dosage ratio of 10:1 (both 10µM+1µM and 25µM+2.5µM) were toxic to ovarian cancer cells. When follicles were exposed to the AC regimen at their IC25 values, the damage to follicles was similar to that seen after exposure to each of the separate chemotherapeutics. Together these studies indicate that doxorubicin and cyclophosphamide caused morphological damage even in a supportive 3D culture matrix.

Addition of gToc to the combination of doxorubicin and cyclophosphamide did not reduce the KGN cell viability after 48 hours but the viability of KGN cells was significantly reduced after 72 hours. Similarly, exposure to the combination of doxorubicin, cyclophosphamide and gToc resulted in similar viable cell numbers to the control, and significantly higher viable cell numbers than in doxorubicin and cyclophosphamide treated follicles in the 2D culture system. In the 3D follicle study using Matrigel for 6 days, the addition of gToc to doxorubicin and cyclophosphamide did not prevent follicle cell death or morphological damage caused by the two chemotherapeutics. This might be because although gToc is an antioxidant that is capable of preventing ROS associated cell death, doxorubicin and cyclophosphamide kill cells in multiple pathways including inhibition of topoisomerase II, breakdown of DNA strands (Kalyanaraman et al. 2002; Kalyanaraman, Perez-Reyes & Mason 1980; Swift et al. 2006; Tewey et al. 1984; Thorn et al. 2011), cross linking of DNA, prevention of RNA transcription and mutation (Arnold, Bourseaux & Brock 1958; Bagley, Bostick & DeVita 1973; Boddy & Yule 2000; De Jonge, Milly E et al. 2005; Emadi, Jones & Brodsky 2009; Siddik 2002; Teicher, B et al. 1996; Yuksel et al. 2015). Also, gToc was not able to maintain estrogen production when doxorubicin and 4OHCYC were present. This could be because these two chemotherapeutics cause morphological damage which compromises hormone synthesis and this mechanism of action is unrelated to ROS related inhibition of hormone production (Diemer et al.

2003; Rodgers et al. 1995; Young et al. 1994) which the antioxidant properties of gToc can counteract.

From this study it appears that chemotherapeutics exerts toxic effects by causing morphological disruption of the follicles which is one of the causes of granulosa cell death and reduction in hormone synthesis. Ultimately reduced proliferation of GCs does not allow the progression of secondary follicles to antral stage to produce a mature oocyte. As a result, premature infertility develops. Hence, the routinely used chemotherapeutics, even at lower doses can cause toxicity in the ovary.

However, gToc has shown cytotoxicity to breast cancer cells but was not toxic to primary derived follicle cells nor to ovarian carcinoma cells. Hence, the co-administration of gToc with doxorubicin and cyclophosphamide to treat breast cancer has the potential to reduce the number of chemotherapy cycles required to eradicate the cancer and the reduction in administration of total load of doxorubicin and cyclophosphamide would reduce toxic effects on the ovary.

The results from this project may therefore contribute to the development of an improved chemotherapeutic regimen for women diagnosed which will reduce the ovo-toxicity associated with doxorubicin and cyclophosphamide. Results from this project confirm the ovo-toxicity of commonly used breast cancer chemotherapeutics and support further evaluation in animal models to determine if the combination of doxorubicin and cyclophosphamide with gamma tocopherol can delay or prevent premature ovarian failure *in vivo.*

8. APPENDICES

Appendix 8.1: Anti Mullerian Hormone (AMH) Production

AMH is a dimeric glycoprotein and a member of the transforming growth factor beta (TGFβ) family (Choi & Rajkovic 2006). The gene expressing AMH is on the short arm of chromosome 19p13.3 and comprises 2750 nucleotides (Broekmans et al. 2008). For biological activity the 5th exon counted from the 3'end must be rich in glycine (Hampl, Snajderova & Mardesic 2011). The molecular weight of AMH is 140kDA and the molecule consists of two identical subunits joined by a disulphide link (Hampl, Snajderova & Mardesic 2011).

AMH is initially produced as a preproprotein of 560 amino acids which is converted to a propeptide (proAMH) after removal of the first 24 amino acids. This form is inactive and does not bind to the AMH receptor. At the site of action ProAMH undergoes enzymatic cleavage between amino acids 450 and 452 to produce a COOH-terminal dimer (AMH_C) and a NH_2-terminal dimer (AMH_N) (Figure 8. 1). The AMH_C is the active receptor binding component of AMH (Pankhurst & McLennan 2013).

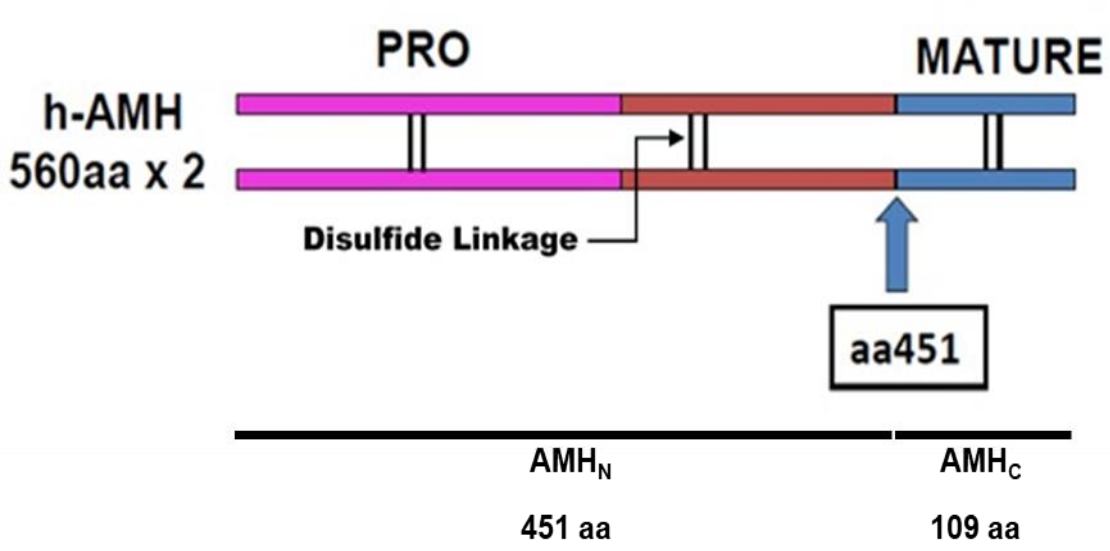


Figure 8. 1: Activation of proAMH.

AMH is a preproprotein of 560 amino acids. Activation of proAMH involves the proteolytic cleavage at amino acid 451 to produce mature and active COOH terminal (AMHC) (adopted from (di Clemente et al. 2010)). AMHN (NH2-terminal dimer) is the pro-region of the preproprotein. aa: Amino Acid.

In the inactive pro AMH the two dimers are bonded to each other non-covalently. When "AMH" was precipitated from blood using two antibodies specific to the NH_2 and COOH terminals, western blot analysis of the precipitated protein showed that AMH_C was precipitated by antibody specific to NH_2 terminal but antibody specific to AMH_C could not precipitate AMH_C from blood, although the same antibody could precipitate recombinant human AMH_C (Figure 8. 2). This indicated that AMH_C remained complexed with AMH_N (proAMH) and the only available form in blood is proAMH and $AMH_{N,C}$.

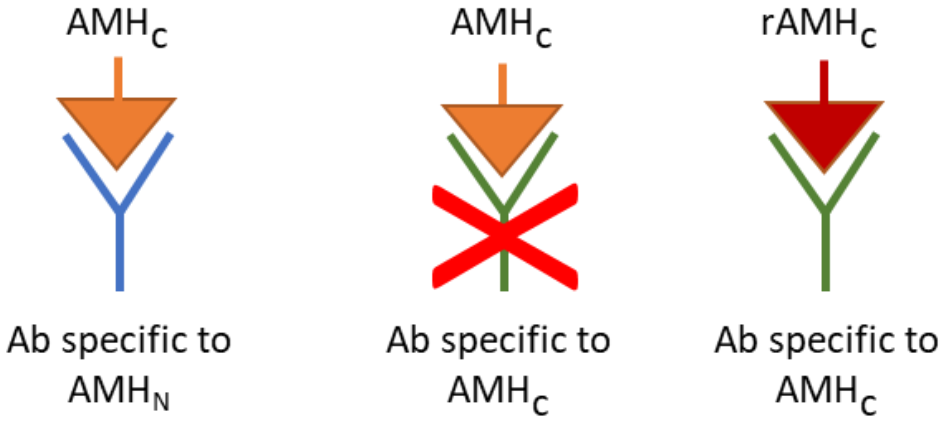


Figure 8. 2: Precipitation of AMH using specific antibody from blood using western blot.

Antibody specific to AMH_N could precipitate AMH_C but antibody specific to AMH_C could not capture AMH_C though it could bind to recombinant AMHC ($rAHM_C$). Ab: antibody.

Furthermore, deglycosylation of proAMH produced two bands indicating that proAMH remained glycosylated in blood (Pankhurst & McLennan 2013). Therefore, it can be concluded that AMH remains glycosylated in the inactive form and cleaves at the site of action either in the mullerian duct or in the ovary. Serum measurements of AMH do not therefore directly estimate AMH bioactivity, which depends on activity of the proteolytic enzyme, as well as receptor expression and transduction.

8.2 AMH Receptors

AMH is the natural ligand of AMH receptors. AMH type II receptors (AMHRII) are specific to AMH (Josso, di Clemente & Gouédard 2001) but type I receptors are non-specific and are shared with bone morphogenetic factors (Josso et al. 2005). The gene expressing AMHRII was found on chromosome 12 and consists of 11 exons and is 8 kbp long. This gene is expressed by Mullerian ducts and the gonads and the corresponding receptor protein has been localised to the Mullerian

duct. In female rat ovaries, AMHRII messenger RNA was expressed by GCs and theca cells of growing follicles (Ingraham et al. 2000; La Marca & Volpe 2006). However, the localization of type I AMH receptors is unclear. There are six type I AMH receptors out of which Alk 3 is responsible for AMH activity in Mullerian ducts whereas the remaining are located in the ovary where AMH binds competitively with TGF-β family members (La Marca & Volpe 2006). To elucidate the receptor binding of AMH, di Clemente et al. (2010) transfected COS cells with human AMHRII and cultured them for 3 days. The cells were then incubated with full length, cleaved (after dissociation with protease from pro-region) or non-cleavable AMH before visualisation using anti AMH mAbs. A strong binding signal was observed with anti-C-terminal mAb 22A2, a faint signal with full length AMH mAb, and no signal for non-cleavable AMH. From these data, di Clemente et al. (2010) proposed that in full length AMH the active C terminal does not orient to bind with the receptor, but after proteolytic cleavage, the C terminal undergoes a conformational change that allows it to bind to AMHRII (Figure 8. 3).

The allosteric binding of cleaved AMH with AMHRII weakens the non-covalent bond between pro-region and C terminal which helps the dissociation of pro-region from the type II receptor-C-terminal complex. The type II receptor-C-terminal complex then stimulates phosphorylation and promotes binding with type I receptor (Hampl, Snajderova & Mardesic 2011). This means AMH binds to type II receptor by ligand and activates type I receptor through phosphorylation which further initiates the cascade of biological responses (Figure 8. 3).

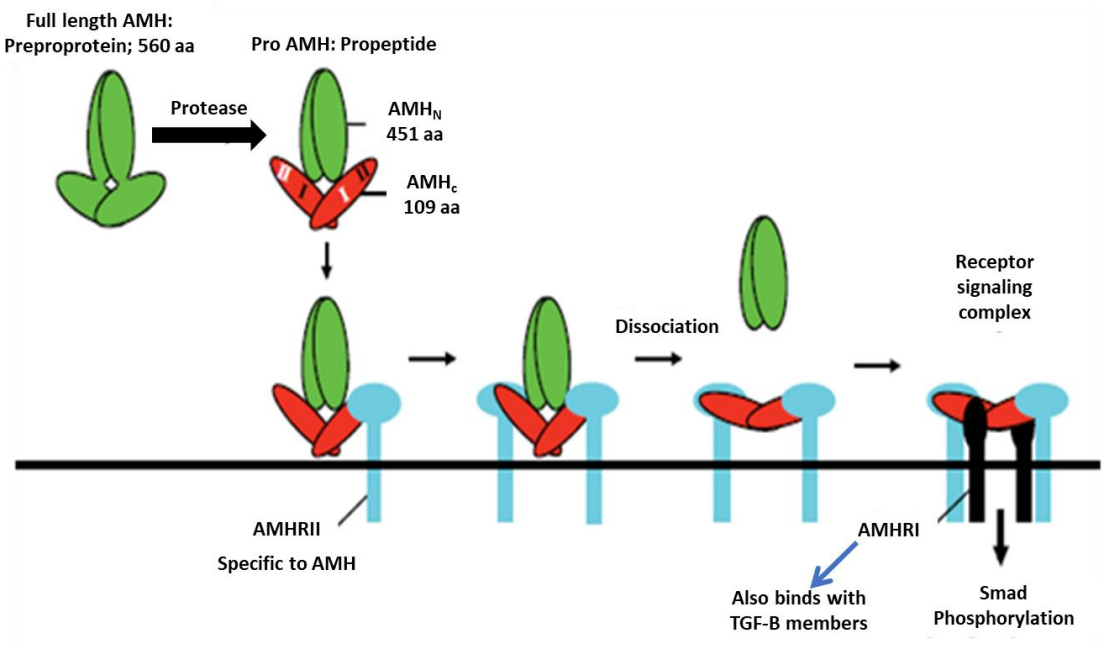


Figure 8. 3: Mechanism of action of AMH receptor binding

Proteolytic cleavage of AMH gives the appropriate orientation for the C terminal to bind with type II receptor, and this in turn facilitates the dissociation of inactive N terminal from the complex. The type II receptor-C-terminal complex then binds with type I receptor by phosphorylation in order to exert biological actions. Adopted from di Clemente et al. (2010).

Appendix 8.2: Quantification of AMH in Serum Samples

The determination of serum concentrations of AMH is important for fertility treatment and reproductive research. There are a number of AMH ELISAs available namely; the DSL AMH ELISA, the Immunotech AMH/MIS kit, the Beckman Coulter Gen II ELISA, the BlueGene ELISA kit and the K-ASSAY (*Table 8. 1*). The Immunotech and DSL ELISAs were combined by Beckman and marketed as the Gen II ELISA. The Beckman Coulter's Gen II ELISA uses the primary antibody from the DSL ELISA kit but the calibration standards from the Immunotech kit (AMH concentrations of 0.16, 0.4, 1.2, 4.0, 10 and 22.5 ng/mL) (Kumar et al. 2010).

Freour et al. (2007) evaluated the Beckman Coulter Gen II ELISA and the DSL AMH ELISA by examining AMH levels in the serum of 69 women aged between 22 and 40 years who were undergoing IVF. Though both the kits followed the same assay procedure and principles the sensitivity of the DSL assay was 0.025 µg/L whereas the Beckman Coulter ELISA sensitivity was 0.40 µg/L (*Table 8. 1*). The determinations of AMH concentrations were almost 4.6-fold lower with the DSL kit than with the Beckman Coulter kit. Bersinger et al. (2007) found less difference between AMH levels obtained by the Beckman Coulter ELISA and the DSL AMH ELISA and demonstrated that the possible cause of difference between them stemmed from residual matrix effects and instabilities of certain antigenic determinants. Streuli et al. (2009) conducted a study using the AMH DSL ELISA to measure AMH in 168 samples from three patient cohorts; 24 ovulatory women, 58 women who had undergone AMH measurement for fertility purposes and 13 women in whom AMH levels were below the detection levels according to the Beckman Coulter ELISA. Streuli et al. (2009) remeasured the AMH levels using DSL kit, but they did not find any differences and they concluded that Beckman Coulter might have solved the problem associated with the Gen II ELISA.

In another multicentre study where serum AMH was measured on 271 patients using DSL ELISA and Gen II AMH ELISA (Wallace et al. 2011) where it was reported that AMH levels were approximately 40% higher when measured with AMH Gen II ELISA instead of DSL AMH ELISA (Wallace et al. 2011). In a later study, however, the Gen II ELISA was compared with the DSL AMH ELISA when measuring serum AMH levels of 5007 women with fertility problems (Rustamov et al. 2012). The Gen II measured AMH levels were 20 percent lower than the DSL AMH ELISA measurements. Also inter-assay variation for the Gen II and DSL AMH ELISAs were 59% and 32% respectively (Rustamov et al. 2012), although the product data sheet claims only 3.57% inter-assay variability for the Gen II ELISA

One disadvantage of the (AMH/MIS) (EIA AMH/MIS) kit from Immunotech is that haemolysed samples cannot be assayed. Also, samples containing other antigen could interfere with the monoclonal antibodies due to steric hindrance of the specific antigen (AMH) to bind to the specific site of the antibody. Also, AMH remains in dimeric form and thus itself can cause limited binding due to its orientation to the binding site of the monoclonal antibody. Limited binding capacity (Single site) of monoclonal antibody used in this ELISA can lead to enormous result (Lipman et al. 2005). Moreover, the intra-assay and inter-assay coefficients of variation for this ELISA were high (*Table 8. 1*) though Anderson and Cameron (2011) reported much lower intra-assay (3.6%) and inter-assay (4%) coefficient of variations while measuring serum AMH levels in 56 breast cancer patients. The sensitivity was reported as 0.05 ng/mL, but only 1 ng/mL by the manufacturer.

Life Sciences Advanced Technologies, Inc, USA produced a Human Mullerian Inhibiting Substance/Anti-Mullerian Hormone (MIS/AMH) ELISA KIT (BlueGene ELISA Kit) which was similar to the AMH Gen II ELISA by Beckman Coulter, Inc, except for the use of a multi-clonal anti-MIS/AMH antibody. Its sensitivity was 0.01 ng/mL which was higher than Gen II AMH ELISA (0.04 ng/mL). There has been no comparative study between these two ELISAs, but it is thought that the increased sensitivity of the Blue Gene ELISA might be due to its polyclonal anti- MIS/AMH antibody (Lipman et al. 2005).

In 2013, the Kamiya Biomedical Company also offered an ELISA kit named K-ASSAY ® for the quantitative determination of human AMH in serum, plasma, tissue homogenates, cell culture supernatants and other biological fluids. The principle was same as that of Life Sciences Advanced Technologies and AMH Gen II ELISA by Beckman Coulter, Inc. This assay kit also used a monoclonal antibody to bind to AMH antigen (*Table 8. 1*). According to the product disclosure data sheet of the respective manufacturers, the sensitivity of Blue gene ELISA kit was 0.01 ng/mL, K-ASSAY ® was 12.2 pg/mL (0.012ng/mL), and EIA AMH / MIS was 0.14 ng/mL (Table 8. 1).

The Anti-Mullerian Hormone / Müllerian Inhibiting Substance (AMH/MIS) kit by Immunotech and the AMH Gen II ELISA by Beckman Coulter, Inc were optimized to measure AMH level in plasma or serum while the other two from Life Sciences Advanced Technologies, Inc, USA and Kamiya Biomedical Company were capable of measuring AMH in serum or blood plasma and cell culture fluid & body fluid & tissue homogenate (Table 8. 1)

Table 8. 1: Comparison of different ELISA methods used for measuring serum AMH

Manufacturer	ELISA kit	Type of ELISA	Primary Antibody against AMH	Visualization	Sensitivity	Measures AMH in	Cross reactivity	Precision Intra-assay and inter-assay Coefficient of Variance	Publications referring to assay
Immunotech	EIA AMH / MIS kit		Monoclonal		1 ng/mL 0.05 ng/mL	Serum		Intra-assay: CoV ≤ 12.3%. Inter-assay: CoV ≤ 14.2%.	(Anderson & Cameron 2011; Kumar et al. 2010)
Beckman Coulter	AMH Gen II ELISA	Enzymatically amplified two-site ELISA	Anti-AMH antibody	HRP conjugate	0.40 µg/L	Serum		Intra-assay: CoV = 2.92 %. Inter-assay: CoV = 3.57 %.	(Bersinger et al. 2007; Freour et al. 2007; Kumar et al. 2010; Pigny et al. 2016; Rustamov et al. 2012; Streuli et al. 2009)
Life Sciences	BlueGene ELISA Kits	Sandwich ELISA	Multi-clonal anti-MIS/AMH antibody	HRP conjugate	0.01 ng/mL	culture media		---	(Hanna et al. 2006)
Kamiya Biomedical Company	K-ASSAY ®	Competitive inhibition enzyme immunoassay	Monoclonal antibody	Biotin	0.012ng/mL	Serum or blood plasma and cell culture fluid & body fluid & tissue homogenate	No significant cross reactivity with AMH analogs	Inter-Assay: not significant	

Appendix 8.3: 3-dimensional (3D) Confocal Microscopy of Stained Follicles

Live-Dead, positive control or DAPI-stained follicles were mounted into a 5 mm^2 grid created by sticking a PVC sheet with 5 mm^2 windows on poly-lysine coated slides. Excess medium was aspirated before adding 5 µL buffered glycerol and a cover slip. Follicles were examined with an SP5 scanning confocal microscope (Leica DMI6500B) using argon laser light for excitation, multi-immersion objective lens and I3 and A filter cubes for Live-Dead and DAPI stained follicles respectively. The deepest plane of the follicle was first brought into focus and the focal plane gradually reduced to the plane closest to the observer. This quantified the distance penetrated by the laser excitation light (Figure 8. 4).

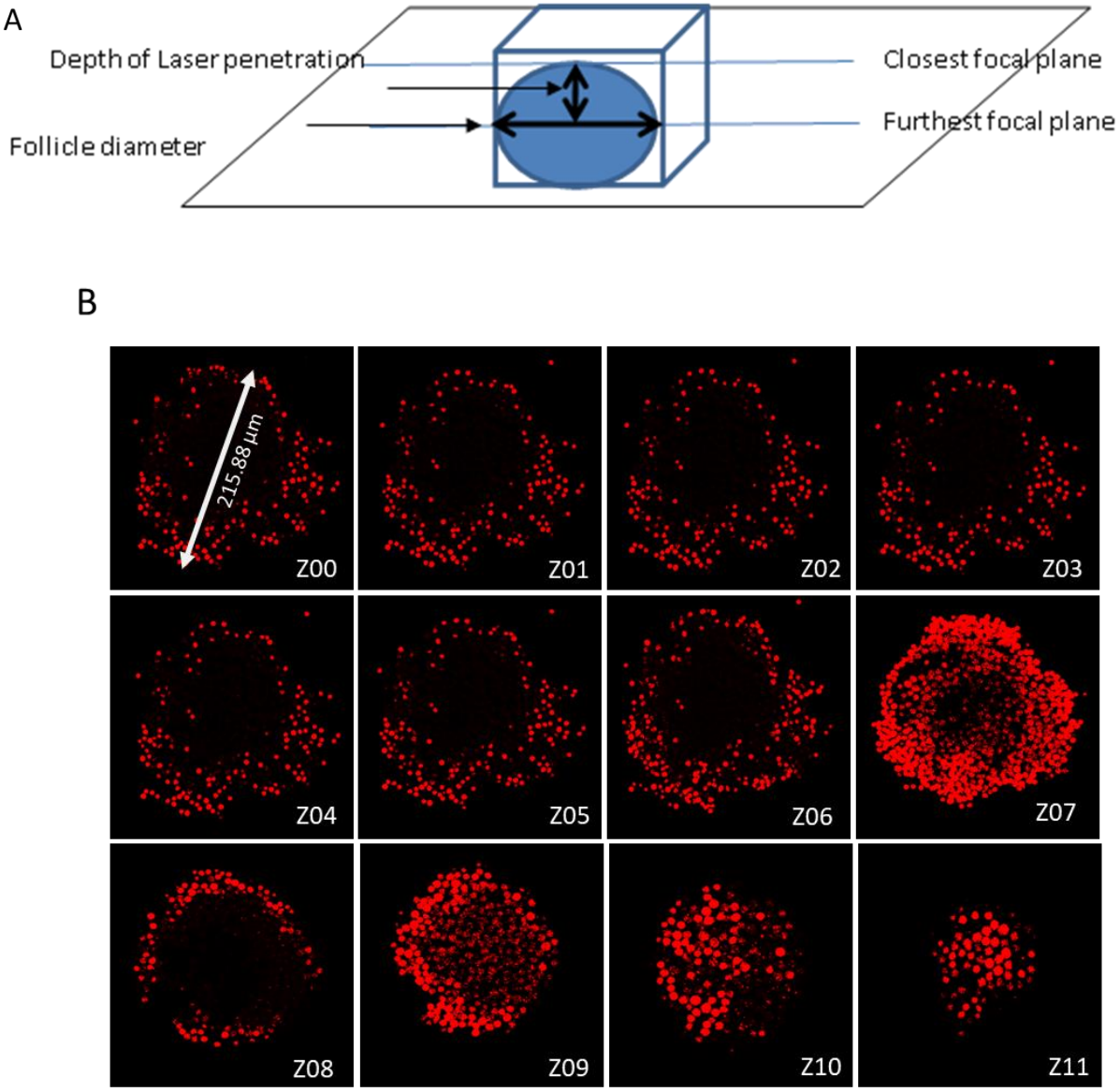


Figure 8. 4: Penetration laser light through murine ovarian secondary follicle.

Murine ovarian secondary follicles were follicles were stained with Live-Dead before fixed and mounted on to a 5 mm^2 area of a PVC sticked pre-coated slide. Stained and mounted follicles were examined under confocal microscope. A. orientation of a follicle under confocal microscope during Z stacking. B: Images of a dead stained follicle at different focal plane (Z stack 00 to 11) where, Z00 is the furthest plane that can be visualized after excitation using argon laser and Z11 is the top plane of the follicle. Difference in depth between each image was 10 µm.

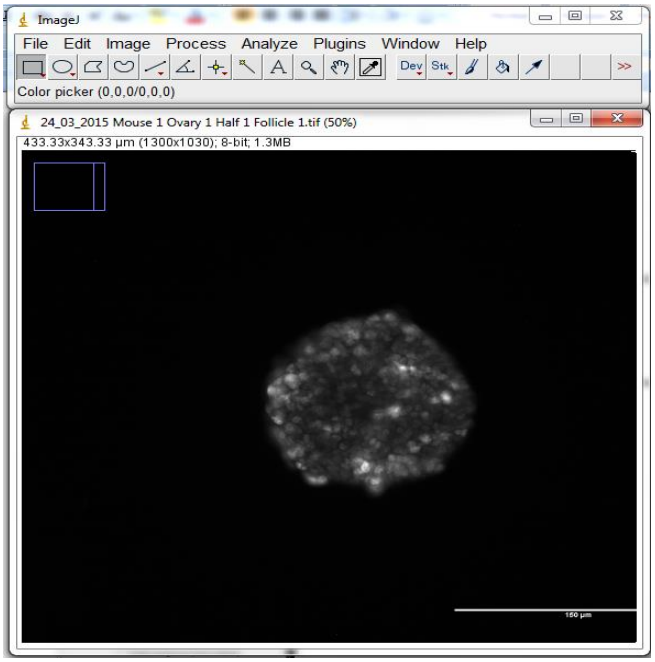
The diameter of the furthest and widest focal plane was 215.88, this means excitation light could pass through the half of the follicle. The average light penetration was about 110 µm. The three staining protocols (DAPI, CalceinAM and EthD-1) were optimized by using confocal microscopy.

Appendix 8.4: Standard Procedure for Quantification of Areas of Fluorescent Staining Using Image J

Step 1:

Open the follicle image in Image J. Go to File → Open

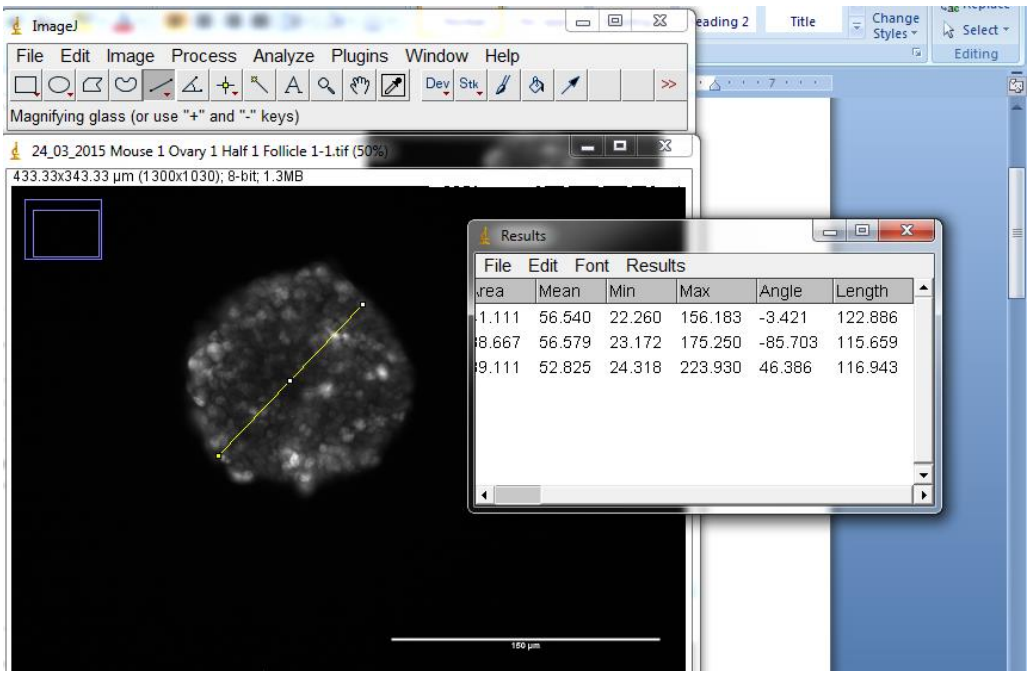
If the follicle image is not visible, Go to Image in the menu bar, select → Adjust→ Brightness/Contrast→ Auto



Step 2:

If the image contains more than one follicle, select follicle that need to be quantified.

(Go to the tool bar and select appropriate tool to mark/select the follicle and then go to image and select crop)



Step 3:

Measure the diameter of the follicle three times by drawing straight line across the follicle. To measure, Draw line and press, Ctrl+M. Do these three times and make average of three measurements.

The images have already calibrated, and a scale has incorporated, so the diameter will come in micro meter.

Setting Scale:

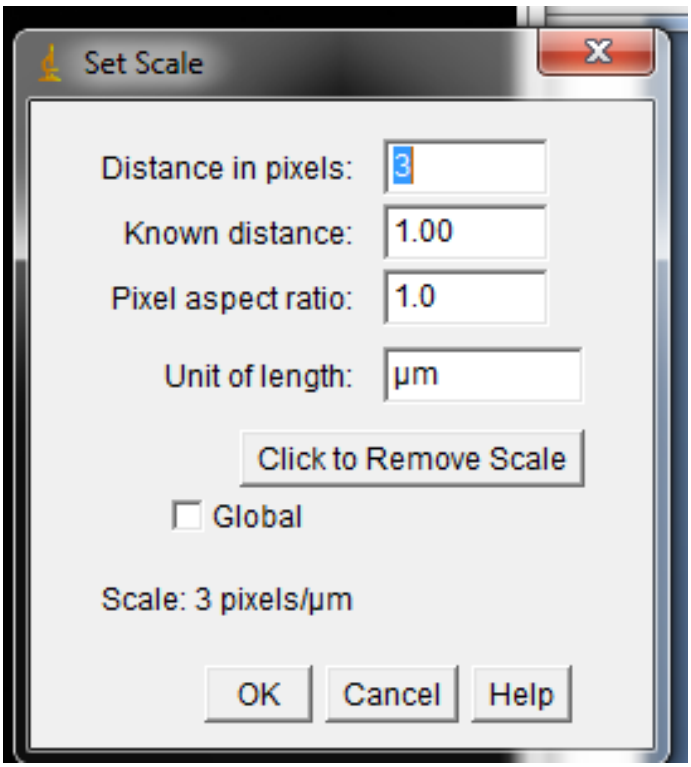
At 20X magnification, pixel size is 0.333 µm. To set scale go to Analyse → Set Scale and put the values in the dialog box as follows:

Distance in Pixels: 3.0 (0.333 µm/pixel equals 3 pixels/µm)

Known distance: 1.00

Pixel aspect ratio: 1.0

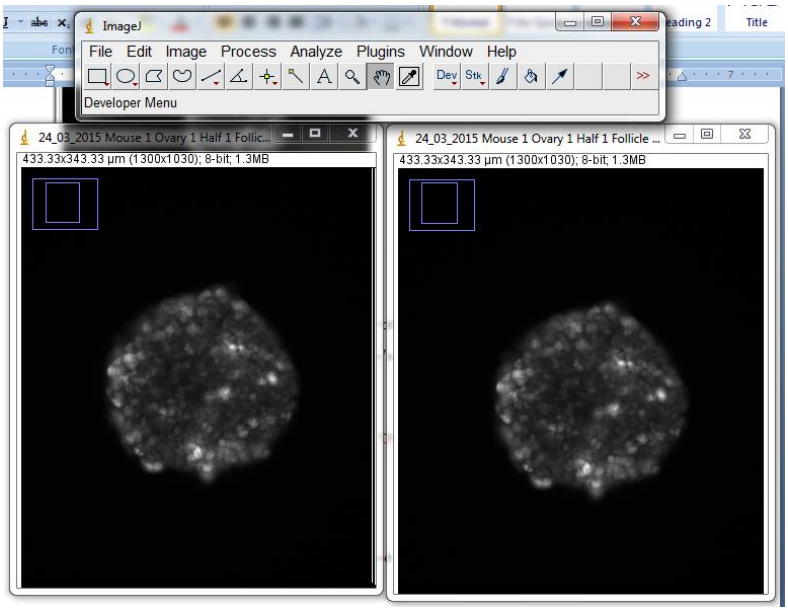
Units of length: µm



Step 4:

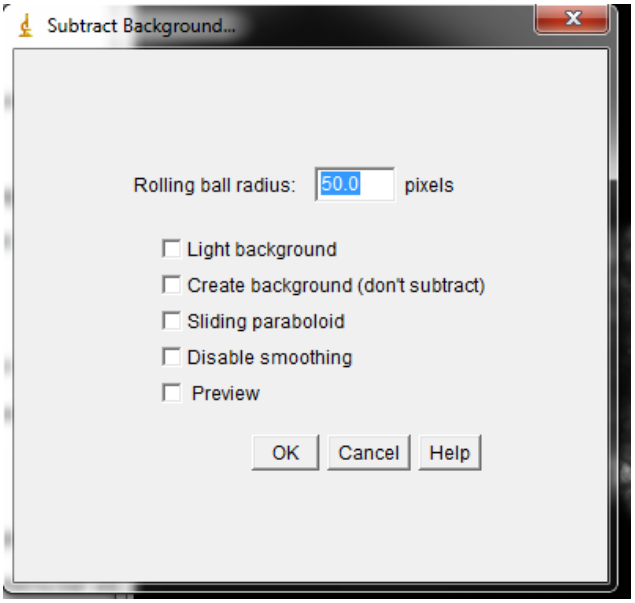
Make a duplicate copy of image. This will help in a later thresholding step.

(Go to image in the menu bar and select Duplicate)



Step 5:

Subtract Background: Go to process in the menu bar and select subtract background.

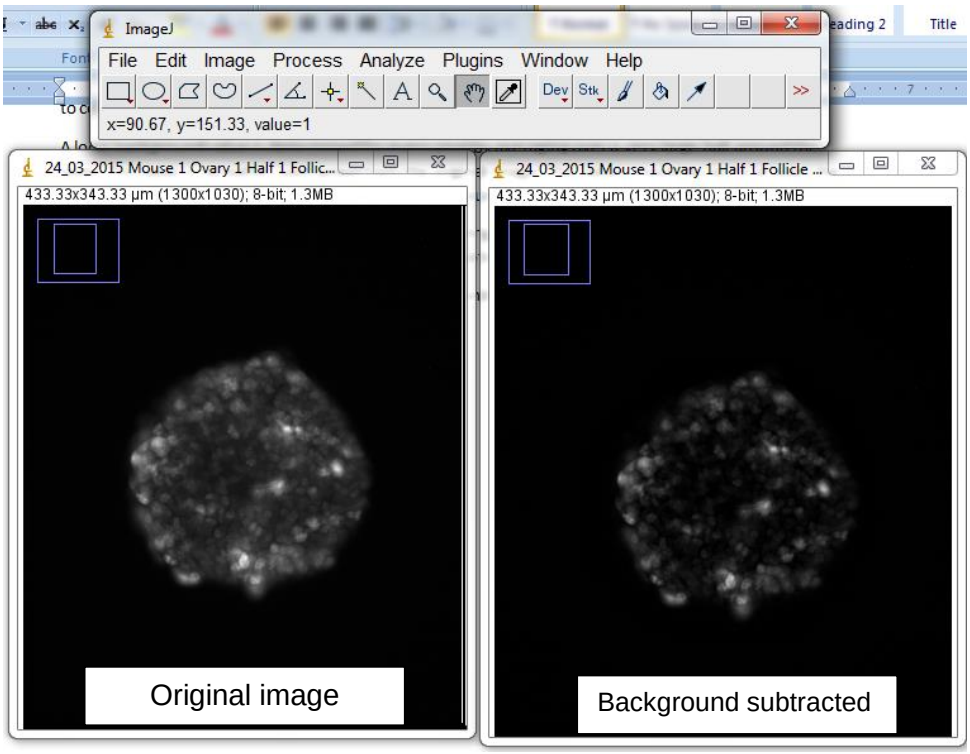


This will subtract background using rolling ball background subtraction method. Image J uses this plugin to correct for an uneven illuminated background by using a "rolling ball" algorithm.

A local background value is determined for every pixel by averaging over a very large ball around the pixel. This value is hereafter subtracted from the original image. The radius should be set to at least the size of the largest object that is not part of the background.

It has a very useful preview capability. Before subtracting check in preview and if necessary compare with the duplicate image if too much background is subtracted.

In general, many images need subtraction of background with a radius of 10-20 pixels. Higher the radius of the rolling ball lower will be background subtracted. For the follicle images, select a rolling ball radius to 20 pixels and keep this radius consistent for all images in an experiment.



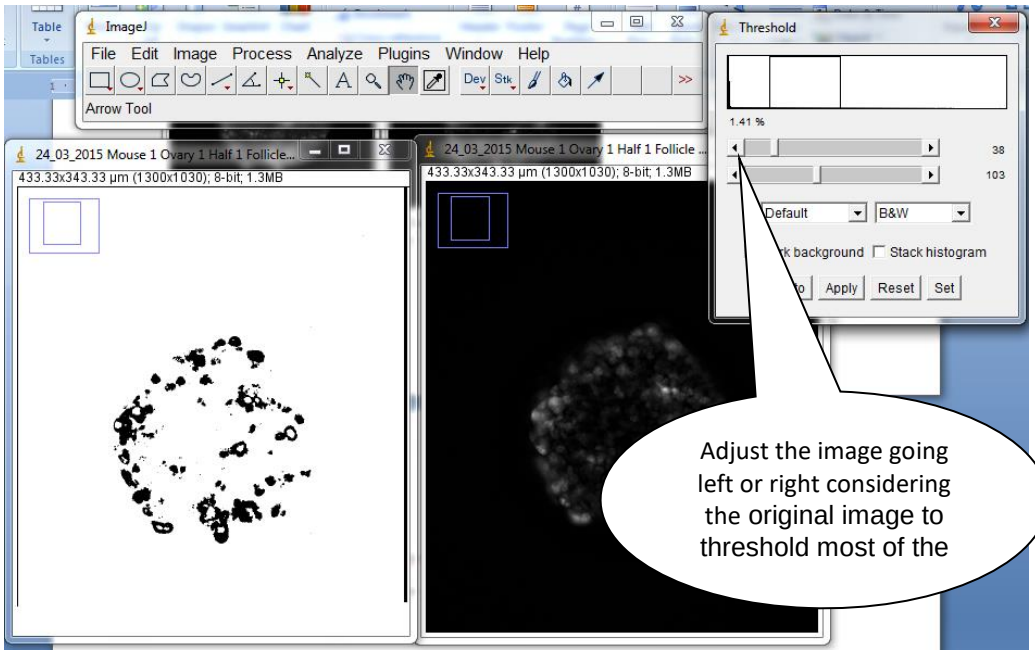
Step 6:

Adjusting threshold: Image →Adjust→ threshold.

You can select colour from red, black and while and over and under. For further processing select black and white.

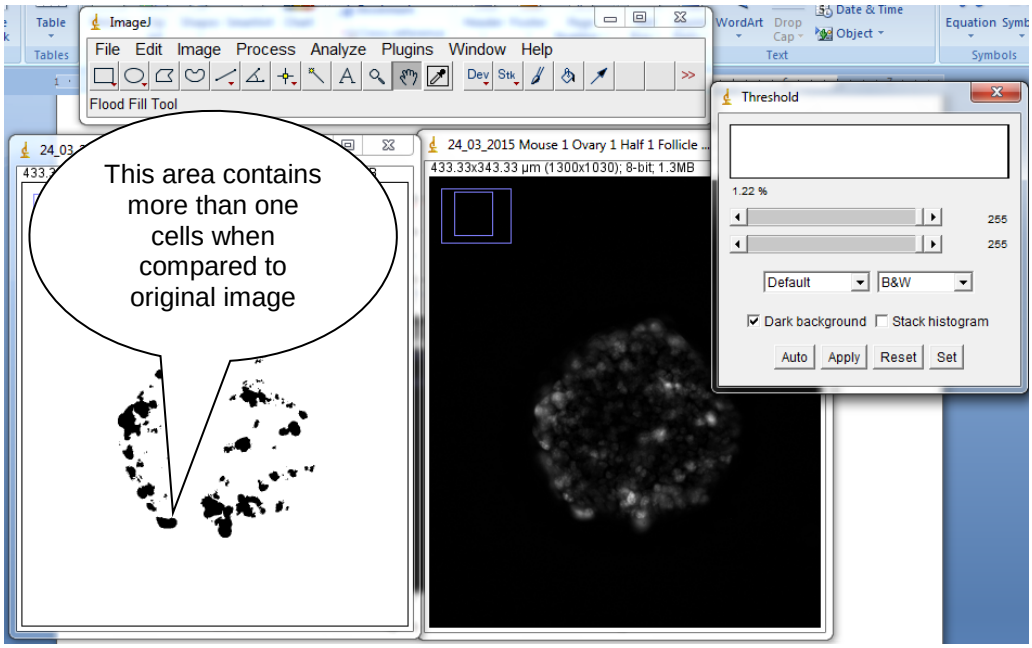
- Zoom in the duplicate image to clearly see the stained particle

- There are two thresholding levels; lower and upper, top bar is for lower thresholding level and the bottom bar is for upper thresholding level
- Increasing the lower/higher level will make more areas black and vice versa
- Comparing the original image make a balance between lower and upper levels to make maximum numbers of stained particles black.
- There might be some white holes in surrounded by black circle and two or more particles joined together, but these cells will be processed in the later stages.



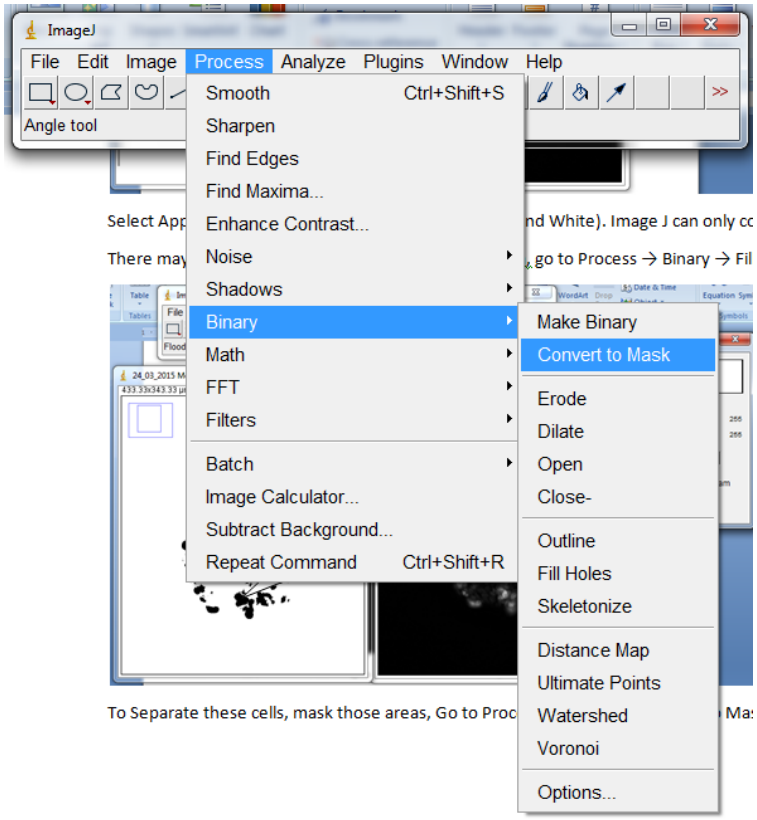
Select Apply and it will convert image to binary (Black and White). Image J can only count black dots.

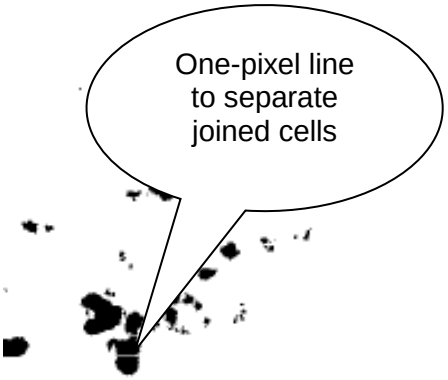
There may be some white holes in the image. Fill them, go to Process → Binary → Fill Holes



To Separate these cells, mask those areas, Go to Process → Binary → Convert to Mask

And then go to Process → Binary → Watershed, this will place a one-pixel line in between cells

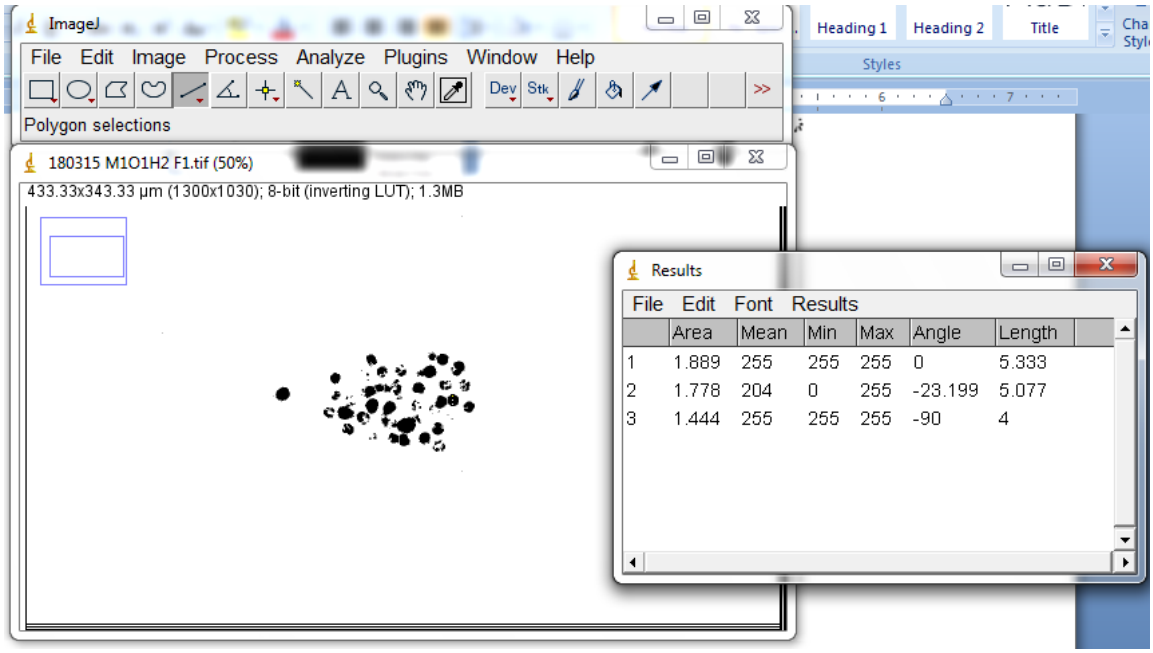




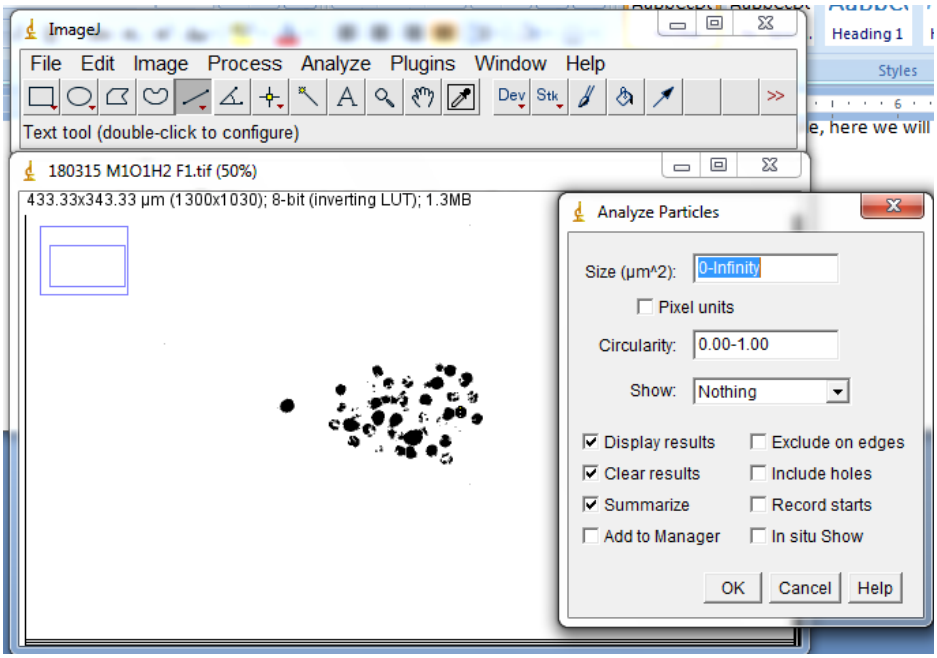
This image is now ready for quantification of GC numbers.

Step 7:

Before quantification of GC numbers, measure smallest three individual particles in the image to be used in the next step. For DAPI stained particles, nuclear size was determined by ethidium homodiamer-1 and that was 1.002 μm^2 and therefore this area was used in estimation process to include and exclude particles in quantification.

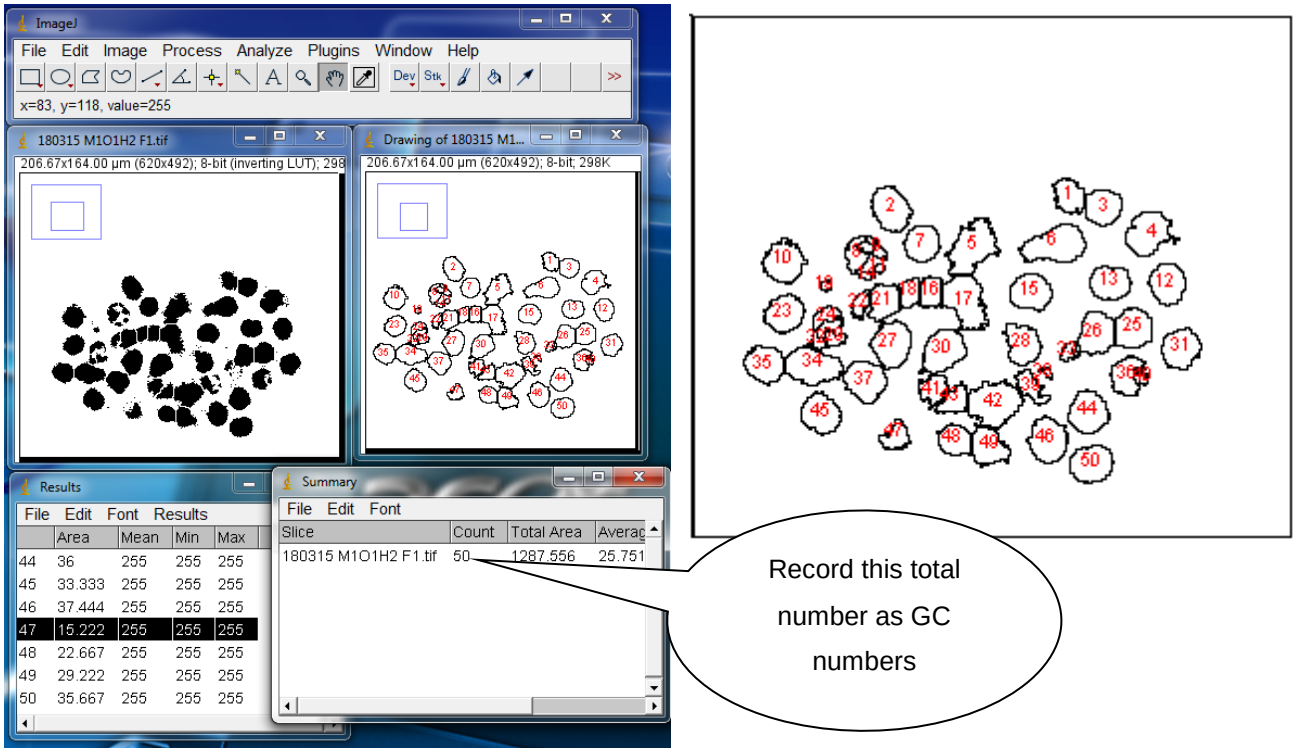


The areas of three different particles are 1.889, 1.778 and 1.444 μm^2 this is the area of the whole nucleus, because DAPI is a nuclear stain. (Diameter of GC ranges from 10 to 12 µm). Take the minimum value to be used in the next step or put a close value to the minimum area of nucleus, for example, here we will use 1 μm^2. Also consider the largest size of a nucleus. Here the largest one was 1.889, and we will set our range up to 2 μ^2. For quantification, go to Analyse → select analyse particle



Set the size from 1 μm^2 to infinity. This will exclude particles that are less than 1 μm^2. The upper limit is set to infinity because there are some attached particles which may are be with higher areas. This will count these large particles as 1 cell. Select outline from the show drop down menu. And enter ok.

This will give the numbers of GC and the outline of the nucleus.



Step 8:

Record the cell numbers with respect to follicular diameter and save the outline.

Go to file→ save as → define location → ok/save

Individual cell areas can be recorded and exported to excel for future use.

Go to the results window → select file → save as → define location, it will save results in excel.

Appendix 8.5: Standard Curves

8.5.1: KGN Cell Viability: CV Standard Curve

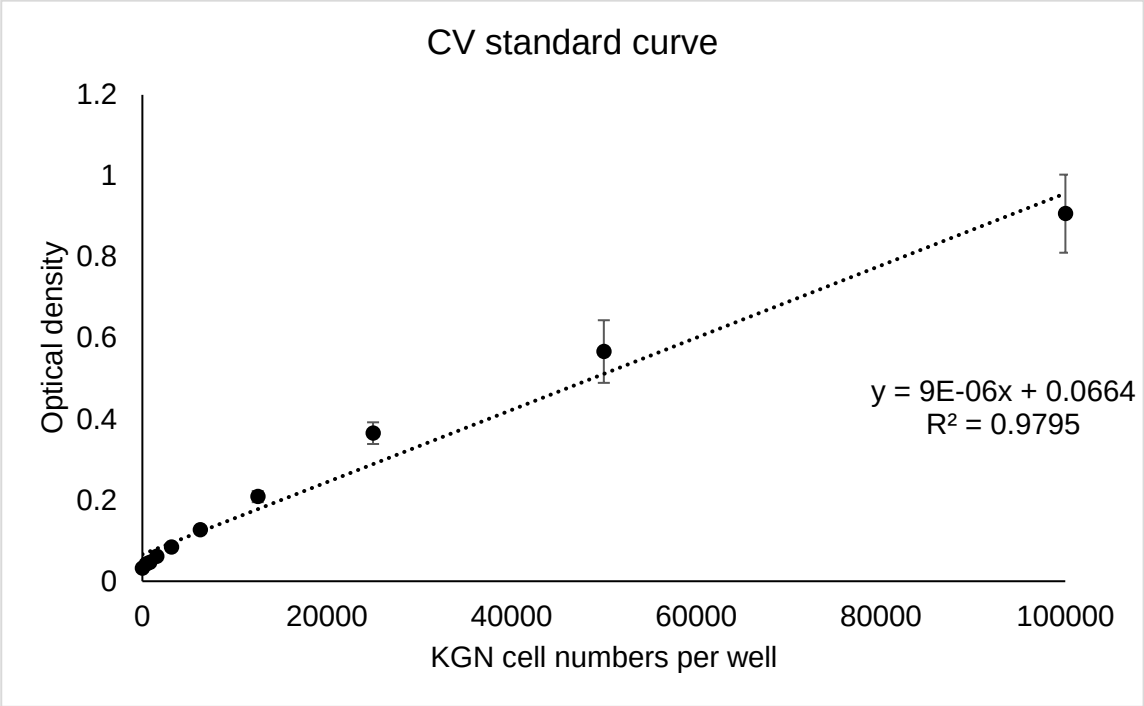


Figure 8. 5: Viable KGN cell standard *curve determined by CV assay.*

KGN cells were cultured in wells of 96 well plate at a density of 0 to 100,000 (six replicate wells for each density) on four separate occasions. Cells were cultured for 24h and thereafter viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

8.5.2: Estrogen Standard Curve

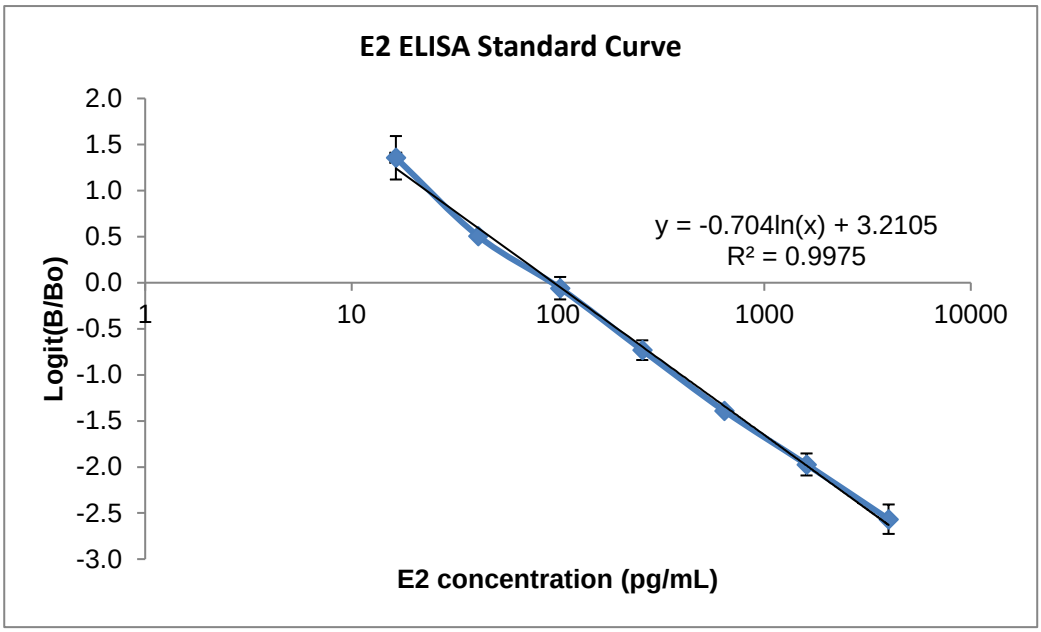


Figure 8. 6: Estrogen standard curve.

Estradiol standard was diluted in the cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. Estradiol standards were examined in a competitive Estradiol (E2) EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. B/B0 represents the OD value (at 412nm) of each concentration calculated by subtracting the average OD for non-specific binding wells and then divided by average OD of the media wells. Separate estrogen standard curve was performed each time and used to calculate the estrogen produced by cells or follicles in conditioned media.

8.5.3: Progesterone Standard Curve

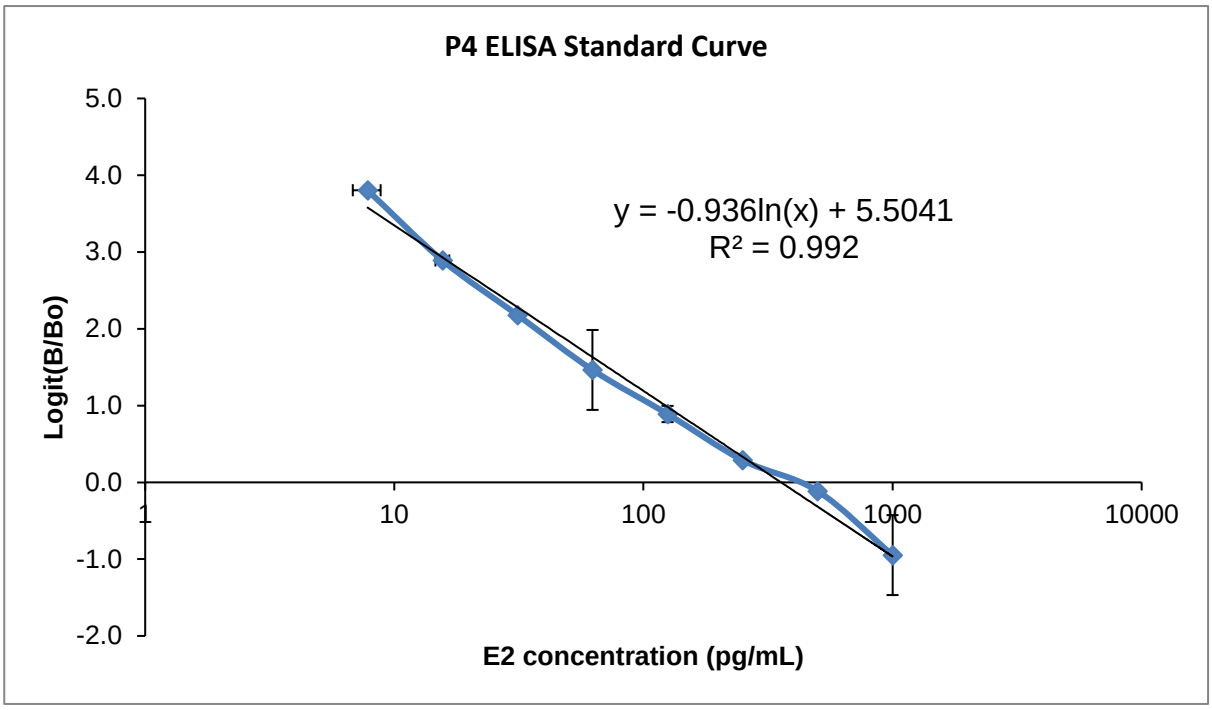


Figure 8. 7: Progesterone standard curve.

Progesterone standard was diluted in the cell culture medium to give concentrations that ranged from 7.18 to 1000 pg/mL. Progesterone standards were examined in a competitive progesterone EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse monoclonal anti rabbit IgG, and an acetylcholinesterase progesterone tracer. B/B0 represents the OD value of each concentration calculated by subtracting the average OD for non-specific binding wells and then divided by average OD of the media wells. Separate progesterone standard curve was performed each time with experiment and used to calculate the progesterone produced by cells or follicles in conditioned media.

8.5.4: AMH Standard Curve

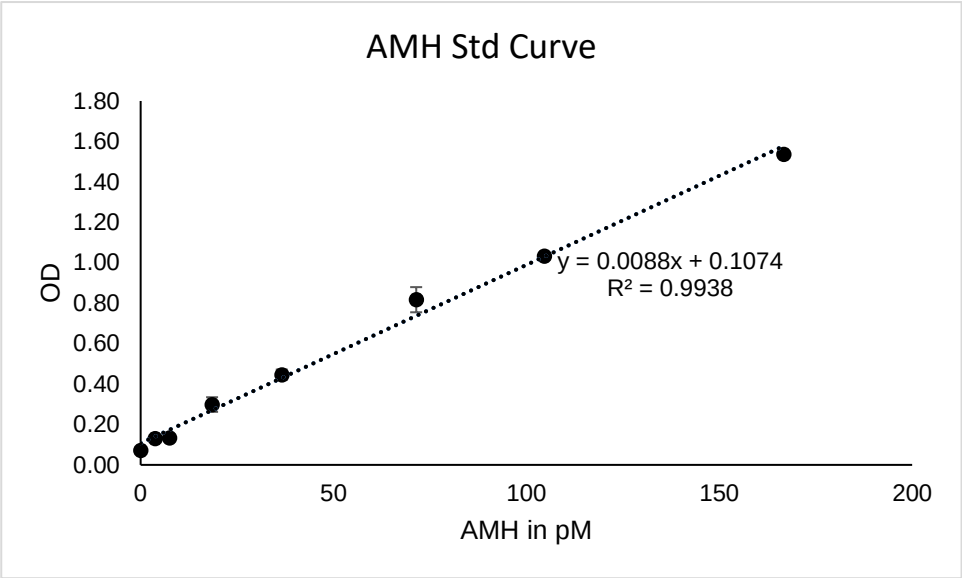
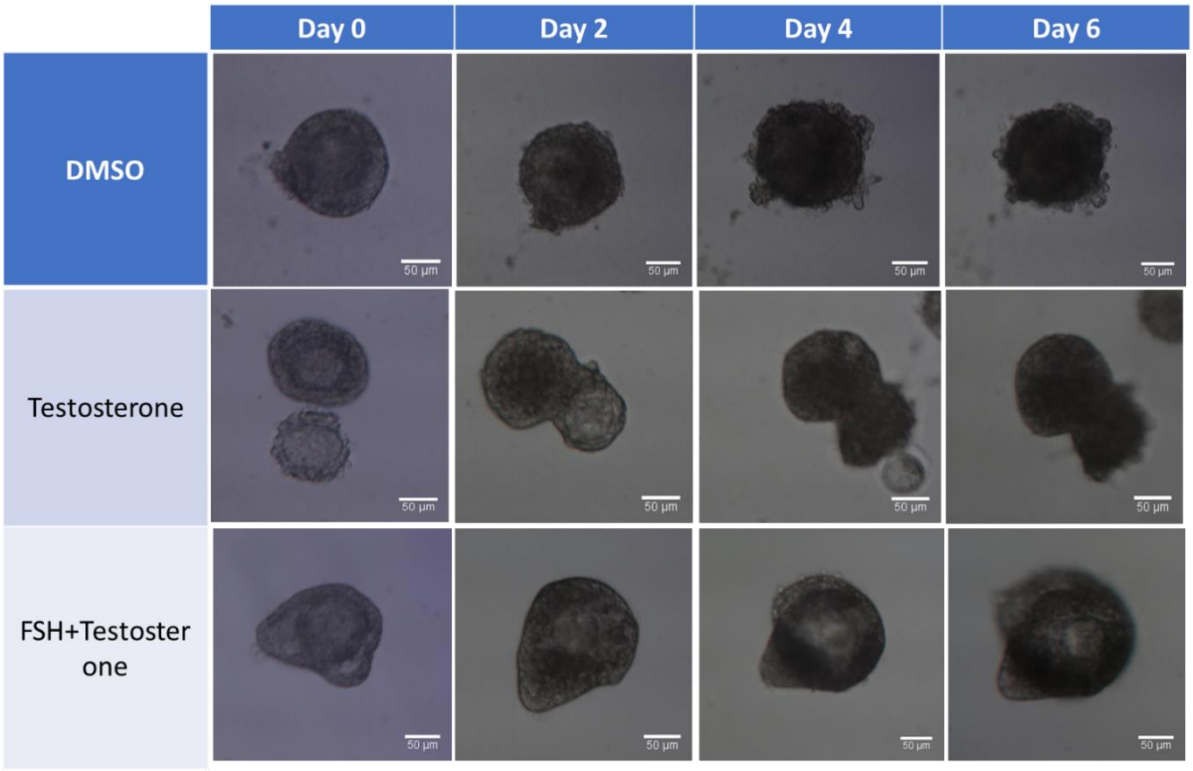


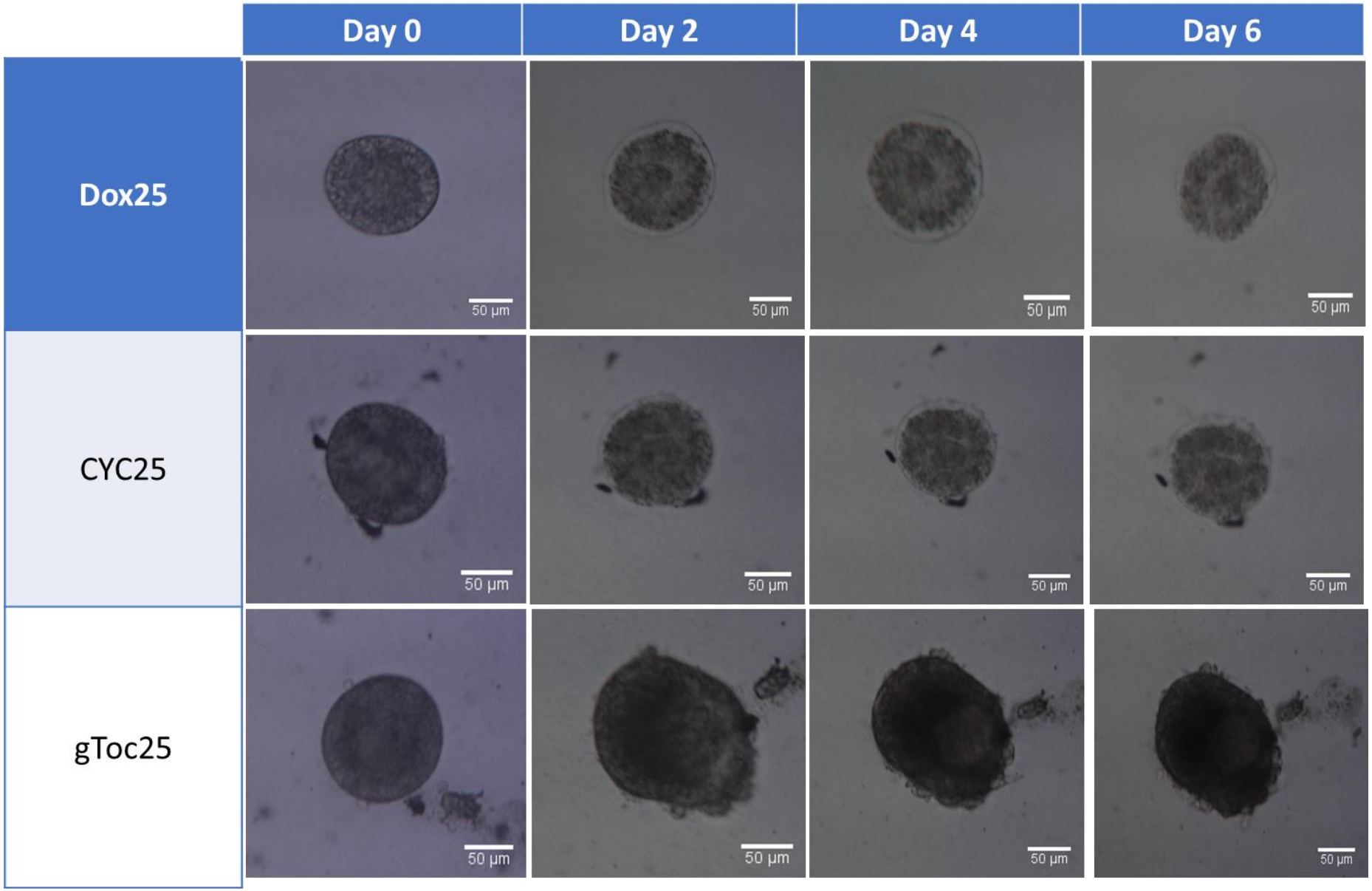
Figure 8. 8*: AMH standard curve.*

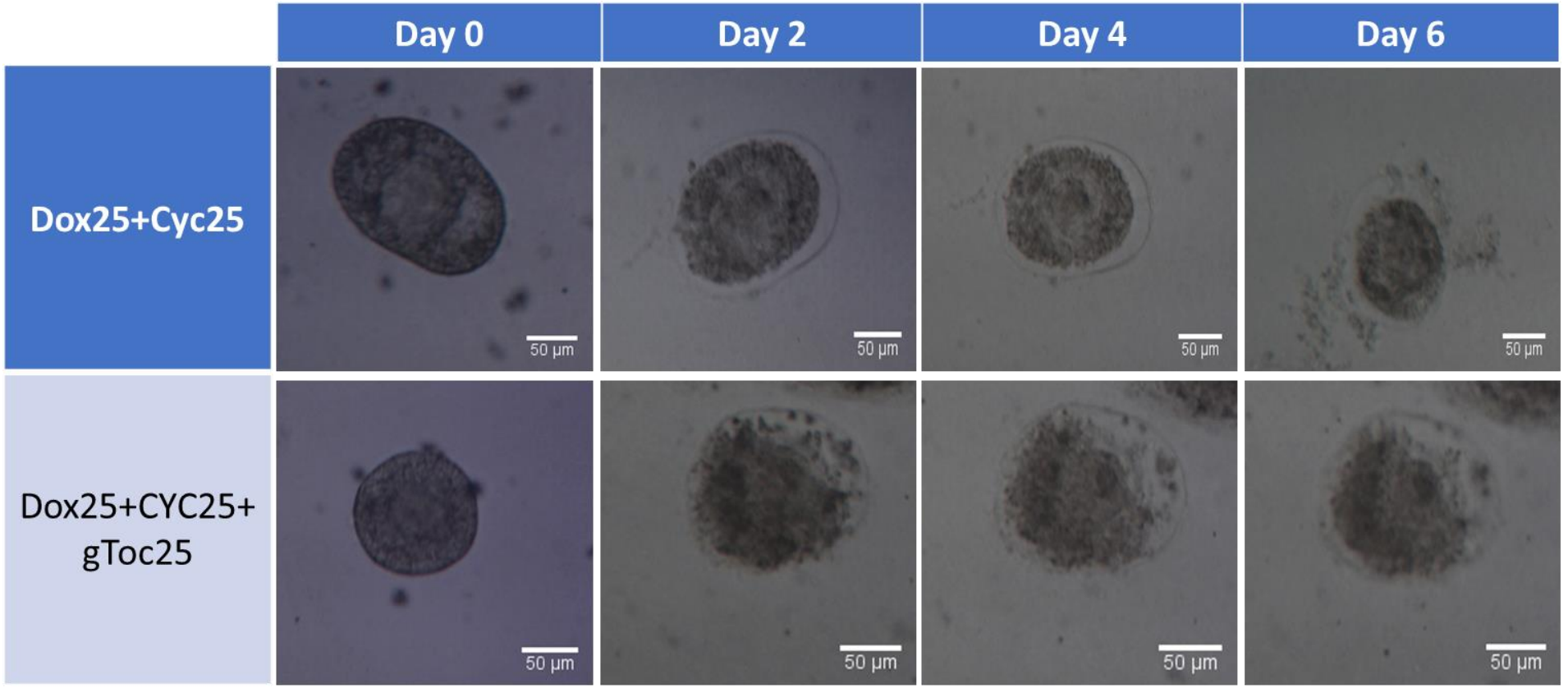
AMH standards were diluted in culture media to give concentrations ranging from 0 to 150 pM. The standards were examined in a two-immunological step sandwich type EIA that uses an anti-AMH monoclonal antibody for capturing AMH, and a biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH in the wells. Mean±SD OD at 450ng plotted against AMH concentrations.

Appendix 8.6: Morphological Change of Follicles Over Time Under Exposure to Treatments

Mechanically isolated morphologically intact (M1/M2) murine follicles (n=48) were cultured in Matrigel with test reagents for 6 days. Six follicles in each culture condition were photographed at commencement of culture (day 0) and every 2 days thereafter. The morphology of each follicle was scored as M1, M2, M3 or M4 according to the integrity of the basal membrane and the loss of GCs. Follicles with M1 and M2 scores were considered to be intact with good, spherical morphology. The experiment was repeated on four separate occasions (n=4).




Day 0
Day 2
Day 4
Day 6
Dox25
CYC25
gToc25
50 µm


Day 0
Day 2
Day 4
Day 6
Dox25+Cyc25
Dox25+CYC25+ gToc25
50 µm

9.0 REFERENCE LIST

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