Development of an *in vitro* assay to assess gap junction activity in cumulus-oocyte complexes (COC) in the rat

Shruti Patel

A thesis

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To my family with love

TE WHARE WĀNANGA O TE ŪPOKO O TE IKA A MĀUI
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6 April 2009

Supervisor's Statement for Shruti Patel

I can affirm that, to the best of my knowledge, Shruti Patel has carried out the research for her Masters thesis according to the requirements of the Victoria University of Wellington statutes as set out in the Calendar.

Shruti has been supervised by myself. I have provided advice about methodology, research, resources and analysis. Shruti carried out her research project independently and therefore the thesis represents her own work.

Ken McNatty
Professor,
Cell Biology and Biotechnology,
School of Biological Sciences

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Special thanks to my brother Sharan for his love and support during this thesis.

ABBRIVATIONS

IVF:	*In vitro* Fertilization
CC:	Cumulus cells
MGC:	Mural Granulosa cells
COC:	Cumulus-oocyte complex
OSF:	Oocyte secreted factor
GVBD:	Germinal Vesicle Break Down
GV:	Germinal Vesicle
MAPK:	Mitogen activated phosphate kinase
ALK:	Activin Like Kinase
PKA:	Phosphate Kinase A
KL:	Kit Ligand
dbcAMP:	dibutaryl cyclic adenosine monophosphate
AC:	Adenyl Cyclase
PDE:	Phosphodiesterase Enzyme
IBMX:	Isobutylmethylxanthine
TGFβ:	Transforming Growth Factor β
BMP15:	Bone morphogenetic protein 15
GDF9:	Growth differentiation factor 9
IGF:	Insulin-like growth factor
IGFRI:	Insulin-like growth factor receptor I
LH:	Luteinising Hormone
LHR:	Luteinising hormone receptor
FSH:	Follicle Stimulating Hormone
GnRH:	Gonadotrophin-releasing Hormone
PMSG:	Pregnant Mare's Serum Gonadotropin

LHR:	Luteinising hormone receptor
ERKO:	ERα Knock out
BERKO:	ERβ Knock out
ER:	Oestrogen Receptor
AR:	Androgen Receptor
HAS2:	Hyaluronic Acid Synthase 2
PTFS2:	Prostaglandin Synthase 2
DMSO:	Dimethyl Sulfoxide
BSA:	Bovine Serum Albumin
PVA:	Polyvinyl Alcohol
PBS:	Phosphate Buffer Serum

ABSTRACT

The capacity of an oocyte to mature during ovarian follicular development is a key process in reproductive biology. Bidirectional communication between mammalian oocytes and their associated follicular somatic cells (cumulus-cells) is essential for oocyte maturation. Historically, studies examining the control of ovarian follicular development focused mainly on the endocrine (external) signalling but recently intraovarian (paracrine) regulation has also been shown to be important. In addition, signalling via gap junctions between follicular cells had also been crucial for oocyte maturation and follicular development. In antral follicles, gap junction activity between the oocyte and adjacent cumulus cells first increase during follicular growth and shortly before ovulation they decrease as the oocyte resumes meiosis once more before ovulation. The range of factors that modulate gap junction activity of oocyte-cumulus cell complexes (COC) is largely unknown.

The aims of these studies were to develop an assay to assess the rate of transfer of low molecular weight materials from cumulus cells to the oocyte via gap junctions. The first objective was to validate a bioassay by which to test the effects of hormones, second messengers, and growth factors on gap junction activity in rat cumulus-oocyte complexes. In this study, COCs were collected from antral follicles of untreated post-pubertal Sprague-Dawley rats. Gap junction activity was measured in the presence or absence of different treatments using the fluorescence dye, Calcein-AM and in the presence of a phosphodiesterase type 3 inhibitor (PDE3) milrinone. Transfer of the calcein dye from cumulus cells into the oocyte was measured at various times using CRAIC fluorescence system.

The results showed that removal of the COCs from their follicular environments disrupted the gap junction activity which recovered over time in culture media. COC were sensitive to changes in pH concentration and gap junction activity could be blocked with

ocatnol-1 but not carbenoxolone. Treating rat COCs with dibutyryl cAMP or agents that maintained or increased intracellular cAMP levels like milrinone or forskolin were unable to modulate gap junction activity. Further, the combined effect of the oocyte-derived growth factors: growth differentiating factor 9 (GDF9) with bone morphogenetic protein 15 (BMP15) was also unable to modulate the rate of calcein dye transfer from cumulus cells to the oocyte. Ovarian steroids such as oestradiol and testosterone by themselves were unable to modulate the gap junction activity of rat COC but the combined treatment of testosterone plus forskolin or testosterone plus forskolin plus insulin-like growth factor 1 (IGF-1) increased the rate of dye transfer from cumulus cells to the oocyte.

In conclusion, a fluorescence dye transfer assay was developed to measure the effects of different treatments on gap junction activity in rat COC. Under *in vitro* conditions, it was established that the combination of steroid and cAMP stimulators or a steroid, cAMP stimulator with IGF1 but not these reagents individually could enhance the recovery of gap junction function in rat COC.

The outcomes of these experiments may help to provide new insights into developing suitable *in vitro* conditions, for the *in vitro* maturation of mammalian oocytes. Also, the newly developed assay may serve as a useful *in vitro* model to evaluate the effects of hormones, nutritional supplements and other factors on COC functions.

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CHAPTER 1 INTRODUCTION

1.1 The Ovary

Ovaries are a pair of female sex glands that are located on either side of the uterus in mammals. The major function of the ovaries is to maintain and mature eggs (i.e. oocytes) for fertilisation and the production of healthy offspring. A second important function is to produce the reproductive hormones mainly oestradiol-17ß and progesterone. The ovary has two principal morphological regions known as cortex and medulla (Figure1). The cortex in the outer region of the ovary is bound by at least one continuous layer of cells known as the surface epithelium. The cortical region is the location of most, if not all, ovarian follicles. These follicles are either present as small non-growing follicles or they are at different stages of development and degeneration (i.e. atretic follicles). Each follicle contains an oocyte which is surrounded by a regular array of somatic cells known as granulosa cells (see Figure 1).

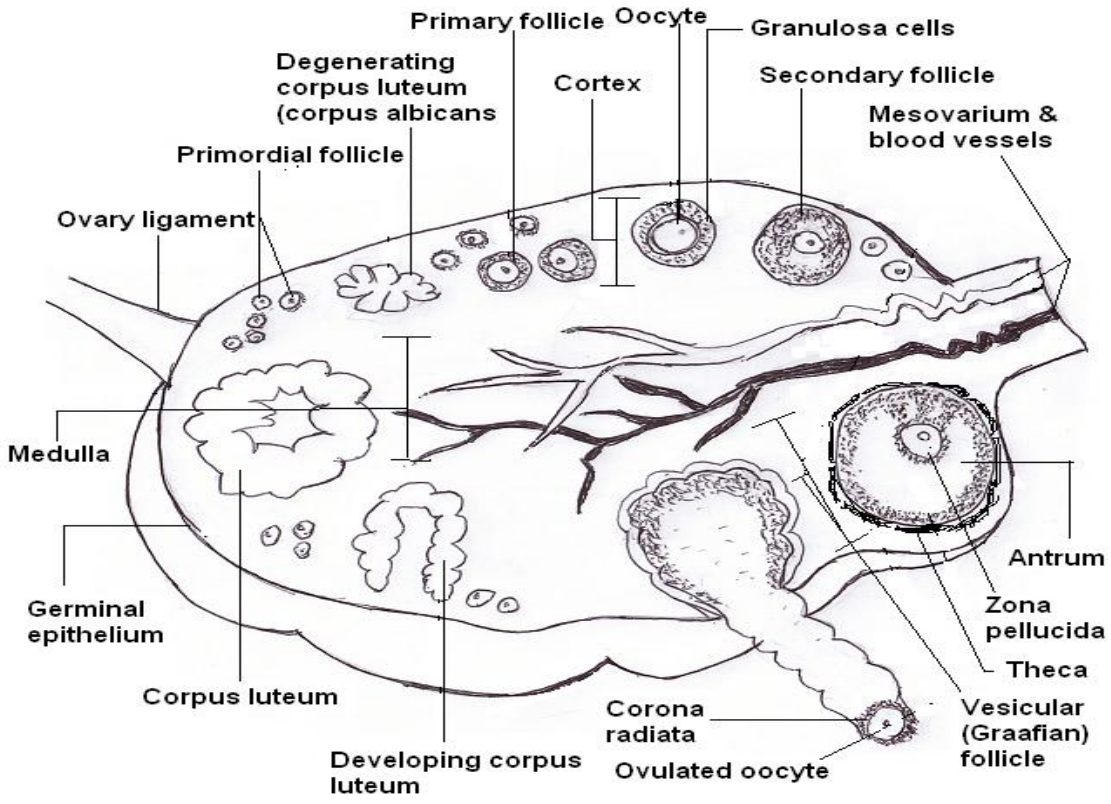

Figure 1 The Ovary [modified from Book: Human Anatomy and Physiology by Marieb Elaie N. (2006)]

1.2 Ovarian follicular development

Most follicles (i.e. > 80%) are present as primordial follicles namely an immature oocyte and a single layer of granulosa cells. Primordial follicles represent the pool from which growing follicles emerge on a regular and continuous basis throughout life (Peters et al, 1975). In some mammals such as humans and rodents, this pool of follicles gets depleted before the life expectancy is reached. In other mammals such as sheep, the pool of primordial follicles is not depleted before the death of the animal (K. McNatty, Unpublished data). Development of the immature primordial follicle into a mature ovulatory follicle is commonly referred to as folliculogenesis. During folliculogenesis, ovarian follicles undergo different developmental stages that are classified as the primary, secondary (pre-antral), tertiary (antral) and preovulatory stages (Lundy et al., 1999) (see Figure 2).

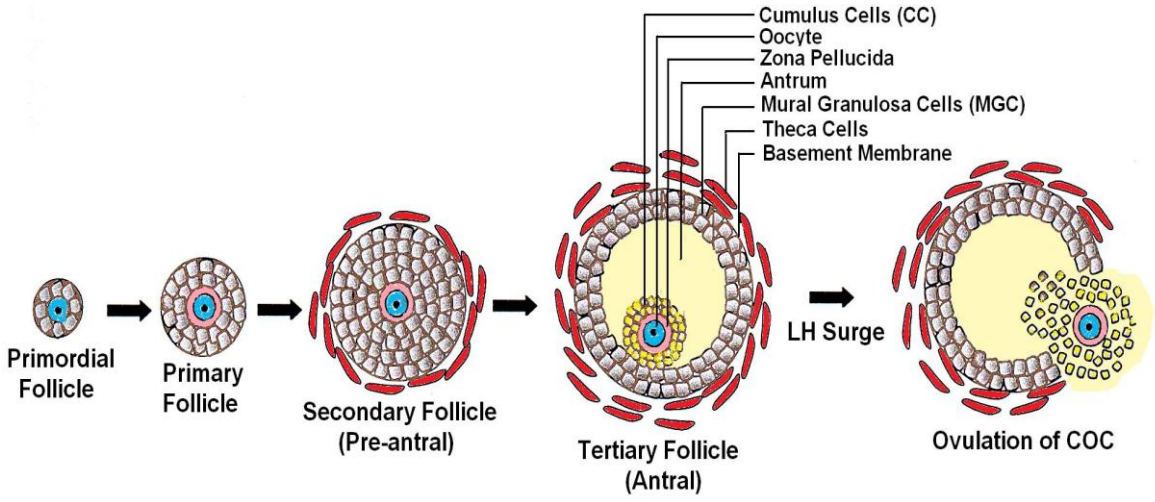

Figure 2: Schematic representation of folliculogenesis and ovulation (Modified from MSc thesis by Rebecca Dragovic (2006), University of Adelaide)

Development of the follicle through these stages is defined by the progressive growth and maturation of the oocyte, proliferation and differentiation of the surrounding

granulosa cells and formation of the theca layer outside the basement membrane of the growing follicle. Follicular development from primordial to the secondary (i.e. early preantral) stage is considered to be pituitary hormone (i.e. gonadotrophin) independent (McNatty et al., 2001). However, from the secondary stage onwards follicular growth is dependent upon the gonadotrophins (follicle-stimulating hormone and luteinising hormone) (Erickson and Ryan, 1976; Halpin et al., 1986; Kumar et al., 1997). At the primordial stage, the nucleus of the oocyte is at the diplotene stage of meiosis and it remains at this stage until shortly before ovulation. As follicles begin to leave the primordial follicle pool and start to grow, granulosa cells change their shape from flattened cells to become cuboidal and thereafter the granulosa cells surrounding the oocyte further proliferate to form multilayer cells surrounding the oocyte. At the early stages of follicular growth, the granulosa cells are connected via desmosomes, but no gap junctions are present (Albertini and Anderson, 1974). During follicular growth, granulosa cells secrete a number of materials including proteoglycans that lead to accumulation of fluid between the cells (Gondos, 1970). Eventually a fluid-filled cavity (i.e. an antrum) forms within the follicle, which is then referred to as antral follicle (Figure 2). Transition of the preantral follicle into an antral follicle is marked by the differentiation of granulosa cells into two different cell types: mural granulosa cells (MGCs), the cells lining the follicle wall and the cumulus cells (CCs), the cells which surround the oocyte (Figure 2). In an antral follicle, cumulus cells surrounding the oocyte are connected to the oocyte via gap junctions, therefore their main function is to provide the oocyte with <1KD molecules like pyruvate, metabolites and protein substrate like amino acid by passing them through the gap junctions (Albertini et al., 2001). Mural granulosa cells have differentiated functions like steroidogenesis and the expression of the luteinizing hormone (LH) receptors (Whitelaw et al., 1992). As the follicle continues to develops, a layer of cells outside the basement membrane differentiates to become part of the follicle. These cells are known as theca cells since the follicle continues to grow, the thickness of theca layer increases markedly. In

some mammals the theca layer differentiates into theca externa and theca interna (Shimasaki et al., 2004). The theca interna is much more vascular than the theca externa.

1.3 The rat oestrous cycle

This research project is focused on oocyte-cumulus cell function of sexually mature Sprague-Dawley rats 21-25 days old. Therefore, it is of value to outline the hormonal and related physiological events that regulate the rat oestrous cycle. An oestrous cycle in rodents can be defined as the repetitive period of sexual receptivity (i.e. heat) separated by specific quiescent intervals. The oestrous cycle of the rat is divided into four stages referred to as pro-oestrus, oestrus, metoestrus and dioestrus. In the pro-oestrous stage, animals are sexually inactive. The ovarian follicles develop towards the preovulatory stage and an increasing endometrial cellular activity occurs at this stage. During the oestrous stage, the female is sexually active and the preovulatory follicles secrete high levels of oestradiol leading to ovulation. Development of the corpus luteum from the ovulated follicles starts in the metoestrus cycle and the release of oestrogen from the ovary is inhibited. Throughout the dioestrus phase, development of corpus luteum continues and progesterone is released. Rodents such as rats and mice undergo regular oestrous cycles throughout the year and are considered to be poly- oestrous. In the rat, the oestrous cycle lasts for 4 to 5 days during which they ovulate but do not form fully functional corpus luteum. Few follicles start to grow towards ovulation at the end of the pro-oestrous stage. Follicular growth increases more than eight fold as it reaches early oestrous stage. Peak oestradiol secretion occurs around 11 am on the day of pro-oestrus and between 11am and midnight the preovulatory surges of FSH, LH and progesterone take place followed by ovulation at 4am the next morning. Corpus luteum forms within 24h in met-oestrus once the oocyte has ovulated. The corpus luteum in the rat persists for 3 days before it regresses to mark the beginning of the next oestrous cycle (see Figure 3).

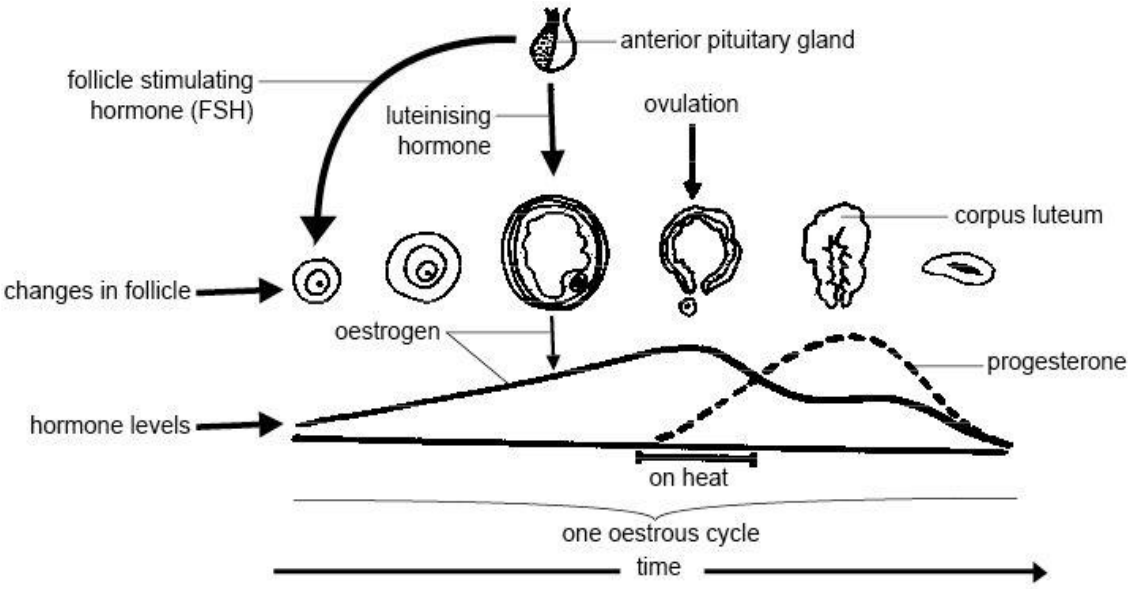

Figure 3: The rat oestrous cycle

1.4 Gonadotrophins and the oestrous cycle

Ovarian follicles during the preantral growth stage develop responsiveness to the gonadotrophins; follicle-stimulating hormone (FSH) and luteinising hormone (LH). Once the follicles develop to the antral stage, they become critically dependent on the gonadotrophins. FSH and LH are synthesised and secreted by the pituitary gland after stimulation by the hypothalamic releasing hormone GnRH (Gonadotrophin-releasing hormone). FSH and LH are glycoproteins with molecular weight of approximately ~30,000Da. Their structures consist of two subunits α and β. The α subunit with a molecular weight of 14,600Da, containing 92 amino-acid residues in the same sequence with five disulphide bonds and two carbohydrate moieties. FSH and LH form the heterodimers through non-covalent interactions. The FSH β subunit is ~15,400Da composed of 111 amino-acid, 6 disulphide bonds and carbohydrate moieties which are N-linked at asparagine residue (Watkin et al., 1987). LH forms non-covalently linked heterodimers with molecular weight of approximately 29,400 Da that contains two non-covalently linked α subunit (MW: 14,600 Da) and a β subunit (MW: 14,000 Da) (Knobil, 1980). FSH and LH hormones signal by binding to the rhodopsin-like G protein coupled

receptor family specifically located on ovarian cells. FSH receptors are located exclusively on granulosa cells whereas LH receptors are initially localised to theca interna cells but shortly before ovulation on granulosa cells. Structurally these receptors consist of a large extracellular N-terminal domain which binds to either FSH or LH. These receptors also contain a seven transmembrane helicase forming a serpentine structure which is responsible for interacting and binding to the G-protein. Binding of FSH or LH to their specific receptor leads to the activation of intracellular signalling pathways including the adenylate cyclase [e.g. cyclic adenosine monophosphate; (cAMP)], inositol phosphate [e.g (IP3)], calcium (Ca^2+) and mitogen activated phosphate kinase (MAPK) pathways (Themmen and Huhtaniemi, 2000).

Within the antral follicle, binding of FSH to the immature granulosa cells leads to an increase in cAMP synthesis which activates a number of genes influencing the differentiation functions of both granulosa cells and cumulus cells and later during preovulatory follicular development, FSH stimulates the expression of LH receptor (Abell et al., 1998). Binding of LH to its receptors located on theca cells stimulates the synthesis and secretions of androgens such as androstenedione and testosterone. These androgens are both secreted into the blood stream but they also diffuse into granulosa cells within the follicle where they are converted into mainly oestradiol by the P450 aromatase enzyme (Whitelaw et al., 1992). Therefore, the production of oestradiol requires both LH stimulation of theca cells and FSH stimulation of granulosa cells. During late follicular development, follicles showing a subsequent induction of LH receptor on granulosa cells in response to FSH are able to undergo ovulation. In contrast, the follicles that fail to acquire LH receptors on granulosa cells undergo atresia without reaching the pre-ovulatory stage (Zeleznik et al 1974; Richards et al 1978; 1979) (see Figure 4).

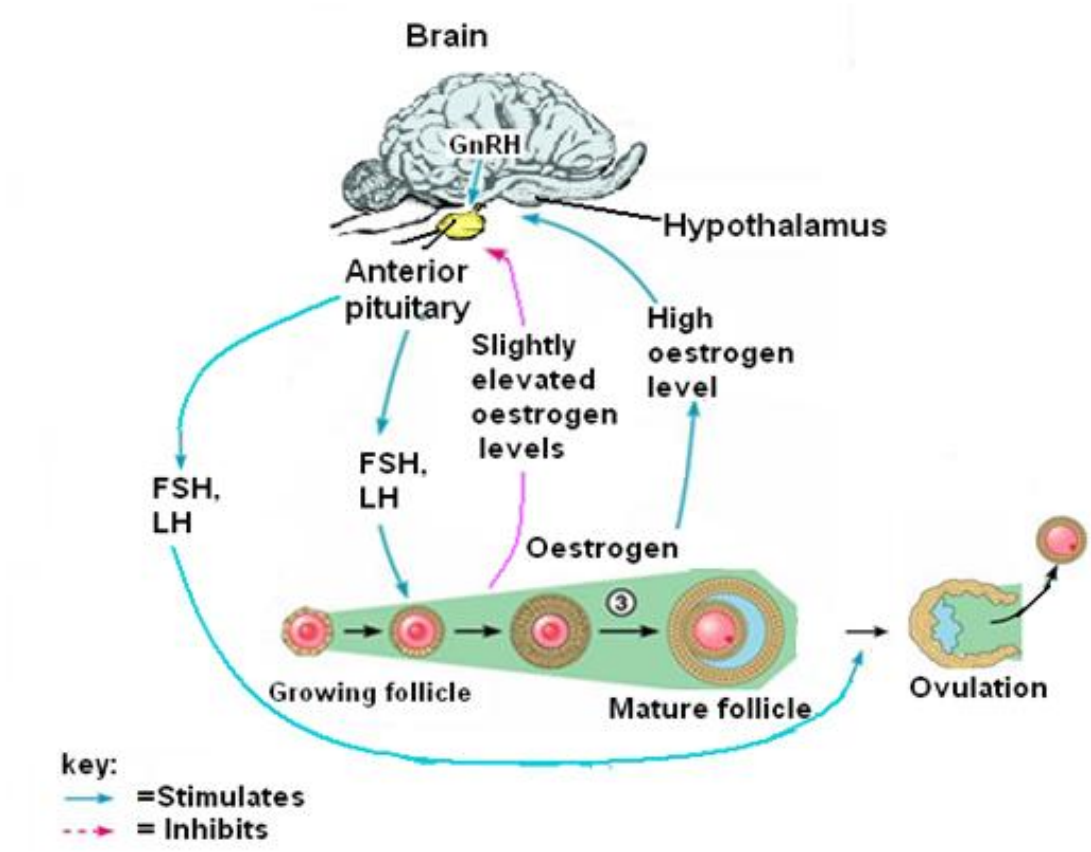

Figure 4: Hormonal regulation of folliculargenesis [Modified from book: Human Anatomy and Physiology by Elaie N. Marieb (2006)]

The expression of gap junction protein Cx43 and mRNA levels are modulated by gonadotrophins like FSH and LH throughout the rat oestrous cycle. Thus, in the developing follicle, granulosa cells are connected by gap junctions with one another and in turn they are connected to cumulus cells and the oocyte to form a synictium that is regulated in part by the pituitary hormones FSH and LH. Studies have shown that Cx43 gap junction protein was present in abundance within cells of the antral follicle when FSH concentration was high (Granot and Dekel, 1997; Wiesen and Midgley, 1993). In contrast, low amounts of Cx43 protein was found during the preovulatory stage when LH concentration was high (Larsen et al., 1981; 1987). Studies reported by Sommersberg (2000) show that FSH leads to an increase in electrical coupling in rat granulosa cells. This effect of FSH on gap junction

abundance was thought to be mediated by the cAMP dependent protein kinase A (PKA) biochemical pathway (Cooke, 1999). A decrease in the gap junction protein Cx43 during the preovulatory surge in the rat Cumulus oocyte complexes (COC) occurs in a two step process. Firstly, LH leads to phosphorylation of the gap junction protein Cx43 via PKA and MAP Kinase pathway (Sherizly et al., 1988) as the phosphorylation of the protein is prerequisite for the activation of the ubiquitination pathway. During the second stage, LH leads to degradation of the Cx43 protein through lysosomal and proteosome pathways (Ciechanover, 1995; Granot and Dekel, 2002). One of the studies done on Cx43 mRNA levels shows that although the Cx43 protein was completely abolished by LH, mRNA levels decreased slightly but were not eliminated (Granot and Dekel, 1994). The results suggest that, the LH effect on the gap junction protein Cx43 protein was mediated at the translational level. This finding was later confirmed by Kalma et al., (2004) that LH inhibits the Cx43 protein translation in rat ovaries.

1.5 TGF Beta Super-family

The Transforming Growth Factor (TGF) β super-family is composed of more than 35 different proteins that include Growth differentiating factor 9 (GDF9), Bone morphogenetic protein 15 (BMP15) and TGF β 1, 2, 3 which play an essential role in follicular development in rodents, sheep, and humans (Elvin et al., 2000; Shimasaki et al., 2004; Juengel and McNatty, 2005). The TGFβ family members are structurally similar and they are synthesised as pre-pro proteins consisting of a large pro-region and small biologically active mature region (Massague, 1990; Chang et al., 2002). The pro-region is cleaved by an endogenous protease, that recognises four amino-acid cleavage site with arginine as the first and last amino acid (RXXR) (Thomas, 2002; Taylor et al., 2003; Rockwell and Thorner, 2004). The pro-region plays an important role in assisting the correct folding and dimerisation of the molecule before the endogenous protease cleaves the active mature region (Shimasaki et al., 2004). A specific structural feature of the TGF β

family is the cysteine knot which is formed from 6 cysteine residues in the mature region (Chang et al., 2002). The TGFβ family members can form homodimers or heterodimers that are covalently attached through another conserved cysteine residue. A few members of the TGFβ superfamily like GDF9 and BMP15 lack this additional conserved cysteine residue. Thus these molecules form homodimers or heterodimers through non-covalent interactions (Shimasaki et al., 2004). TGFβ family members activate the cellular processes through two different types of receptors: type I and type II. There are seven type I receptors known as Activin like kinase (ALK 1-7) and five type II receptors known as Activin receptor (R) II, Activin RIIB, BMPRII, TGFβ RII and AMPRII (de Caestecker, 2004; ten Dijke and Hill, 2004). It is the binding of specific TGFβ ligands to these different sites of receptors that results in the activation of specific signalling pathways. In the cellular cytoplasm, downstream signalling occurs via specific Smad molecules. There are eight known Smad molecules known as Smad 1-8 of which Smad 2 and 3 are generally thought to be activated by the TGF β subfamily and Smad 1, 5 and 8 by the BMP subfamily. Recent studies with BMPs however suggest this may not always be the case (Ken McNatty personal communication). Smad 4 is considered to be a common Smad that forms a complex with other phosphorylated signalling Smad molecules to enable them to enter the nucleus to regulate transcription, whereas Smad 7 is considered to be inhibitory which thereby blocks signalling by TGF β family members at the cytoplasmic level (Derynck and Zhang et al., 2003).

In general the TGFβ1 and 2 subfamily members binds to a type II receptor which in turn leads to the recruitment of a type I receptor and the activation of the cytoplasmic signalling molecules Smad 2 and 3. On the other hand, the BMP subfamily of ligands such as BMP15 produced by the oocyte is thought to bind to a type I receptor which in turn recruits a type II receptor that can phosphorylate cytoplasmic Smad molecules 1, 5 and 8 (Moore et al., 2003; Mc Natty et al., 2004).

However GDF9, being a member of BMP15 subfamily binds and phosphorylate the type I receptor first, which in turn activate the type II receptor and the activated type II receptor further activates the cytoplasmic Smad 2 and 3 instead of type II Smad molecules 1, 5, 8 (Mazerbourg et al., 2004; Kaivo-Oja et al., 2003; Roh et al., 2003) (Figure5).

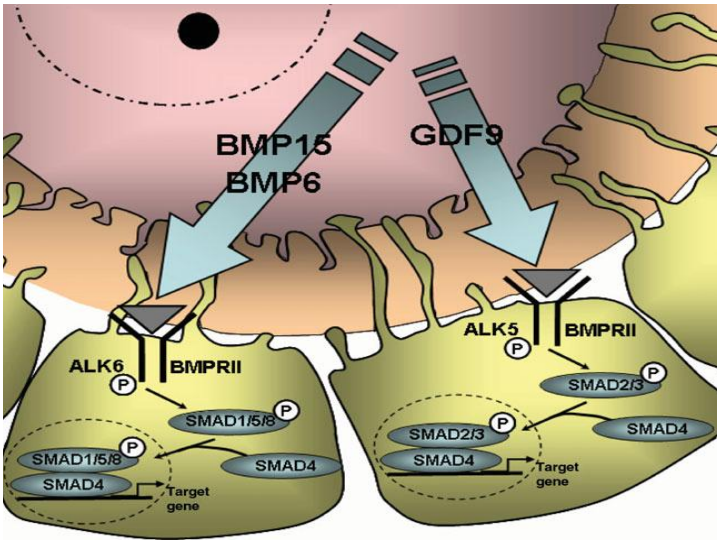

Figure 5: Growth factor GDF9 and BMP15 signalling pathway (From Gilchrist et al., 2008 with permission)

GDF9 and BMP 15 mRNA and protein have been found in the oocyte of different species (McGrath et al., 1995; Dube et al., 1998). The patterns of GDF9 and BMP15 protein expression differ within different stages of folliculogenesis and also between different species. GDF9 and BMP15 mRNA and proteins were first observed either in primordial or primary follicles in rats, sheep, cows and humans (McGrath et al., 1995; Jaatinen et al., 1999; Bodensteiner et al., 1999; Elvin et al., 2000; McNatty et al., 2001; Juengel and McNatty, 2005). Homozygous GDF9 knock out mice or GDF9 inactive sheep were infertile as the follicular development was arrested in the primary stage which showed impaired granulosa cell proliferation and abnormal oocyte development (Dong et al., 1996; Hanrahan et al., 2004). In comparison to the above results, the homozygous BMP15 knockout mice did not have arrested follicular development (Solloway et al.,

1998). In contrast to that in sheep naturally occurring point mutations (Inverdale and Hanna) leading to an inactivation of the BMP 15 gene, display abnormal follicular development with the follicles arrested at or before the primary stage of growth (Davis et al., 1992; Galloway et al., 2000). This and more recent findings for point mutations in women suggest that BMP 15 is also critically important for early follicular development in sheep and humans (Juengel & McNatty, 2005).

In the early growing o ocyte, GDF9 stimulates the granulosa cells to secrete Kit ligand (KL). In turn, KL interacts with the oocyte via a c-Kit receptor to promote oocyte growth until it reaches a meiotically mature state (Joyce et al., 1999). In the meiotically mature oocyte, GDF9 suppresses Kit ligand expression in granulosa cells thereby terminating oocyte growth (Eppig, 2001). Meiotically mature oocytes secrete oocyte derived growth factors via paracrine actions which regulates a number of somatic cell functions such as proliferation, differentiation and luteinisation. Oocyte-derived growth factors such as BMP15 and 6 are thought to generate a concentration gradient from the oocyte to the granulosa cells. This localised gradient of BMPs protects COC to some extent from apoptosis (Hussein et al., 2005). In rodents, recombinant BMP15 suppresses the action of the FSH receptor on the granulosa cells *in vitro* thus BMP15 regulates the FSH actions on granulosa cells (Otsuka et al., 2000; 2001). It seems more likely that BMP15 might regulate the responsiveness of ovine follicles to LH (Ken McNatty, personal communication). Collectively, these results show that GDF9 plays an important role in early follicular development in rodents and sheep and that GDF9 as well as BMP15 each play important roles in sheep.

It remains uncertain at this time whether GDF9 is important for follicular development in women but studies in infertile women have indicated associations with point mutations in GDF9 (Dixit et al., 2005; Kovanci et al., 2007). GDF9 helps to maintain the cumulus cell phenotype (Elvin et al., 2000; Vitt et al., 2000). In mice, BMP15 and GDF9 can inhibit the expression of the luteinizing hormone receptor (LHR) on the

granulosa cells immediately surrounding the oocyte. This is consistent with the view of the oocyte secreted factors generating a concentration gradient so that the granulosa cells immediately adjacent to the oocyte undergo a different pattern of differentiation than the granulosa cells. Therefore, the oocyte via the secretions of BMP15 and GDF9 induces a cumulus cell phenotype (no LHR) in the granulosa cells immediately around the oocyte whereas the mural granulosa cells that lie near the follicular wall can be induced to express the LHR. In this way, the cells surrounding the oocyte are unable to respond to LH (You-Qiang et al., 2004; Joyce et al., 1999). In mice, GDF9 maintains and regulates the inner connections between oocyte and adjacent somatic cells and also regulates the amino acid transport from cumulus cells via gap junction to the oocyte (Carabatsos et al., 1998; Eppig, 2004). Recombinant GDF9 leads to cumulus cell proliferation followed by mural cell proliferation (Hayashi et al., 1999). This has been proved by in vivo studies in the rat with [^{3}H] thymidine, in which radiolabeling in an antral follicles was higher in the COC than in the proximal mural granulosa cells on the distal side of the follicle (Hirshfield, 1986). *In vitro* combined administration of ovine (o) GDF9 and (o) BMP15 growth factors in rat granulosa cells were able to stimulate (4-9 fold) (3H) thymidine uptake. Thus, the above result shows that the combined effect of GDF9 and BMP15 has a mitogenic effect on rat granulosa cells (McNatty and Juengel 2005). BMPs can also inhibit progesterone synthesis by granulosa cells *in vitro* (Shimasaki et al., 2004; Juengel et al., 2006). Progesterone is a marker of luteinization of granulosa cells. This shows that oocyte-derived growth factors including BMP15 or GDF9 may act as luteinization inhibitors during follicular development (Channing et al., 1970; Eppig et al., 1997a, b).

GDF9 and BMP15 in antral follicles up-regulates the genes encoding hyaluronic acid synthase 2 (HAS2) and prostaglandin synthase 2 (PTFS2) in cumulus cells (Elvin et al., 1999; Pangas et al., 2004). Hyaluronic acid is a non-sulphylated glycosaminoglycan which remains associated with the cumulus cells expanding the spaces between the cells and embedding them in a mucineous matrix. Thus, the oocyte-derived growth factors,

GDF9 and BMP15 have been shown to have a major role in the cumulus expansion process. In summary, oocyte-secreted factors like GDF9 and BMP15 play essential roles in somatic cell development through most of folliculogenesis (You-Qiang et al., 2004; Hussein et al., 2005) (see Figure 6).

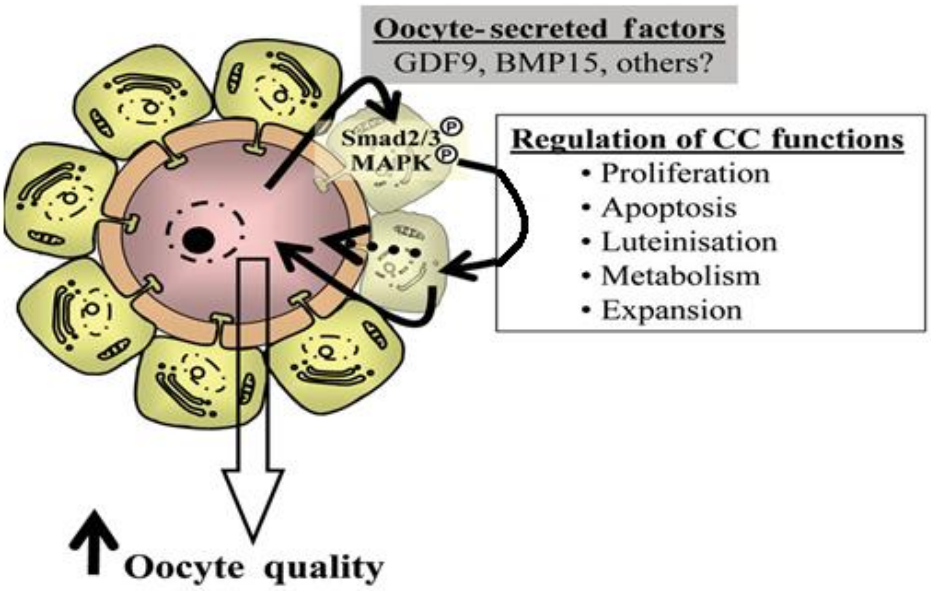

Figure 6: GDF9 and BMP 15 and ovarian development [Modified from Gilchrist et al., 2008 with permission]

1.6 Insulin-like growth factor-1 (IGF-1)

Insulin like growth factors are known to play an important role in the development of the rat ovary. There are two kinds of IGF proteins namely, type I and type II. IGF I was the predominant IGF found in the rat ovary. IGFII is produced by neonatal ovaries but the levels decline postnatally (Levy et al., 1992; Davoren et al., 1986; Adashi et al., 1989). IGFI binds to the extracellular domain of a membrane-bound receptor 1 (IGFRI) which forms a tetramer structure composed of two α subunits and two β subunits that are linked by interchain disulphide bonds (Ullrich et al., 1986). The α subunit forms the extracellular ligand-binding domain and the hydrophobic β subunit traverses the cell membrane. The IGFII ligand binds to a type II receptor, IGFRII. The IGFRII is a single chain glycoprotein

with around 90% of the protein in the extracellular region and a small cytoplasmic domain containing several phosphorylation sites (Morgan et al., 1987). Binding of IGFI ligand to its receptor activates the tyrosine kinase on β subunit through auto-phosphorylation. Downstream activation of the IGFI signal is caused by tyrosine kinase receptor activation whereas downstream activation of IGFII receptor activates the Gi2 proteins (Nishimoto et al., 1989). In situ hybridization studies revealed that IGF I mRNA was detected in the pre-antral and antral granulose cells and later it was also found in the cells of cumulus oophorus. IGF I mRNA was absent in atretic follicle and corpus luteum cells (Oliver et al., 1989). In the rat, an important action of IGFI is to enhance the action of FSH or LH on ovarian follicular development. IGFI knockout mice studies done by Jian et al., (1997) showed that IGFI and FSH receptors are co-expressed in the follicle. IGFI and FSH knockout studies have revealed that the IGFI creates a positive feedback loop to enhance FSH action and FSH enhances IGFI action through complementary regulation (see Figure 7).

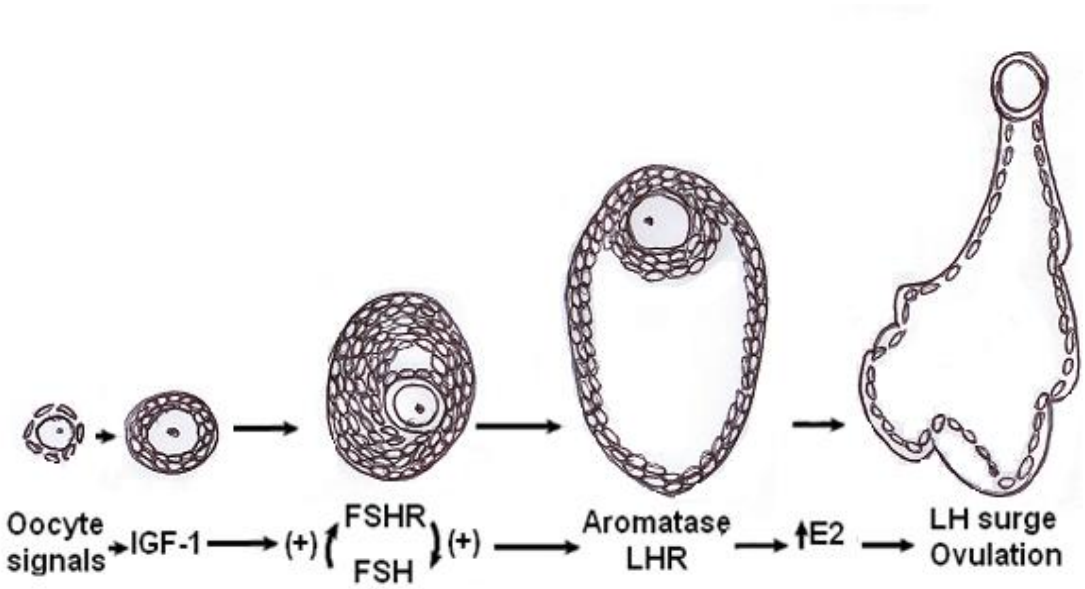

Figure 7: Role of IGF in folliculogenesis (modified from Jian et al., 1997)

IGFI enhances FSH-induced granulosa cell proliferation and differentiation in a pre-antral rat follicle (Adashi et al., 1990). In addition, IGFI enhances FSH-induced aromatase activity, LHR expression and inhibin secretion by cultured rat granulosa cells (Adashi et al., 1985a, b; Bicsak et al., 1986). IGFI also stimulates proteoglycan production in granulosa cells which may play a role in antrum formation (Adashi et al., 1986). In theca cells, IGFI enhances the expression of P450 site chain cleavage gene expression thereby enhancing the production of androgen which is then converted to oestrodiol by granulosa cells (Magoffin et al., 1990). In addition, IGFI directly augments LH-induced androgen production by theca cells (Cara and Rosenfield, 1988). In late follicular development, IGF increases FSH-stimulated production of progesterone and 20α hydroxy-pregn-4-en-3-one (Adashi et al., 1984, 1985). Morphological studies on preantral rat follicles cultured with IGFI resulted in an increase in the number of gap junctions between granulosa cells and between the oocytes and granulosa cells. Thus this result shows that IGF-1 assists in follicular development by enhancing the gap junctions within rat pre-antral follicles (Zhao et al., 2001).

1.7 Role of steroids oestradiol and testosterone in ovarian follicle

One of the major functions of the ovaries is to synthesise steroid hormones. The principal oestrogen is oestradiol-17β, a product of androgen aromatisation which occurs in a cooperative process between theca cells and granulosa cells known as “two cells two gonadotrophins theory”. In antral follicles, LH stimulates androgen production by the theca cells that transverse the basement membrane to the neighbouring granulosa cells. In granulosa cells, aromatase enzyme P450 converts androgen into oestradiol 17β under the influence of FSH (see Figure 8). The capacity of a follicle to make oestrogen is first apparent in the late preantral stage. Even though aromatase activity is present in small antral follicles, oestrogen production at this stage of development is limited by an inability to produce androgen substrate for aromatisation to oestrogen (Carson et al., 1981).

Preovulatory follicles have the highest amount of oestradiol 17β primarily due to the size of their granulosa cell population and their capacity for androgen aromatisation (Hillier 1981, McNatty, 1982). Androgen protein expression and mRNA are abundantly present in granulosa cells and theca cells of early antral follicles in rodent ovaries. Immunostaining result shows that AR staining was found to be more intense in rat granulosa cells and moderate in the theca cells (Hirai et al., 1994; Gill et al., 2004).

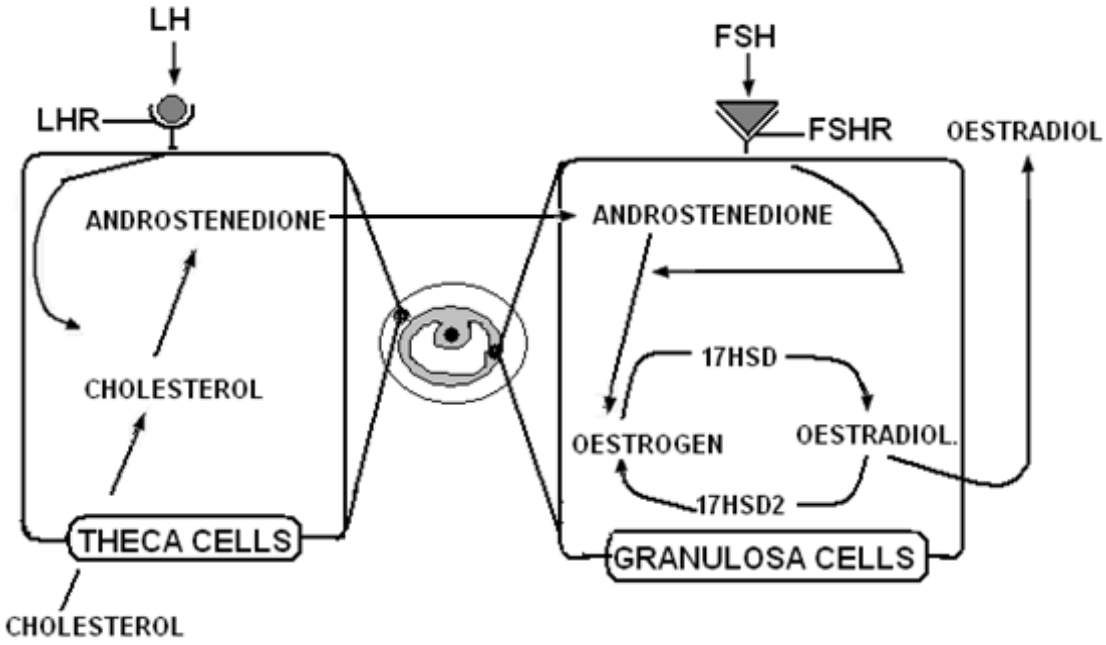

Figure 8: The two cell-two gonadotrophin theory

AR expression in the ovary declines as the follicle grows (Tetsuka and Hillier, 1996). In the late antral follicle, AR immunostaining decreases in the mural granulosa cells and strong positive AR staining is seen in the cumulus cells surrounding the oocyte (Szoltys and Slomczynska, 2000). This result suggests that the expression of AR was influenced by the oocyte secreted factors (Tetsuka and Hillier, 1996). Indirectly this shows that testosterone has a major role in regulating the early follicular development. Androgen acts via androgen receptors (AR) which are the members of the nuclear receptor superfamily, encoded on the X chromosomal gene (Lubahn at el., 1988). Downstream of

receptor activation, androgen acts via a sequence of processes that includes nuclear translocation, DNA-binding and complex formation with transcription factors to activate specific genes (Quigley et al., 1995). *In vitro* culture of mouse pre-antral follicles for four days in the presence of testosterone resulted in a significant increase in mouse follicle size. Further, the increase in granulosa cell proliferation was also observed within this follicle using bromodeoxyuredine dye as an indicator of cellular proliferation (Wang et al., 2001). Androgen also regulates the granulosa cell differentiation as indicated by an increase in progesterone and oestrogen synthesis (Hillier et al., 1981; Weil et al., 1999). Testosterone increases murine follicular responsiveness to FSH by increasing the level of cAMP (Wang et al., 2001). *In vitro* culture of a primate's antral follicles in the presence of testosterone leads to an increase in follicular IGF-I and IGF-IR receptor mRNA expression (Vendola et al., 1999a and b). This result suggests that testosterone plays an important role in follicular development directly and indirectly by enhancing production of other factors. Mouse pre-antral follicles cultured in the presence of an anti-androgen antibody and AR antagonist (bi-calutamide) failed to develop into pre-ovulatory follicles (Murray et al., 1998). In later stages of follicular development, testosterone completely reverses the IBMX inhibition of GVBD by elevating the intracellular cAMP. This result shows that testosterone stimulates *in vitro* oocyte maturation in later stages of follicular development (Gill, et al., 2004). As ovarian follicles grow towards ovulation and develop the capacity to synthesise oestrogen, the concentrations of this steroid increase both in peripheral plasma and also in follicular fluid (Hillier, 1981; McNatty, 1982). Thus, the follicles that go on to ovulate to release oocytes for fertilisation are those that secrete significant amounts of oestradiol.

Oestrogen acts on target cells via the specific receptors ERα and ERβ. These receptors act as nuclear transcription factors to activate the expression of specific genes (Jensen and DeSombre, 1973). The ovary contains both types of receptors. However, ERβ receptor is dominantly present in granulosa cells (Drummond and Findlay, 1999). Oestrogen and oestrogen receptor knock-out studies in mice have proven invaluable for

understanding the role of oestrogen in follicular development. Knocking out any one oestrogen receptor ERα (ERKO) or ERβ (BERKO) leads to infertility in mice. Mice lacking the ER α receptor are acyclic and infertile. Follicular development in these mice was arrested at the early antral stage (Krege et al., 1998). Therefore, these results show that the final stages of follicular growth are dependent upon the presence of the ERα receptor. ERβ receptor knockout mice (BERKO) show a partial loss of fertility due to more numbers of primordial follicles and fewer growing follicles with fewer oocytes being released at ovulation. Morphologically the ovaries are smaller in size (Emmen et al., 2005; Couse et al., 2005) and there is an increase in the expression of the androgen receptor during late antral follicle development that results in follicular atresia due to premature exhaustion of the follicle. Finally, ArKO mice (on an oestrogen free diet) an androgen deficient mouse which results in an absence of downstream oestrogen synthesis, has an increased number of morphologically immature secondary and antral follicles at six weeks of age. Collectively, these studies in mice show that oestrogen is important for the development of the normal follicle (Britt et al., 2000; Fisher et al., 1998). Isotype-selective oestrogen receptor studies also suggest that ERα inhibits ovulation and increases proliferation of granulosa cells while ERβ causes differentiation of the granulosa cells and an increase in the number of oocytes released during ovulation (Drummond and Findlay, 1999). Recent study done by Bley et al (1997), indicated that the FSH and oestrogen or androgen and FSH can stimulate granulosa cell proliferation *in vitro* and that this effect can be enhanced by insulin and IGF-1. Oestrogen causes an induction of FSH, LH and prolactin receptors and also influences post receptor mechanisms. Oestrogen stimulates the production of LH and FSH ligands and also causes cAMP accumulation and increases the cAMP binding sites on granulosa cells (Richards and Rolfes, 1980). However, it is not fully understood how these factors affect oocyte-cumulus cell function. Steroids like oestrogen and progesterone are thought to play an important role in regulation of the Cx43 gap junction protein. It is thought that by treating immature rats with oestrogen leads to an increase in

ovarian Cx43 mRNA expression and protein synthesis (Wiesen and Midgley, 1994; Granot et al., 2002). Yet no studies have been done in ovaries which show the pathway through which oestrogen increases the Cx43 gene expression. Oestrogen increases Cx43 expression by activating the Cfos and Cjun proteins (AP-1 complex) in myometrium cell line. In contrast, an increase in progesterone during the preovulatory surge reduces the expression of the Cx43 gap junction protein by decreasing the expression of Cfos and Cjun transcription factors (Wiesen and Midgley, 1994; Granot et al., 2002).

1.8 Cycle AMP, milrinone and forskolin

Secondary messenger cyclic adenosine monophosphate (cAMP) plays an important role in regulation of the follicular phase development and lutenization in the mammalian ovary. Differential regulation of the cAMP in the surrounding somatic cells and oocyte play an important role in oocyte maturation. Accumulation of cAMP is regulated by two major enzymes; membrane bound adenylyl cyclase (AC) and phosphodiesterase (PDE) that catalyse the synthesis and degradation of cAMP respectively. Adenyl cyclase is responsible for catalysing the formation of cAMP from ATP. The phosphodiesterase (PDE) enzyme hydrolyses the bond between the phosphate and the 3-hydroxyl group of the ribose sugar in cAMP. PDE enzyme is encoded by 21 different genes which are organised into 11 different families depending on the biochemical and pharmacological properties, like substrate affinity and sensitivity to specific inhibitors (Beavo, 1995; Soderling and Beavo, 2000; Michaeli et al., 1993). There are 4 types of PDE genes present in the rat mouse and human ovary. In the mammalian ovary, PDE 3 gene is expressed in the oocyte and PDE 4 is expressed in the granulosa cells (Wang et al., 2007; Park et al., 2003). The presence of different mRNA variants shows that more than one protein is derived from each gene. Different PDEs control the cAMP levels in the granulosa cells and in the oocyte which regulates the oocyte meiotic maturation (Conti et al., 1995; Monaco et al., 1994). Secondary messenger cAMP has different effects on the cellular events like metabolism,

growth stimulation, meiotic arrest, cell differentiation and proliferation. Furthermore, it mediates the action on different genes by acting through a cAMP response element. cAMP is generated locally within the oocyte or is transferred from the surrounding cumulus cells via gap junctions (Dekel and Beers 1978; Dekel et al., 1981; Lindner et al., 1974). cAMP acts by activating the cellular type I and II PKA pathway thereby regulating the oocyte meiotic maturation (Gelerstein et al., 1988). The enzyme adenyl cyclase is composed of three main proteins: hormone receptor, guanine nucleotide regulatory protein and catalytic protein (Ross and Gilman, 1980). Downs et al., (1985), reported that using a site selective cAMP analogue, activation of type I PKA led to the inhibition of meiosis. On the other hand, activation of type II PKA resulted in the cumulus cell expansion and germinal vesicle breakdown (GVBD). Different agents are responsible for activating the adenyl cyclase enzyme which acts on the whole cell. For example, hormones like gonadotrophins act via receptor to activate adenyl cyclase. Forskolin (cAMP stimulator) acts by activating the catalytic subunit (Seamon et al., 1981; Seamon and Daly, 1981a). Milrinone inhibits the PDE 3 enzyme in the oocyte which inhibits the meiosis. Rolipram inhibits the PDE4 enzyme in the granulosa cells by activation of type II PKA which results in either inhibition or activation of meiosis depending on the oocyte cAMP levels (Tsafriri et al., 1996).

1.9 Gap junctions in the rat ovary

Gap junctions play a pivotal role in the female reproductive tract. Direct cell to cell communication between the oocyte and surrounding follicular cells play an important role in oocyte maturation by transferring small molecules like cAMP that maintain the oocyte in the diactyalate satge of meosis (Sela-Abramovich et al., 2006; Dekel and Beers, 1978; Dekel et al 1981; Lindner et al 1974) and corpus luteum formation (Reynolds and Redmer 1999). The mammalian oocyte utilises the glucose as an energy substrate but it is deficient in carrying out glycolysis, thus cumulus cells surrounding the oocyte provides the

oocyte with nutrients like amino acid, metabolites, and glucose by transferring them through the gap junctions that connects the two cell-types (Sugiura et al., 2005). As mentioned earlier, gap junctions begin to form before antrum formation in the antral follicle, connecting the granulosa cells (Anderson, 1971; Merk et al., 1972).

Gap junctions are cell-to-cell communication channels formed by the docking of two half channels known as connexons around an aqueous pore (Kidder and Mhawi, 2002). Connexons are integral membrane proteins that are formed by combining six different connexin proteins (Figure 9). Various different connexins are present in different organisms and within different tissues in the same organism (Bruzzone et al., 1996 a, b). These channels formed by different connexins play a distinctive role in transporting specific molecules across the cells. Gap junctions are known to be present in developing follicles. Expression of gap junction mRNA and proteins increase as the follicle develops.

Ovarian gap junctions have an ability to form homotypic (formed by identical connexons) and heterotypic (formed by two different connexons) channels. These heterotypic and homotypic channels lead to an increase in variation of gap junction functions. However, different gap junction channels have similar overall structures consisting of four membrane spanning domains, two extracellular loops, a cytoplasmic loop and cytoplasmic C and N termini (Gershon et al., 2008; Bruzzone et al., 1996 a, b) (see Figure 9). Sequence similarity among the family members is concentrated in the trans-membrane domain and extracellular loops. Extracellular loops form the contact domain that allows docking of connexons from adjacent cells. Most of the variation is seen in the cytoplasmic loops and this accounts for the distinctive permeability of specific molecules through gap junctions (Bruzzone et al., 1996 a, b). Docking of homotypic or heterotypic connexons from adjacent cells is facilitated by the extracellular loop.

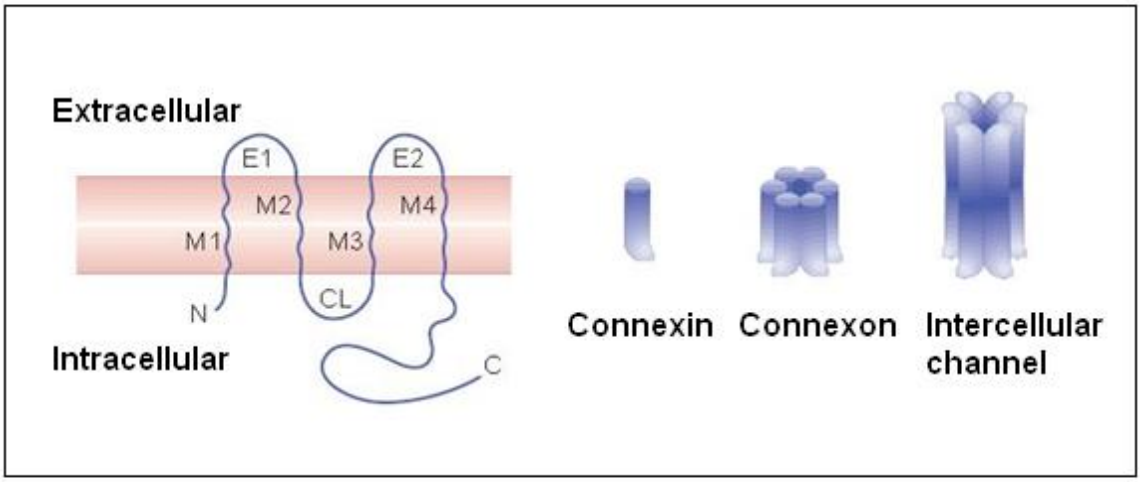

Figure 9: The structure of gap junctions (modified from Mhawi and Kidder, 2002)

There are 21 different connexins sequences found and more are being characterised. For example, a Cx43 connexin: where 43 refer to the molecular weight of the protein. Different connexins such as connexin 43 (Cx43), (Cx32), (Cx57), (Cx45) and (Cx37) are expressed in the rat ovary (Valdimarsson et al., 1993). The presence of Cx43 mRNA and protein product was detected from the primary stages of growth to antral follicles (Juneja et al., 1999). Gap junctions formed by Cx45 were observed in granulosa cells of an adult rat ovary, co-localised with gap junctions formed by Cx43 proteins (Kidder and Mhawi, 2002; Okuma et al., 1996; Alcolea et al., 1999; Wright et al., 2001). Further, the presence of Cx 30.3 was observed in the theca cells and Cx57 was observed in granulosa cells of the adult rat ovary (Manthey et al., 1999; Itahana et al., 1996, 1998). Therefore, in the rat ovary, gap junctions formed by connexin Cx32, 37, 43, 45, 57 are involved in some way with folliculogenesis (see Table 1).

Presence of Connexins in rat ovary	
Cx 32	**Granulosa cells**
Cx 43	**Granulosa cells**
Cx 45	**Granulosa cells**
Cx 37	**Oocyte-Cumulus cells**
Cx 30	**Theca cells**

Table 1: Presence of connexin gap junction proteins in the rat ovary

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Of these connexins, Cx37 is expressed in oocytes from the primary stage of follicular development (Simon et al., 1997). In the cumulus oocyte complex, cumulus cell derived Cx43 forms a homotypic gap junction with Cx43 connexon on the oocyte surface and it also forms a heterotypic gap junction with oocyte-derived Cx37 connexons (Simon et al., 1997) (see Figure 10). Connexin knockout studies in mice have provided the evidence for the essential role of connexin Cx37 and Cx43 in ovarian folliculogenesis. Rats lacking the gene Gja4 that accounts for Cx37 protein product shows that ovarian folliculogenesis is normal until the preantral stage (Simon et al., 1997, Carabatsos et al., 2000).

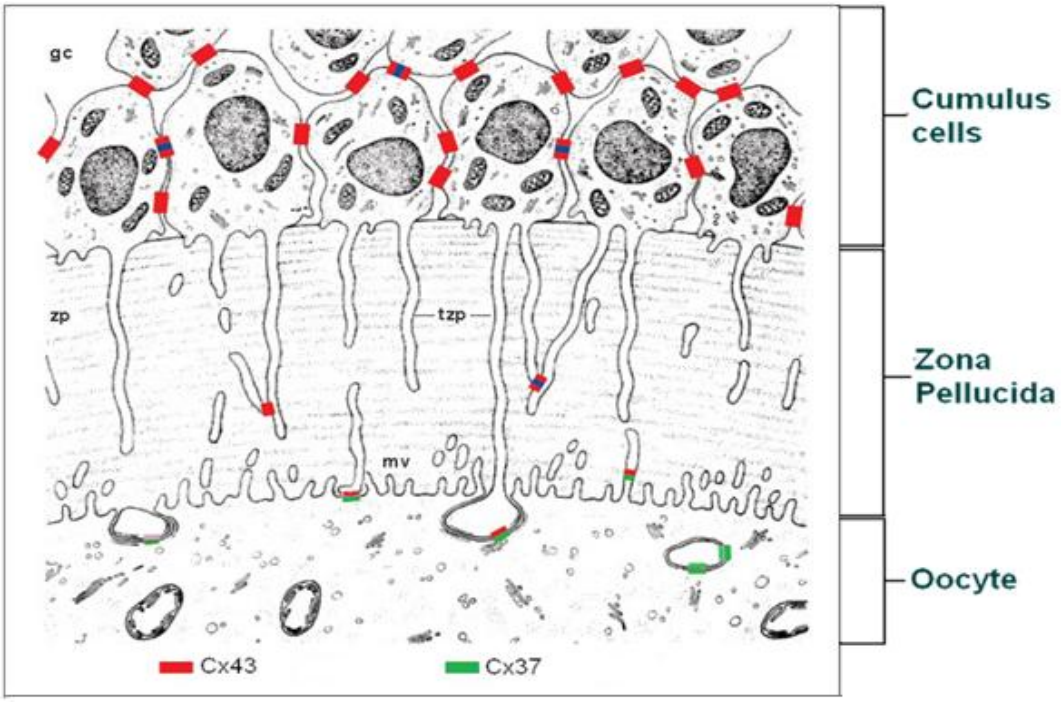


Figure 10: Distribution of gap junction protein connexin in COC (Modified from Mhawi and Kidder, 2002)

Oocytes in these follicles fail to achieve meiotic maturation and ovulation could not be induced by gonadotrophin stimulation (Carabatsos et al., 2000). In $Gja^{-/-}$ mice, the gap junctions between the cumulus cells and oocyte were lost impairing the transfer of nutrients from cumulus cells into the oocyte (Carabatsos et al., 2000). According to Juneja et al., (1999) deletion of the gene encoding Cx43 connexin (Gja1) resulted in the arrest of rat pre-antral follicular development due to failure of proliferation and differentiation of granulosa cells. Thus, these follicles are developmentally abnormal with meiotically incompetent oocytes that fail to be fertilised by the sperm (Kidder and Mhawi, 2002). According to Granot, (2002), Cx43 mRNA and protein in a rat oocyte is localised at the inner side of the zona pellucida. Thus this result shows that both Cx37, Cx43 mRNA and protein product are present in the oocyte.

1.10 Meiosis

Meiosis is the process by which oogonia enters during a final round of mitosis to produce primary oocytes. The oogonium is a mitotically dividing cell synthesising DNA during the S (synthesis) phase of interphase of the cell cycle. The dividing oogonium then proceeds through a short prophase and metaphase to the mitotic division. During this process, germ cells increase in number exponentially until their transformation into oocytes. During this transformation, the oogonia enter meiosis but the cell cycle is altered whereby the prophase is arrested and becomes markedly prolonged. Thereafter, this arrested phase overcome just before ovulation respectively by two subsequent nuclear divisions. The time taken for the transformation of the first and last oogonia into oocytes is known as oogenesis. The duration of oogenesis varies in different species. For example, it takes two days in the rat. The diploid nucleus in the primary oocyte contains two sets of chromosomes from each parent. Primary oocytes enter the meiotic process at different times. Some germ cells have already entered meiosis while others continue to divide mitotically. During meiosis, the primary oocyte undergoes two divisional processes to form a mature oocyte called meiosis I and meiosis II. Each consists of a series of stages. Meiosis I results in the reduction of the number of chromosomes from diploid to haploid and meiosis II results in the separation of sister chromatids.

Different stages of meiosis are recognised by the arrangement of the chromosomes. Meiosis I consist of four stages, prophase I, metaphase I, anaphase I and telophase I. Prophase is further divided into four stages leptotene, zygotene, pachytene (transient stages) and diplotene (stationary stage). In mammals, when an oocyte enters the diplotene stage it remains in this stage for weeks, months or years before ovulation takes place. The nuclear envelope of the oocyte is still intact during the diplotene stage. In vivo, oocytes arrested in the diplotene stage do not undergo GVB (germinal vesicle breakdown) until they are stimulated by the external hormonal signals (Baker, 1982).

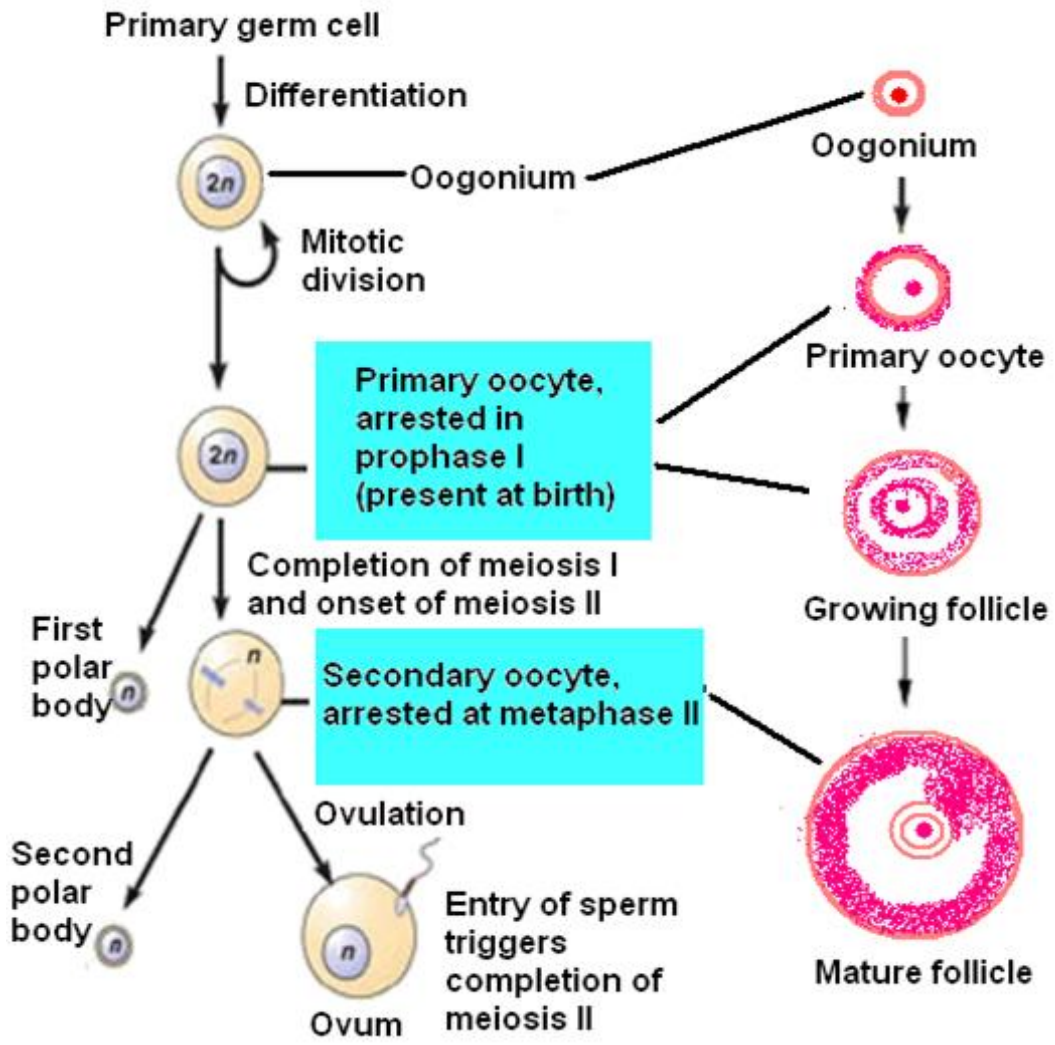


Figure 11: Different stages of meiosis

Thus, certain factors present in the follicle maintain the oocytes in their meiotic arrested stage. This effect is mediated by transferring the meiotic arresting factors from surrounding somatic (cumulus) cells into the oocyte via gap junctions (Dekel 1988; Leibfried and First 1980; Tsafriri and Channing 1975). In vivo factors such as cyclic adenosine monophosphate (cAMP) (Bornslaeger et al., 1986; Cho et al., 1974; Dekel and Beers et al., 1978; Magnusson and Hillensjo et al., 1977), purines such as hypoxanthine, guanosine, and adenosine (Downs, 1985; Eppig et al., 1985; Salustri et al., 1988) (only in rodents) have all been implicated in the maintenance of meiotic arrest. This was supported by *in vitro* studies showing that an oocyte removed from an antral follicle will resume

meiosis spontaneously in the absence of any hormonal signals (Pincus and Enzmann, 1935). Further in the diakinesis (late prophase) stage, the nuclear envelope starts to breakdown and spindle fibres start assembling. The end result of meiosis part I produces two cells with a haploid number of chromosomes. One cell is small which is called polar body and the other cell is large which is called secondary oocyte. Meiosis part II is similar to the mitotic division of the somatic cell which results in the formation of two cells, one small cell known as polar body and one large cell known as the ovum (oocyte) (see Figure 11). In our study, we have looked at the germinal vesicle stage oocyte during which the oocyte was arrested in the prophase I stage and the gap junctions were still intact in the cumulus-oocyte complexes (COC).

1.11 General Introduction

Different methods have been used to measure the gap junction activity, for example radioactive nucleotide transfer and non radioactive methods like metabolic co-operation, fluorescence dye microinjection, scrape loading, dual whole cell patch clump electrophysiology, FRAP (fluorescence recovery after photo bleaching) and flow cytometry. Flow cytometry is a method for characterising and analysing particles (usually cells) when they pass through the laser beam on a single cell suspension. Flow cytometry technique allows analysis of a large number of cells with high sensitivity that allows the detection of minor differences in dye coupling. However, the main disadvantage of this assay is that only cell suspension solutions are suitable for flow cytometry. Different cell sizes, difference in the number of functional gap junctions and difference in connexin expression may interfere with efficient dye transfer (Paula, 2006). In comparison the dye transfer gap junction method we have developed using Calcein-AM dye has allowed us to measure gap junction activity in cell culture in any cultured media. Another method used to measure the gap junction activity is FRAP (Fluorescence recovery after photo-bleaching). The main disadvantage of using FRAP methods is the free radical disturbance

of the cells caused by the photo bleaching. This technique is also inconsistant as it cannot measure concentration, diffusion, or co-localisation and requires a higher concentration of fluorophores. Further radio active methods like radio active nucleotide transfer assay to measure the gap junction activity is hazardous to health.

Furthermore, the calcein dye we have used in this assay has a good retention in target cells and low pH sensitivity. There is no stain transfer among the cells. Calcein produces an intense green signal which is easily detected by CRAIC technology. It is non toxic to live cells and cells could be incubated in the presence of calcein for longer periods. In comparison to that, Lucifer yellow dye used to measure gap junction activity becomes incorporated into cellular organelles with time, making fluorescence particulate. It is only available for the transfer to the adjacent cells for short periods of time.

In this study, we have observed the gap junction activity within COC obtained from sexually immature 21-25 day-old Sprague-Dawley rats without any hormonal treatments because of the easy availability of the rat tissue from the Psychology Department of Victoria University of Wellington. Sexually immature rats were used in this study as they are not yet sensitised to the gonadotrophins hormones FSH and LH. Gonadotrophin hormone FSH elevates the gap junction protein in the antral follicle in sexually mature rats and LH surge reduces the gap junction protein level in the pre-ovulatory follicle. Studies done by Merk et al., (1972) reported that exogenous administration of pregnant mare's serum gonadotrophin (PMSG) led to an increase in the number of gap junctions in association with follicular development in the rat. Thus in this study, rats were not pre-treated with (PMSG) hormone.

In this study, gap junction activity was observed in rat antral follicles because in early antral follicles no gap junctions were present (Albertini and Anderson, 1974). In preovulatory follicles, LH surge leads to down regulation of the ovarian Cx43 gap junction protein (Wiesen and Midgley, 1993). Thus it was necessary to observe the effect of gap junction before the pre-ovulatory gonadotrophin surge.

CHAPTER 2 MATERIALS AND METHODOLOGY

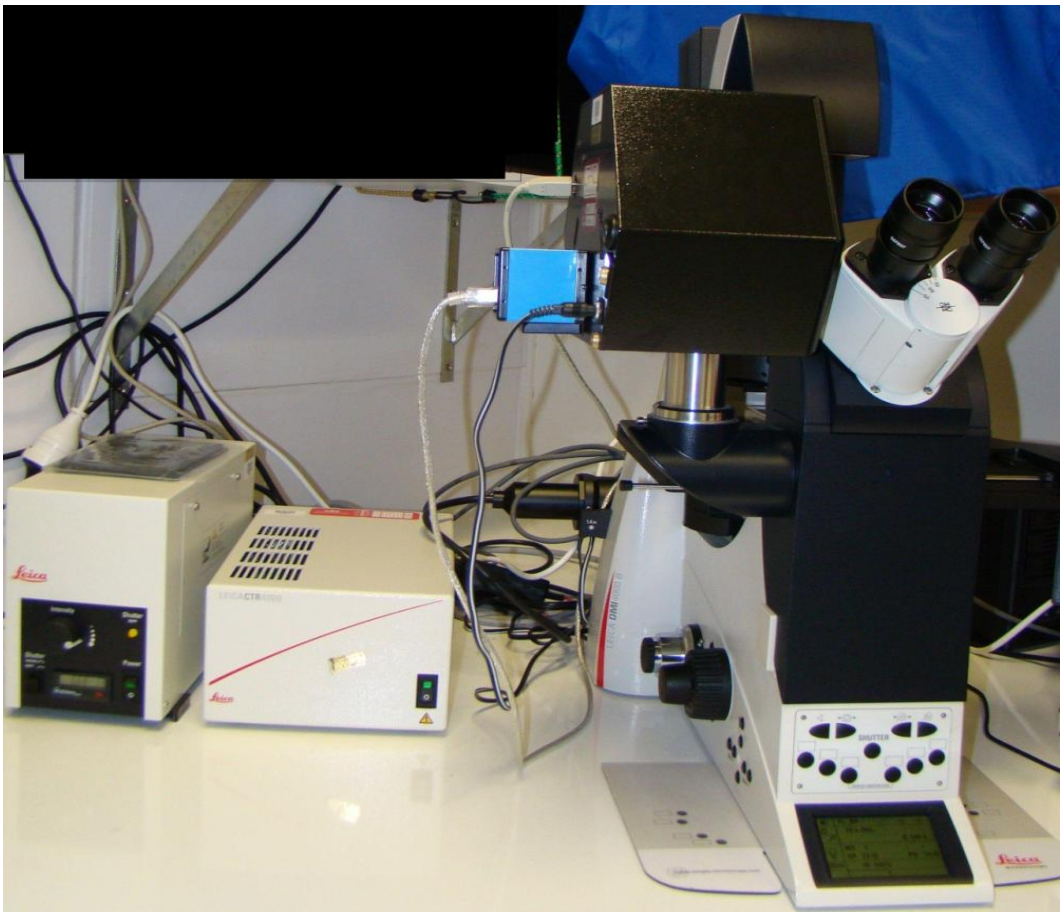


Figure 12 The inverted microscope and the microscope spectrophotometer (MSP) (CRAIC fluorescence system (black box on the side of microscope by the eye-pieces)

2.1 Animals and recovery of ovaries

The animals used in these studies were 21-25 day-old sexually immature Sprague-Dawley rats provided by the Psychology Department of the Victoria University of Wellington. Ms Norma Hudson recovered the ovaries on my behalf (School of Biological Sciences) from animals were culled from the breeding colony. The animals were sacrificed using CO_2. The ovaries, were dissected and collected into 1ml L-15 medium supplemented with 100µM milrinone and kept at 37ºC in a water-bath until further dissection.

2.2 Equipment

Zeiss stereo-zoom dissection microscope (47 52 65 model, West Germany) was used for the ovarian dissections and to recover COC. Oocytes denuded from their cumulus cells were observed using a Leica inverted microscope DMI4000 at 100x magnification (see Figure 12). The fluorescence intensity of the denuded oocytes was measured using a QDI 302 Microscope Spectrophotometer (MSP) software (San Dimas California CRAIC technology), filter I3 and stored on a hard drive (see Figure 12). The settings for these measurements were, sampling time 50 milliseconds, scans to average (NS) 40, left limit 400, right limit 700 and 10x objective.

2.3 Stock solutions

A 1mM phosphodiesterase (PDE) inhibitor milrinone (Sigma-Aldrich) stock solution was stored in M199 media at -20ºC. This was used routinely in the gap-junction assay at a final concentration of 100µM. The 10mM dbcAMP stimulator, 10mM forskolin (Sigma-Aldrich) was prepared as a stock solution in M199 and stored at -20ºC. A control medium containing the identical concentration of solvent was also stored in the same manner. The 10mM fluorescent dye calcein–acetoxymethyl (AM) (Invitrogen) used to measure the gap-junction activity was stored in DMSO at <20ºC and used routinely at a final concentration of 1µM. Stock solution of 10,000IU Penstrep (Invitrogen) was stored at -20ºC. 1000mM Hepes buffer stock solution (Sigma-Aldrich) was stored at 4ºC. 0.23mM Sodium Pyruvate (Sigma-Aldrich) stock was stored at room temperature. Stock solution of 200mM Glutamax (Invitrogen) was stored at 4ºC. 0.3mg/ml Polyvinyl alcohol (Sigma-Aldrich) stock was stored at room temperature. Steroids like 3.65mM 17βoestradiol and 3.47mM testosterone (Sigma-Aldrich) stock solutions were prepared in M199 and were stored at 4ºC. 0.131mM Insulin-like growth factor 1 (IGF-1) (Novazymes) stock solution was made in M199 media and stored at 4ºC. Recombinant ovine BMP15 (Batch NZ 321) and GDF9 (Batch NZ 317) were kindly supplied by Prof Ken McNatty. Each of these

growth factors were present in spent media from transfected 293H cells. Protein was present in serum-free, dimethyl minimum essential medium/Hams F10 (1/1, v/v) containing L-glutamine, and penicillin (100U/ml) and streptomycin (100µg/ml). The concentration of BMP15 in the spent media was 0.12µg/ml and the concentration for GDF9 was 1.1µg/ml. The control medium used for the studies with BMP15 + GDF9 was spent media from untransfected 293H cells (Batch NZ307). The gap junction blockers 2000mM 1-octanol (Sigma-Aldrich) and 0.3mM carbenoxolone (Invitrogen) stock solution were also prepared in M199 and stored at -20ºC..

2.4 Media compositions

2.4.1 L-15 Lebowitz media with phenol red (Invitrogen) was used as the dissection medium

The L-15 medium (Invitrogen) was supplemented with 0.1% BSA (v/v: ICP Bio Ltd., Auckland, NZ), 100IU/ml penstrep (Invitrogen) and 15mM hepes buffer (Sigma-Aldrich) and milrinone (Sigma-Aldrich) was added to the final concentration of 100µM.

2.4.2 Incubation medium A

This consisted of a phenol red-free M199, a bicarbonate buffered medium supplemented with 100IU/ml penstrep (Invitrogen), 0.4% BSA (v/v: ICP Bio Ltd., Auckland, NZ), 0.23mM sodium pyruvate (Sigma-Aldrich), 2mM glutamax (Invitrogen) and milrinone (Sigma-Aldrich) was added to the final concentration of 100µM.

2.4.3 Incubation medium B

This consisted of a phenol red-free M199 medium (Invitrogen) supplemented with 0.3mg/ml polyvinyl alcohol (PVA) (Sigma-Aldrich). Milrinone (Sigma-Aldrich) was added to the final concentration of 100µM.

2.4.4 Incubation medium C

This consist of a phenol red free M199 medium (Invitrogen) supplemented with 0.3mg/ml polyvinyl alcohol (PVA) (Sigma-Aldrich) plus phosphate buffer saline (PBS) (Sigma-Aldrich) and milrinone (Sigma-Aldrich) was added to the final concentration of 100µM.

2.4.5 Wash medium

The wash solution consisted of a phenol red-free M199, a bicarbonate buffered medium (Invitrogen) identical in composition to incubation medium A and contained 0.4% BSA (v/v: ICP Bio Ltd., Auckland, NZ).

2.5 Cumulus-oocyte complexes (COC) collection

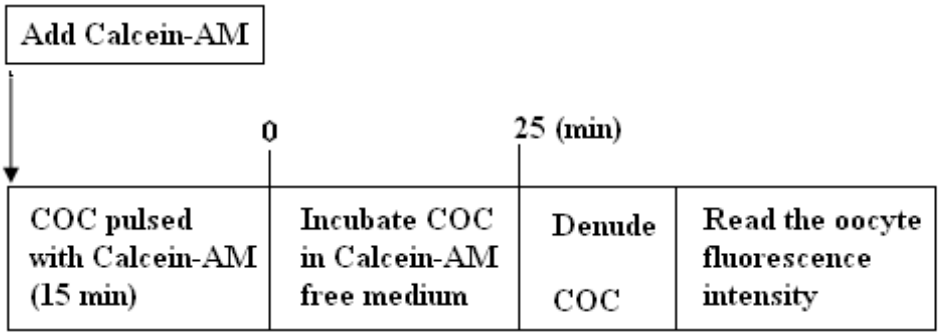
An effort was made to recover the COC within 2 hours of the recovery of the ovaries. To collect COC, the ovaries were cleaned of their surrounding fat layer and were kept in petri dish containing 3ml L-15 dissection medium. The surface visible ovarian follicles were punctured using a 1ml syringe fitted with a 20G needle. Intact COC with two or more layers of cumulus cells surrounding the oocyte were selected. On average about 60 COC were recovered from a pair of ovaries. The isolated COC were transferred into a new petri dish containing 3 ml fresh L15 dissection medium and kept at 37^0C temperature at all times.

2.6 The gap junction assay

The gap junction activity between an oocyte (egg) and the surrounding somatic cumulus cells was measured during an *in vitro* culture of the rat COC in the presence or absence of different treatments. The gap junction activity was measured using the acetoxymethyl (AM) ester derivative of the fluorescent indicator calcein (Calcein-AM, 3-6 Di amino methyl) fluorescein, tetraacetoxy methyl ester, C-3100; Molecular Probes and Eugene or Calcein-AM (MW: 994.87). Calcein-AM is non-fluorescent, electrically neutral and a highly lipophilic agent. The lipophilic characteristic of Calcein-AM is due to its lipophilic acetoxymethyl group (AM) which allows Calcein-AM to easily permeate the plasma membrane (Wang et al., 1993; Lichtenfels et al., 1994; Papadopoulos et al., 1994; Thomas et al., 2004) Once the dye enters the cumulus cells, non-specific endogenous esterase cleaves the lipophilic acetoxymethyl groups producing the lower molecular weight calcein dye (MW: 622.54). The cleaved calcein is a fluorescent, negatively charged molecule unable to pass through the cell membrane except via gap junctions. Therefore, the gap junctions in the cumulus-oocyte complex were measured using the spectrophotometer (CRAIC fluorescence technology system) (see Figure 12) assessed by the amount of calcein dye transferred to the rat oocyte from the surrounding cumulus cells through the gap junctions (Thomas et al., 2004). In each method prior to the recording of the fluorescent intensity of the denuded oocyte, a dark scan was taken as a zero limit reference. Another reference factor is not required for this procedure according to the manufacturer's guideline.

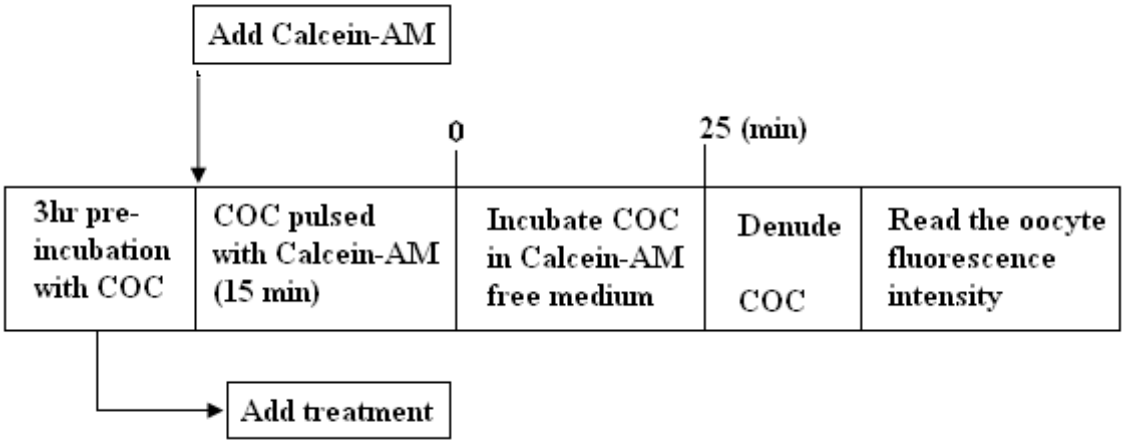
A number of different strategies were employed to evaluate the calcein dye transfer and treatment effects. The methods evaluated are outlined below:

2.6.1 Method A (Figure 13)



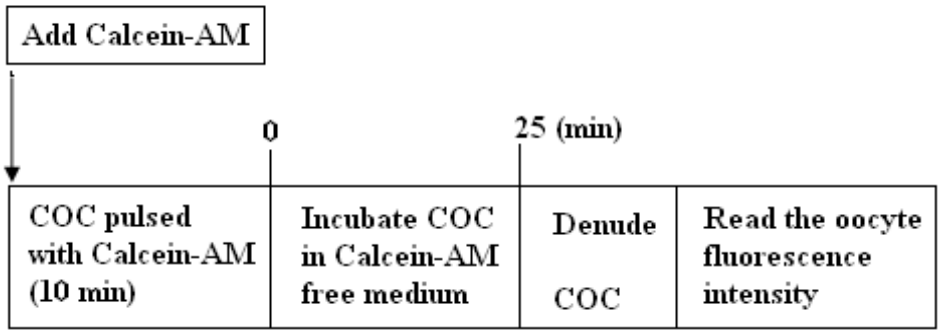
In method A (Figure 13), the COC were transferred into wells (10 COC/well) labelled T0 and T25 containing 400µl of L-15 dissection medium. The complexes were then transferred twice into the new wells each with 400µl phenol red-free M199 medium to remove any excess phenol red medium. The washing was performed because the phenol red medium was thought to interfere with the fluorescence readings for calcein. The COC were transferred into a new well containing 400µl of incubation medium B and pulsed with 1µM Calcein-AM dye, then incubated for 15 minute at 37^{o}C gassed with 5% CO_2 in air 96% humidity. Thereafter, the COC in the T0 labelled wells were washed three times in 200µl incubation medium B. The COC were denuded of their surrounding cumulus cells by vigorous vortexing for 5 minute. The fluorescence intensity in denuded oocytes of this treatment was assessed using the microscope spectrophotometer (CRAIC technology fluorescence system) to provide a zero time reference point. The COC in the T25 labelled wells were incubated in 200µl of the Calcein-AM free incubation medium B for 25 minute at 37^{0}C to allow transfer of calcein dye from cumulus cells into the oocyte. Thereafter, COC was washed twice in 200µl of incubation medium B and denuded by vigorous vortexing for 5 minute. The fluorescence intensity of denuded oocytes was measured. Thus, an increase in fluorescence intensity at T25 relative to T0 is a measure of the level of dye transferred from the cumulus cells to the oocyte over a defined period of time.

2.6.2 Method B (Figure 14)



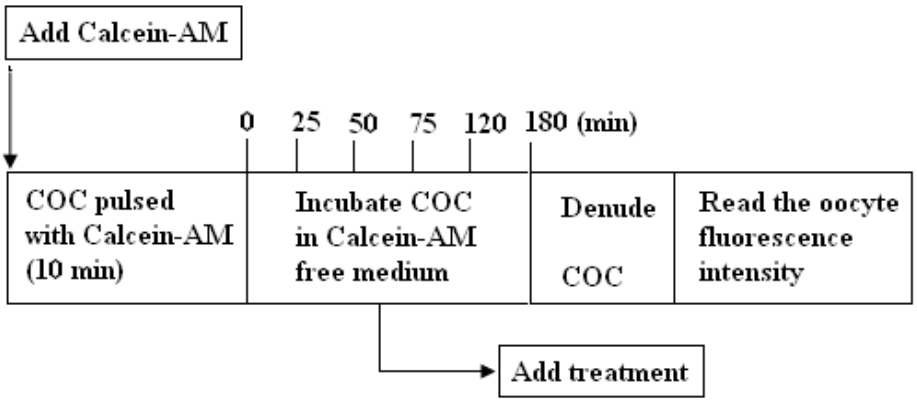
Method B, (Figure 14) is the same as method A except that the COC were pre-incubated for 3 hours in 400µl of incubation medium A with or without different treatments.

2.6.3 Method C (Figure 15)



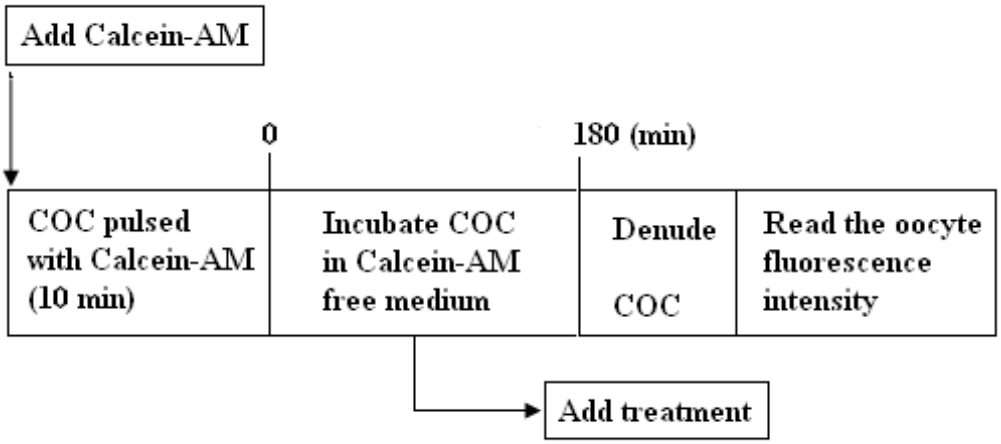
Method C (Figure 15) is similar to method A except that the COC were pulsed with Calcein-AM dye for 10 minute instead of 15 minute.

2.6.4 Method D (Figure 16)



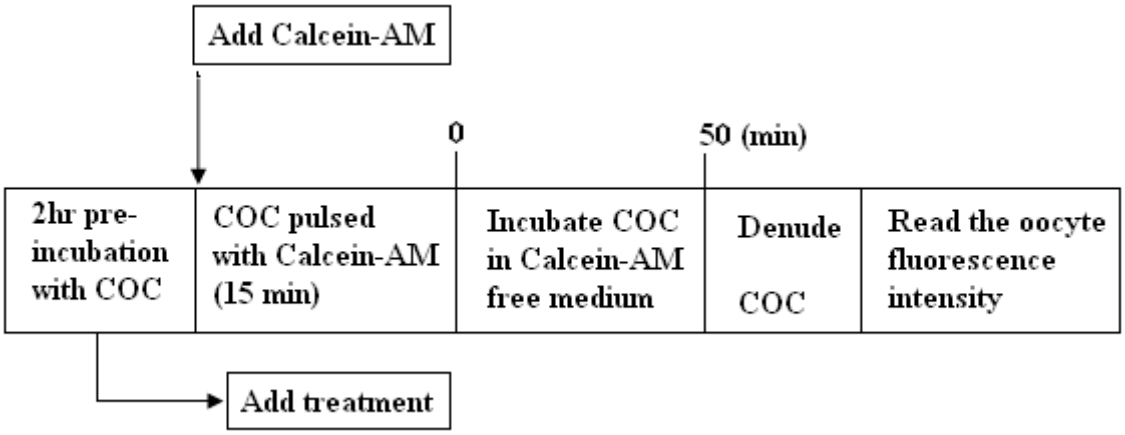
For method D, (Figure 16), the COC were added to the wells (10 COC/well) labelled T0, T25, T50, T75, T120 and T180 with each well containing 400µl of L-15 dissection medium. The COC from each well were washed twice to remove any excess phenol red medium carried from a previous well by transferring into successive new wells with 400µl phenol red-free M199 wash medium. After washing, the COC were transferred into new wells containing 200µl of incubation medium B and 1µM Calcein-AM. The complexes were pulsed with Calcein-AM for 10 minute at 37^{o}C in an incubator with 5% CO_2 in air and 96% humidity. After a 10 minute calcein pulse, the COC were transferred into new wells containing 200µl of incubation medium B with and without different treatments. At the appropriate times the COC from T0, T25, T50, T75, T120 and T180 wells were washed twice in 200µl incubation medium B to remove any excess un-incorporated calcein dye. Following the denuding, the isolated oocytes were identified under the inverted microscope at 100x magnification and the fluorescent intensity was measured. This method enabled us to assess gap junction activity over a three hour time period in the presence and absence of different treatments.

2.6.5 Method E (Figure 17)



As previously outlined for the other methods, in method E 10 COC were added to each well labelled T0 and T180 containing 400µl of L-15 phenol red dissection medium. The COC were washed twice in 400µl of non-phenol red M199 wash medium. Then the COC were washed twice in wash medium and transferred into the next well containing 200µl of incubation medium B and pulsed with 1µM Calcein-AM for 10 minute. After the 10 minute calcein pulse, the COC were transferred into another well containing 200µl of incubation medium B with and without different treatments. The COC from the T0 wells were washed twice in 200µl incubation medium B. After washing, the COC were denuded and the fluorescence intensity of denuded oocytes was read using the microscope spectrophotometer (CRAIC fluorescence technology system). The COC from T180 labelled well were incubated for a further 180 minute in 200µl incubation medium B with and without different treatments. Incubating the complexes for 180 minute allowed the transfer of calcein dye from cumulus cells into the oocyte. The complexes were denuded off their surrounding cumulus cells by vortexing for 5 minute and the fluorescent intensity of the denuded oocyte was read using the microscope spectrophotometer (CRAIC technology) (see Figure 17).

2.6.6 Method F (Figure 18)



In method F 10, COC were added to the wells labelled T0 and T50 each containing 400µl of L-15 dissection medium. The COC were then washed twice by transferring to new wells containing 400µl phenol red-free M199 wash medium. The COC were then incubated for 2 hour in the next well containing 200µl of incubation medium B with and without different treatment. After 2 hour incubation was completed, the COC from each well were transferred into the corresponding well containing 200µl of incubation medium B and 1µM Calcein-AM. The complexes were pulsed with the Calcein-AM for 10 minute at 37ºC temperature, 5% CO^2 and 96% humidity. After a 10 minute calcein pulse, the COC were transferred into new wells containing 200µl of incubation medium B with and without different treatments. The COC from the T0 and T50 labelled well were denuded straight after calcein exposure and 50 minute after calcein exposure respectively. The fluorescence intensity of denuded oocytes was read using microscope spectrophotometer (CRAIC technology) (see Figure 18).

2.7 Staining Protocol

Attempts were made to stain the germinal vesicle of the denuded oocyte. The staining protocol is presented in Appendix A.

2.8 Statistical analysis

Within each experiment, 6-10 COC were treated with different treatments in different ways and the fluorescence intensity within each oocyte was measured using a spectrophotometer. Large variances were seen between the fluorescence readings of individual oocytes in each experiment. Therefore, the fluorescent readings of an individual sample (oocyte) in each experiment were log transformed and the geometric means were calculated. To obtain the geometric mean, the antilog of the mean of the log value was calculated. Thereafter the mean for all the replicate experiments were averaged. In most instances the non-parametric Mann-Whitney test for 2 groups was performed using the statistics program SPSS 17.0 to compare the control versus treatment groups at times 0 and 180 minute. The effect of gap junction activity was measured at various time points 0, 25, 50, 75, 120, 180 minute in the presence of incubation medium A, medium B, cAMP and forskolin. Linear regressions were performed for all the replicate experiments to obtain the slopes of the fitted lines and Mann-Whitney tests were used to compare the slopes of control versus treatment groups. In this thesis, the data are mostly represented in a table format or Box and Whisker plots that shows comparison between the control and treatment groups at only 0 and 180 minute and line graphs were also plotted which shows, comparison of mean fluorescence intensity of control and treatment groups over various time points 0, 25, 50, 75, 120, 180 minute.

Data obtained from the testosterone treatment alone and the combined treatments of testosterone + forskolin and testosterone + forskolin + IGF-1 were log transformed. Levene's test for homogeneity of variances revealed that the results for the above treatment groups were normally distributed. Therefore mean results of control versus treatment groups were compared using Analysis of variance (ANOVA) and the Post hoc Tukey test for comparisons between treatments. The effects of testosterone and the combined effect of testosterone + forskolin and testosterone + forskolin + IGF-1 are represented as a bar graph in this thesis.

CHAPTER 3 RESULTS

3.1 The fluorescent technique using Calcein-AM dye

The aim of this study was to observe the effects of different growth factors, hormones, steroids and chemicals on the gap junction activity within the cumulus-oocyte complex of the rats. To undertake this study, cumulus-oocyte complexes were recovered from their follicular environment (see Figure 19a) and after an appropriate incubation time, the cumulus cells were stripped off the oocyte by vortexing for 5 minute at 20 second intervals without disrupting the integrity of the oocyte (see Figure 19b). In this study, the rat COC was pulsed for various intervals in the presence of Calcein-AM dye. During this time, Calcein-AM enters the cumulus cells and to some extent also the oocyte. Endogenous esterase's within the cumulus cells break the acetoxy methyl group (AM) producing the green fluorescent calcein (Figure 19c). After a subsequent incubation interval in the absence of Calcein-AM, the oocytes were denuded from their surrounding somatic cells as described above and check was made that the fluorescent intensity of only completely denuded oocyte was taken by observing the oocyte under the inverted microscope. Fluorescence intensity of the individual oocytes (see Figure 19d) was measured using the microscope spectrophotometer (CRAIG technology). The fluorescence intensity of the denuded oocytes relative to the background intensity or dark scan give a measure of gap junction activity for the rat COC.

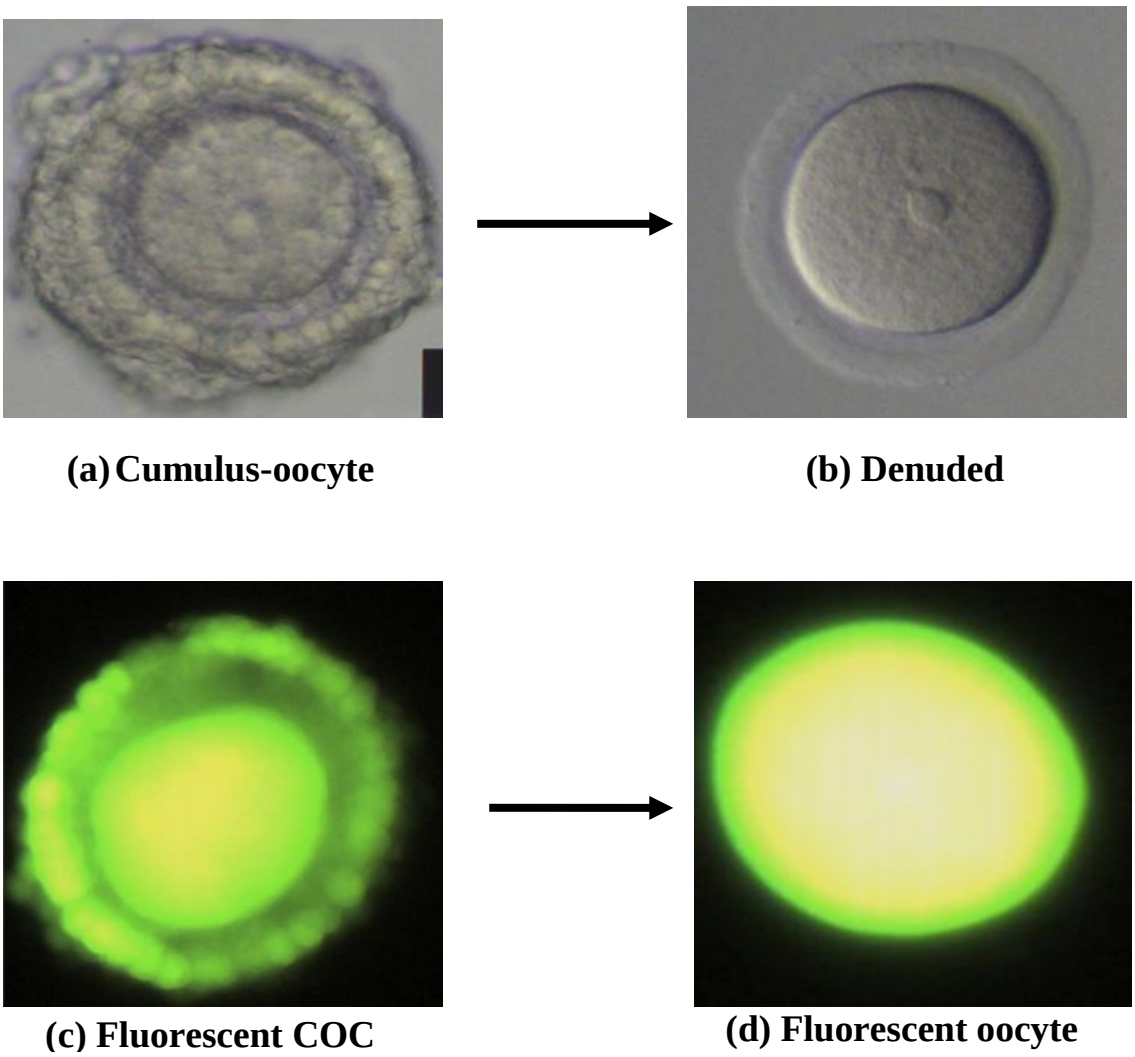


Figure 19 (a): A rat cumulus-oocyte complex (COC). (b). A rat oocyte denuded of the surrounding cumulus cells. (c). A cumulus-oocyte complex labelled with the fluorescent calcein dye. (d) A denuded oocyte labelled with the fluorescent calcein dye.

3.2 Effect of a 3hr pre-incubation of the rat cumulus-oocyte-complexes (COC) on gap junction activity

In order to explore assay conditions for measuring the gap junction activity in the rat COC, the effect of 3 hour pre incubation was investigated (Method B; see Materials and Methods). The results from these studies are to be compared with those where COC were not subjected to pre-incubation (Method A). The results show that the fluorescence reading in denuded oocytes after a 3 hour pre-incubation was significantly higher ($P<0.01$) than that of the fluorescent value in oocytes without a pre-incubation period at time 25 minute (see Figure 20). No significant differences were observed in the values at 0 minute between the control and pre-incubation treatments. Therefore, a pre-incubation time of 3 hours significantly increased the calcein transfer rate from the surrounding cumulus cells to the oocyte. Values of 0 minute and 25 minute results are significantly ($p<0.05$) different from each other in the presence of pre-incubation treatment and there is no difference between the 0 minute and 25 minute values in the control result.

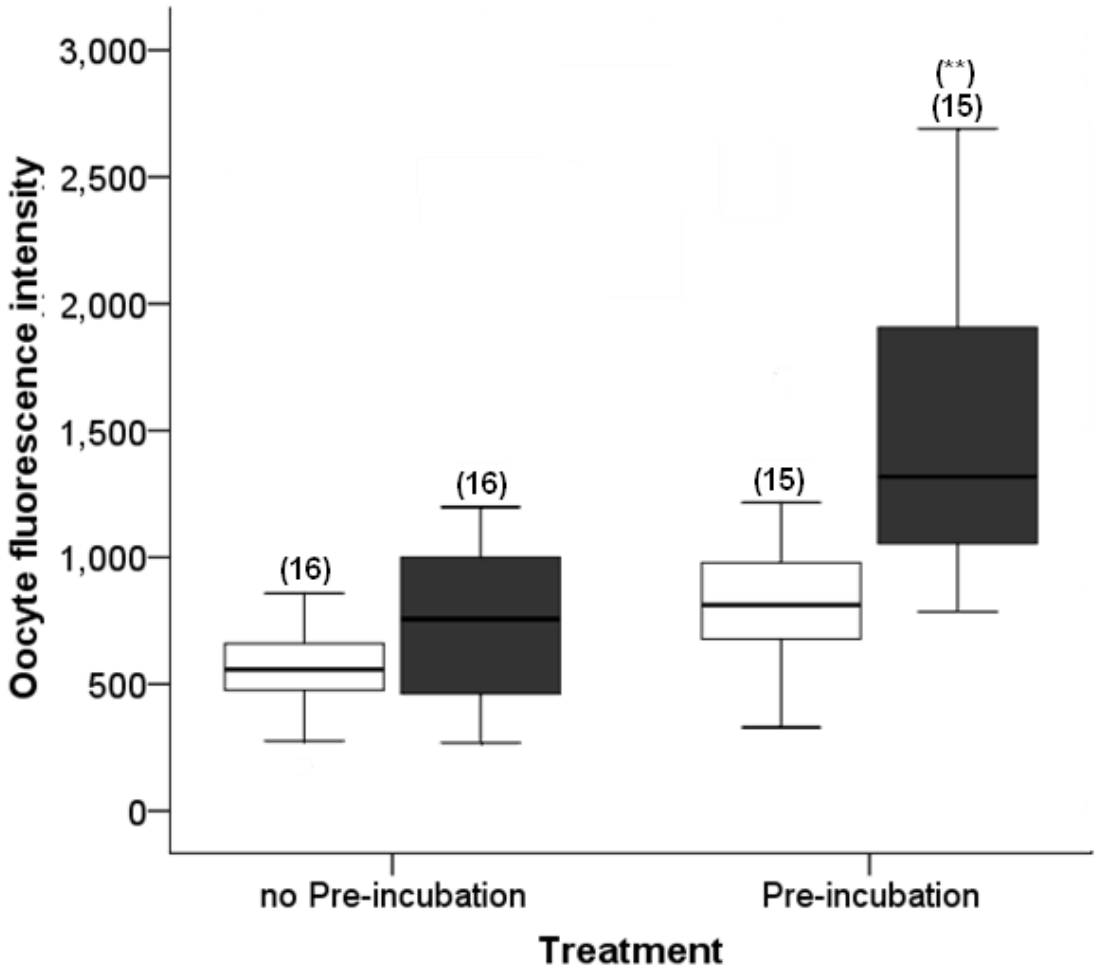


Figure 20: Effect of 3 hour pre-incubation on the gap junction assay of rat COC

Fluorescent values were represented by Box and Whisker plots. The fluorescence values at 0 minute after a 15 minute Calcein-AM pulse are shown in white and the values at 25 minute after a 15 minute Calcein-AM pulse are shown in black. Values in the bracket refer to the number of independent experiments with each being done with 6-15 COC per individual experiment. The asterisk sign indicates that the pre-incubation data represented by the black Box and Whisker plot was significantly different from the black Box and Whisker result showing no incubation treatment by Mann-Whitney test ($P<0.01$).

3.3 Effect of PDE inhibitor 3 (Milrinone)

In this experiment, the COC were pre-incubated for three hours in the presence of milrinone (Method B). Then the complexes were then pulsed with Calcein-AM for 15 minute. The fluorescence intensity of the denuded oocytes was read at 0 minute and 25 minute (Method B) after the three hour pre-incubation interval. The result in Figure 21 showed that the fluorescence value for milrinone was not statistically significant from control (no milrinone treatment) when comparisons were made at either 0 or 25 minute. The value of the 25 minute fluorescence reading in the milrinone treatment was significantly higher than that at 0 minute ($P<0.01$). The fluorescence value at 25 minute in the control (i.e., without milrinone) was statistically insignificant from the 0 minute value as calculated by the Mann-Whitney test. The above results represents that the addition of the milrinone had no effect on the gap junction activity of the rat COC.

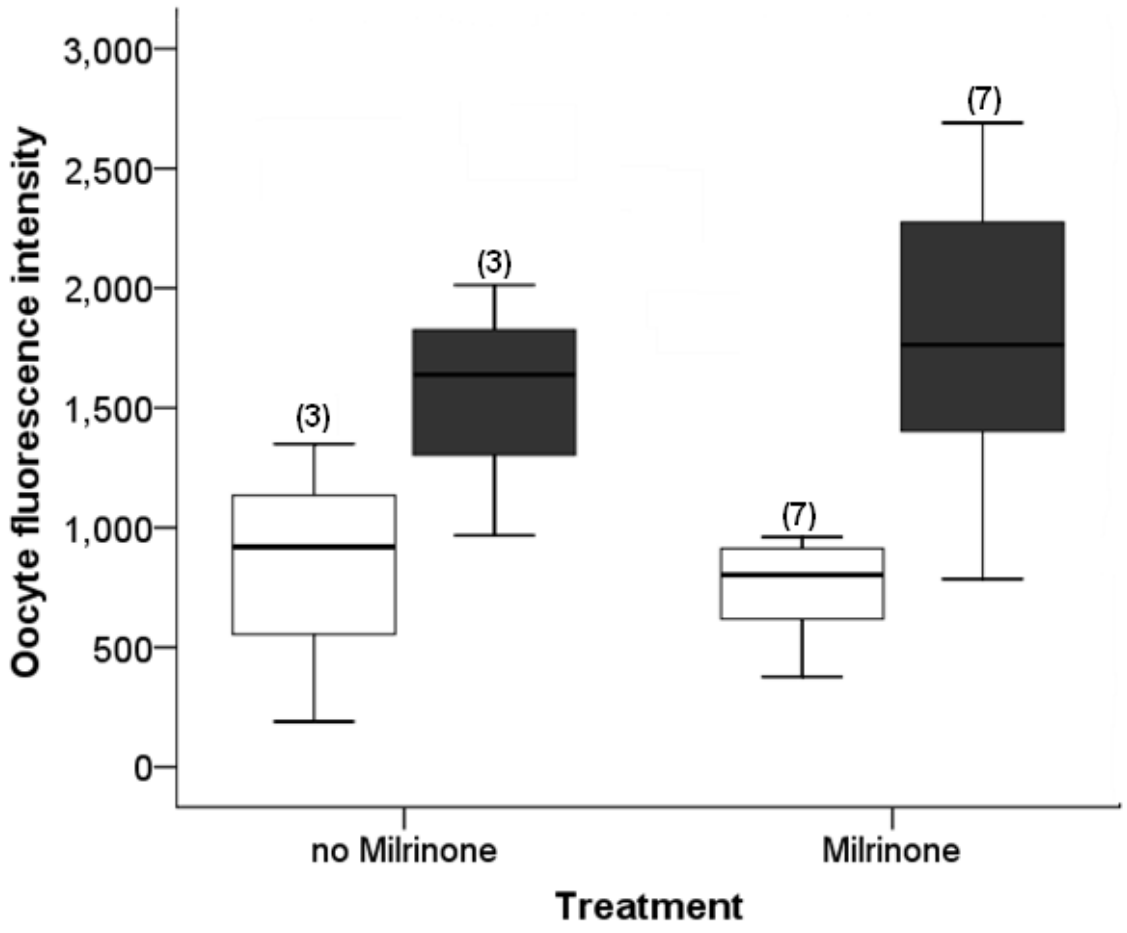


Figure 21: Effect of milrinone on gap junction activity of rat COC

Box and Whisker plots show the fluorescence values of 0 minute (white) and 25 minute after a 3 hour preincubation period and a 15 minute pulse of Calcein-AM (black) with respect to milrinone or no milrinone treatment. Values in the brackets refer to the number of independent experiments with 6-15 COCs per individual experiment.

3.4 Effect of Calcein pulse duration

The aim of this experiment was to identify an optimal time to pulse COC with Calcein-AM in order to minimise the amount of dye entering the oocyte during exposure to the dye but maximising the transfer into the surrounding cumulus cells. In this study, the effect of 10 minute or 15 minute Calcein-AM exposure time was investigated. After being pulsed with Calcein-AM for 10 or 15 minute, the COC were denuded of cumulus cells immediately (0 minute) and further incubated without Calcein-AM for 25 minute. The fluorescence intensity of the denuded oocytes was read at 0 minute and after 25 minute following the Calcein-AM pulses (Method A). The results obtained from the Mann-Whitney test showed that the values of 15 minute pulse of Calcein-AM led to significantly higher fluorescence readings compared to 10 minute exposure at both 0 and 25 minute (both $P<0.05$) (Figure 22). However, the fluorescence values after 25 minute were not different from those at 0 minute for either treatment. Nevertheless, the lower fluorescence reading at 0 minute and after 10 minute Calcein-AM pulse suggests that a shorter incubation time would be preferable to minimise background fluorescence.

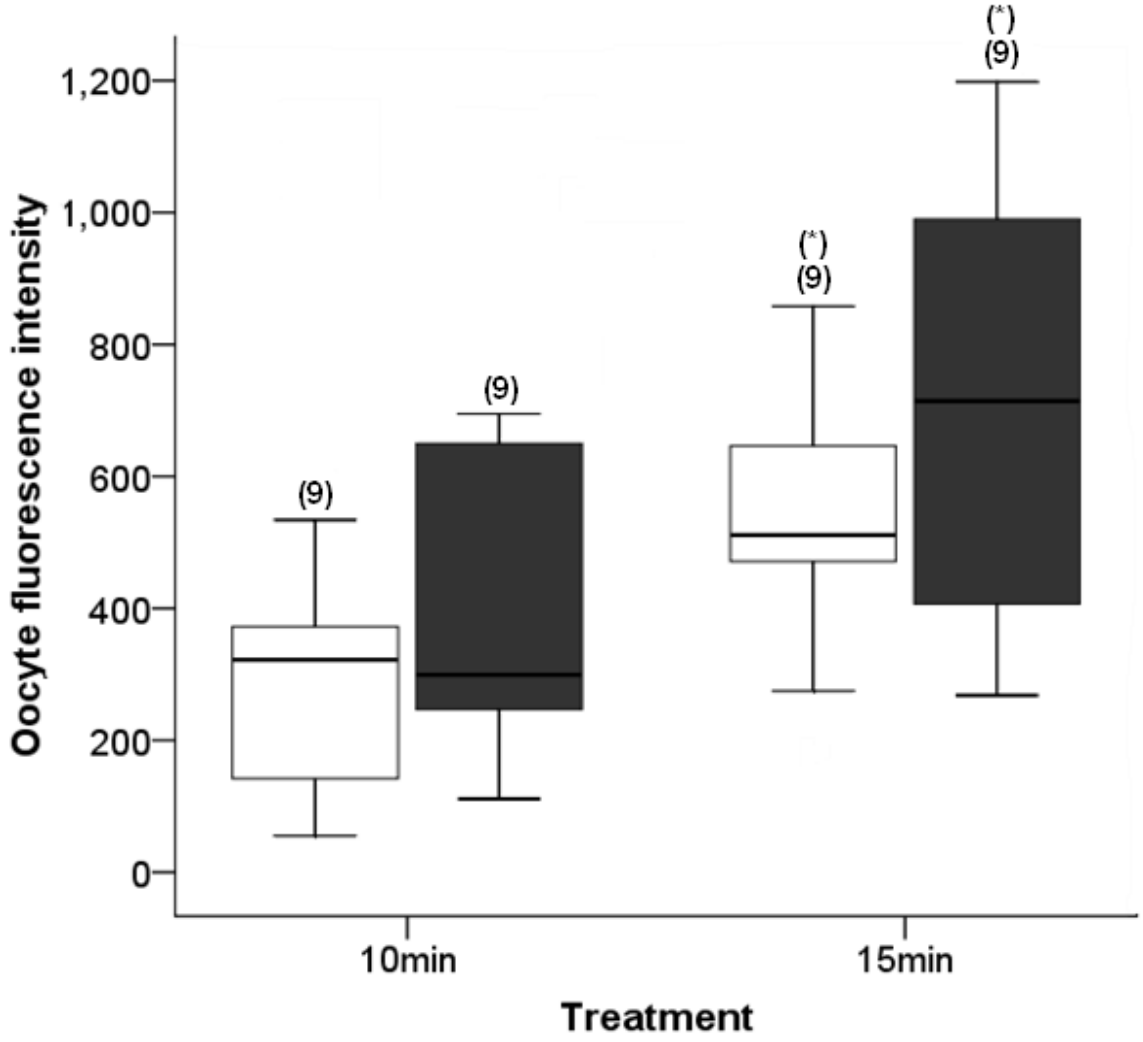


Figure 22: Effect of Calcein pulse duration on fluorescent intensity in denuded oocytes

The data were represented by Box and Whisker plots. The 0 minute values are obtained immediately after replacement of the Calcein-AM media with the calcein-free media and those in black represent the values after a further 25 minute of incubation. Values in the brackets refer to the number of independent experiments performed using 6-10 COC complexes per individual experiment. Box plots with asterisk (*) marks are significantly different ($P<0.05$) from the 15 minute vs. 10 minute comparisons at 0 minute (white box-plot comparisons) and 25 minute (black box-plot comparisons) respectively as by Mann-Whitney test.

3.5 Effect of different incubation media on gap junction activity

In order to develop the gap junction assay further, the effects of different incubation media on gap junction activity of the rat COC were examined. Medium A was composed of glutamax, sodium pyruvate and M199, medium B was composed of polyvinyl alcohol and M199 and medium C was composed of polyvinyl alcohol plus PBS (see Materials and Methods for details). To observe the effects of media A and B, COC were pulsed with Calcein-AM for 10 minute and then incubated in the presence of the same incubation media A or B respectively in absence of Calcein-AM at 0, 25, 50, 75, 120, 180 minute. Thereafter the oocytes were denuded and the fluorescence intensity of the denuded oocytes was recorded (see Method D). To observe the effect of incubation medium C, COC were pulsed with Calcein-AM for 10 minute and then incubated in Calcein-AM free medium for 180 minute only. The fluorescence intensity of the denuded oocytes was read at 0 minute and 180 minute following a 10 minute pulse of Calcein-AM. A summary of the results from the different incubation media are shown only for the readings at 0 and 180 minute (Figure 23a). The result shows that the respective values at 0 minute for medium A were significantly lower than those in medium B and C and values at 180 minute for incubation medium A is significantly lower then incubation medium B but not medium C. Further, the results also show that the mean values at 0 and 180 minute were significantly different from one another in both incubation medium B and C but not when medium A was used. These findings demonstrate that dye transfer was enhanced when rat COC were incubated in medium B but not in medium A or C. The results, for medium A and B at sequential time points from 0 to 180 minute are shown in Figure 23b. When the time series results were subjected to the linear regression analysis the mean slopes for dye transfer for COCs cultured in medium B was greater than that for medium A ($p<0.05$) indicating a greater rate of transfer in medium B (see Figure 23b).

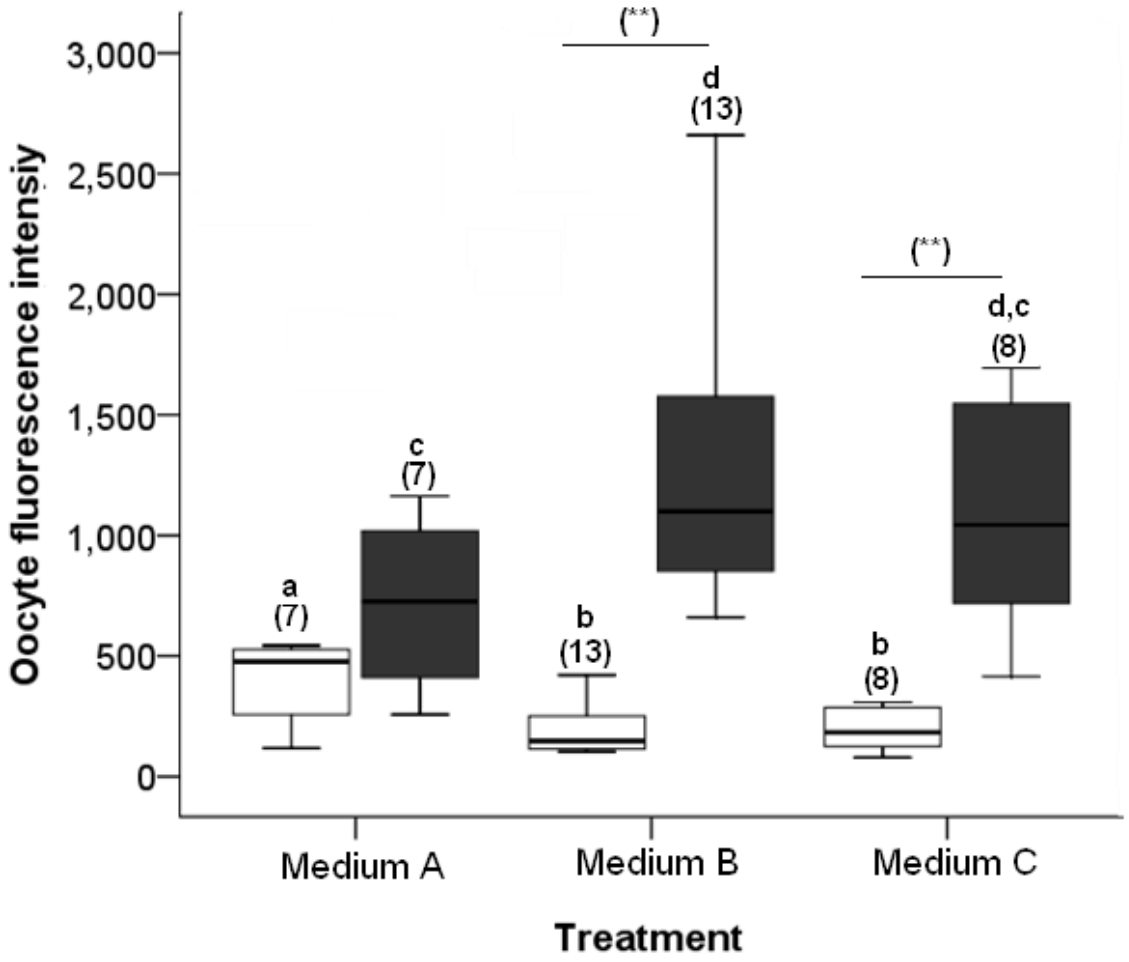


Figure 23a Effect of different incubation media on the gap junction assay of rat COC

Results for each treatment were expressed as Box and Whisker plots. Black boxes represent fluorescence values after 180 minute whereas white boxes represent values at 0 minute following the Calcein-AM pulse. Values in the brackets refer to the number of independent experiments performed using 6-15 COC per individual experiment. In Figure 23, the Box and Whisker plots which do not share a common alphabetical letter are significantly different from one another at 0 minute ($p<0.05$). Also at 180 minute, Box and Whisker plots not sharing a common alphabetical letter are statistically different from one another ($p<0.05$) as calculated by a Mann Whitney test. The asterisk (**) sign indicates that 0 minute and 180 minute results are significantly different ($p<0.01$) in medium B and C.

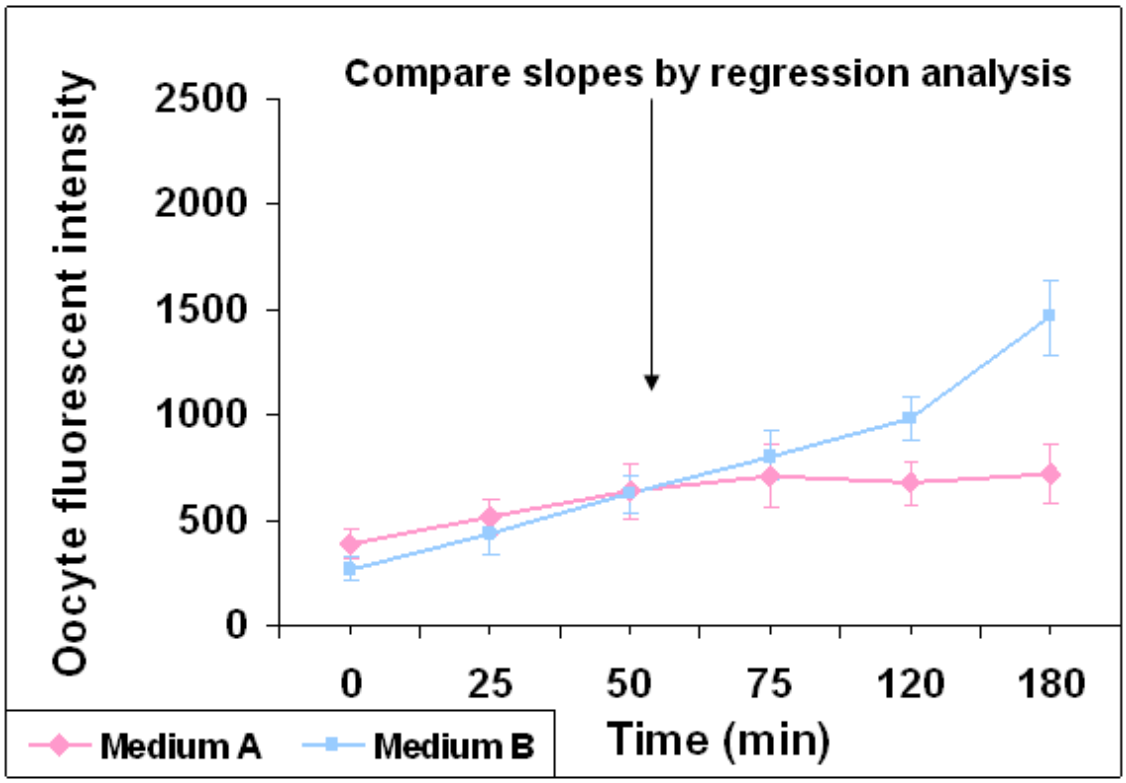


Figure 23b Effect of medium A and B on Calcein dye transfer from cumulus cells to oocyte with respect to time

Value for medium A and B at 0, 25, 50, 75, 120, 180 minute are represented as a line graph. Seven independent experiments were performed for medium A and five experiments were performed for media B using 6 -10 COC per individual experiments at each time points. Result obtained by comparing the slopes of lines with regression analysis shows that the slope of medium A is statistically significant ($p<0.05$) from the slope of medium B.

3.6 Effect of pH on gap junction activity

The regulation of pH machinery is a critical process in all mammalian cells. Ovarian cells contain the regulatory machinery to maintain the cells at a pH of 7.0 - 7.2. The factors regulating pH in the granulosa cells are responsible for maintaining the pH of the oocyte which is achieved by transferring molecules to the oocyte via gap junctions. In this study, the effect of different medium pH on the gap junction activity in the rat COC was examined. The COC were pulsed with Calcein-AM for 10 minute and then incubated in calcein-free medium for a further 3 hours (see Method E). The pH of the medium was maintained at pH 6.5, 7.2, 8.0 or 8.5 throughout these experiments. The fluorescence intensity of the denuded oocytes was read at 0 and 180 minute following the pulse of Calcein-AM. The results are summarised in Figure 24 and show that the fluorescence value of denuded oocytes at 0 minute in the presence of pH 6.5 was significantly different from those at pH 7.2, 8.0 and 8.5. After 3 hours incubation, the fluorescence readings at pH 8.5 were significantly lower than those at pH 8.0, 7.2 and 6.5 ($P<0.05$) as calculated by the Mann-Whitney test. Further results show that in presence of all the pH 6.5, 7.5, 8.0 and 8.5 fluorescence reading at 0 minute and 180 minute are statistically significant ($p<0.05$). These data suggest that at pH 6.5, the oocytes more readily take up dye without gap junction transfer and that over a 3 hour interval the transfer of calcein dye at very alkaline pH was impaired.

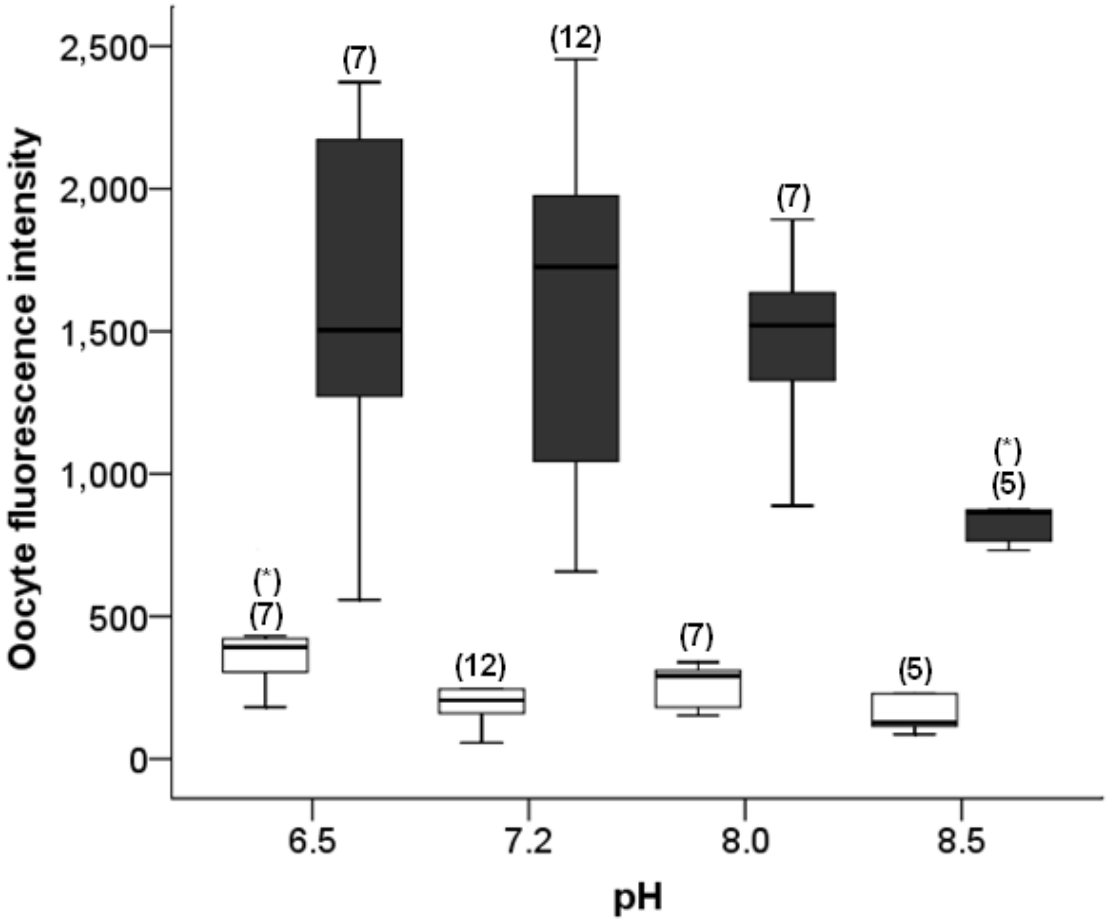


Figure 24: Effect of pH on the gap junction activity of the rat COC

The results are summarised as Box and Whisker plots at both 0 (white) and 180 (black) minute following a 10 minute exposure to Calcein-AM. Values in the brackets refer to the number of independent experiments performed using 6 - 10 COC per individual experiment. Box plots with asterisk (*) at 0 or 180 minute respectively were significantly different from the other treatment groups measured at the same time by Mann-Whitney test ($P<0.05$).

3.7 Effect of 1-octanol (a gap junction blocker)

Studies with 1-octanol concentrations at 4mM or greater indicated that since the cumulus cells dissociated from their oocytes the studies of dye transfer were not possible at this or higher concentrations (see Figure 25). However, at 2 mM the COC remained intact throughout the incubation period (Figure 26). The results for 2 mM 1-octanol are summarised in Table 2. In this study, recovered rats COC were pre-incubated in the presence of 1-octanol for 2 hours. Then the COC were pulsed with Calcein-AM dye for 10 minute. After 10 minute Calcein-AM incubation, fluorescence intensity of the denuded oocytes was read at 0 and 50 minute using the microscope spectrophotometer (CRAIC technology) (see Method F). Table 2 represents that the fluorescence reading of the control value is statistically significant ($p<0.05$) from the fluorescence reading of 1-octanol at both 0 minute and 180 minute as calculated by the Mann-Whitney test. Further results show that the 0 minute value is significantly lower then the 180 minute value in the control. In comparison to that in the presence of 1-octanol (gap junction blocker) the fluorescent value at 0 minute is significantly higher then the fluorescent value at 180 minute. These results show that at 2mM, 1-octanol was more readily transferred directly into oocytes without traversing the cumulus-oocyte gap junctions and that over 3 hours additional dye transfer did not occur. We have also observed the effect of gap junction blocker carbenoxolone but due to lack of time to explore a wide range of concentrations of carbenoxolone we were unable to demonstrate the effect of carbenoxolone on the gap junction activity of the rat COC.

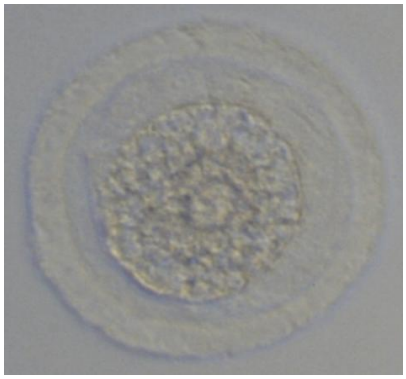


Figure 25: An oocyte free of cumulus cells after incubation in 4mM 1-octanol

The above Figure shows the morphological appearance of the denuded rat oocyte at 10X in presence of 4mM 1-octanol after 50 minute of incubation. At 4mM 1-octanol there was no evidence for persistent attachment of cumulus cells to oocytes. The cytoplasm of this oocyte is intensely granulated and there is evidence that the zona pellucida has hardened.

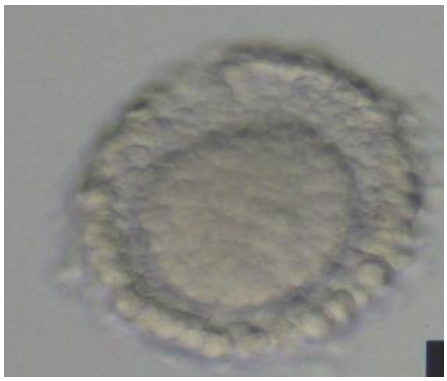


Figure 26: An intact cumulus-oocyte complex in presence of 2mM 1-octanol 10x

At 2mM 1-octanol, cumulus cells remained attached to their oocyte after 180minute incubation interval.

Treatment	Incubation time (minute)	
	0 minute	**180 minute**
Control	620 ± 63 (7)	1202 ± 129 (7)
1-octanol (2mM)	1344 ± 216* (8)	707 ± 180* (8)

Table 2 Effect of 1-octanol (gap junction blocker) on the gap junction activity

Numbers in the brackets refer to the number of independent experiments performed using 6 - 15 COC per individual experiment. Within columns, the asterisks indicate significant differences compared to the controls ($P<0.05$). Within each of the rows, the values are significantly different from one another as calculated by Mann-Whitney test ($P<0.05$).

3.8 Effects of dbcAMP and forskolin

An increase in cAMP levels within the oocyte are known to maintain the oocyte in meiotic arrest. In this experiment, the effects of dbcAMP and forskolin on the gap junction activity of the rat COC were examined. The COC were pulsed with Calcein-AM for 10 minute followed by a 3 hour incubation in the presence of no additive (Control), dbcAMP or forskolin. The fluorescence intensity of the denuded oocytes was recorded at 0, 25, 50, 75, 120 and 180 minute (see Method D). The results for the 0 and 180 minute time points are summarized in Figure 27a. The results show that the values for the control, dbcAMP and forskolin were not statistically different from one another, either at 0 or 180 minute, as calculated by the Mann-Whitney test. Further results also show that 0 minute value is statistically significant ($p<0.01$) from the 180 minute value in presence of control, dbcAMP and forskolin treatment. Therefore, the above result suggests that dbcAMP and forskolin were unable to modulate the gap junction activity of the rat COC. Figure 27b represents the dbcAMP and forskolin results at different time points over the period of a 3 hour incubation. These results show the levels of gap-junction activity as assessed by dye transfer to oocytes at specific time points. Figure 27b shows that there is a gradual increase in the gap junction activity in the presence of dbcAMP, forskolin and the controls from 0 minute to 180 minute incubation. When the comparison was made between slope of control and treatment results using a regression analysis, results obtained show that there is no significant difference between the slope of cAMP and the forskolin treatment from the slope of the control result. Even the error bars on the line graph at each point show that cAMP and forskolin treatment are not statistically significant from the control results.

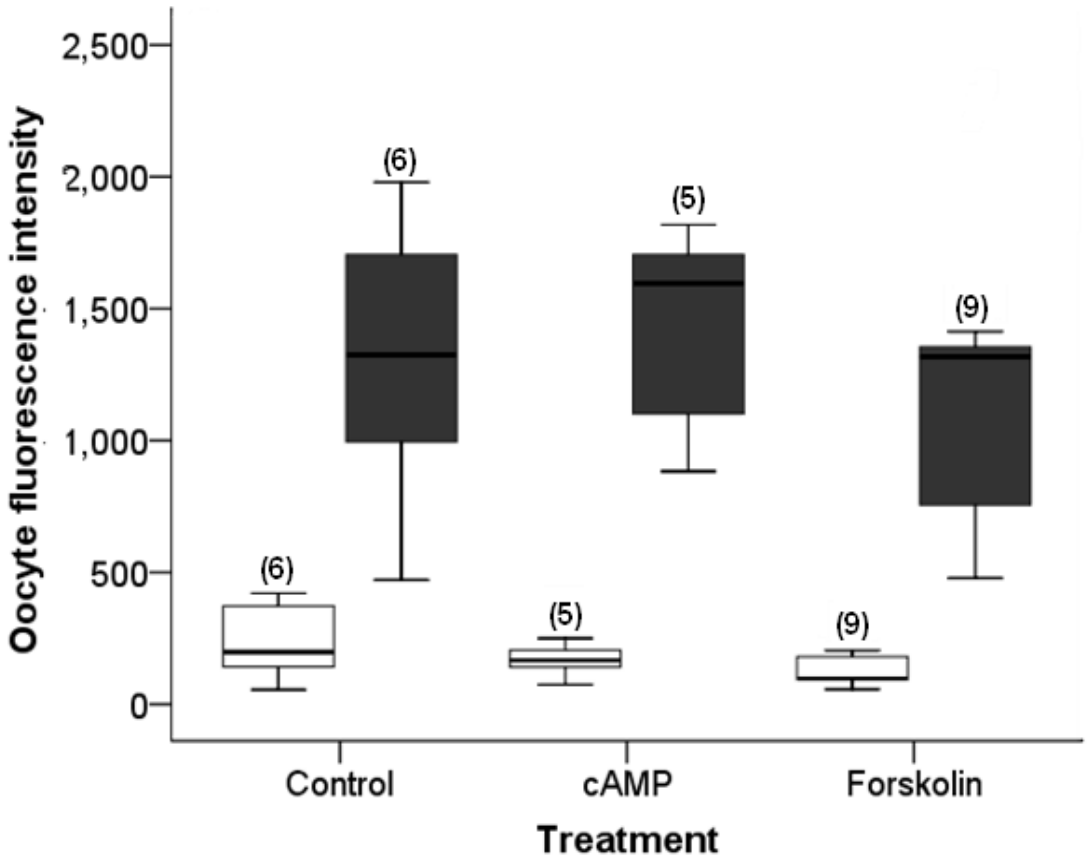


Figure 27a Effects of dbcAMP, forskolin and no treatment (Control) on gap junction activities of the rat COC

Values are shown as Box and Whisker plots at 0 minute (White) or after 180 minute (Black). The value in the brackets refers to the number of independent experiments performed using 6-15 COC per individual experiment.

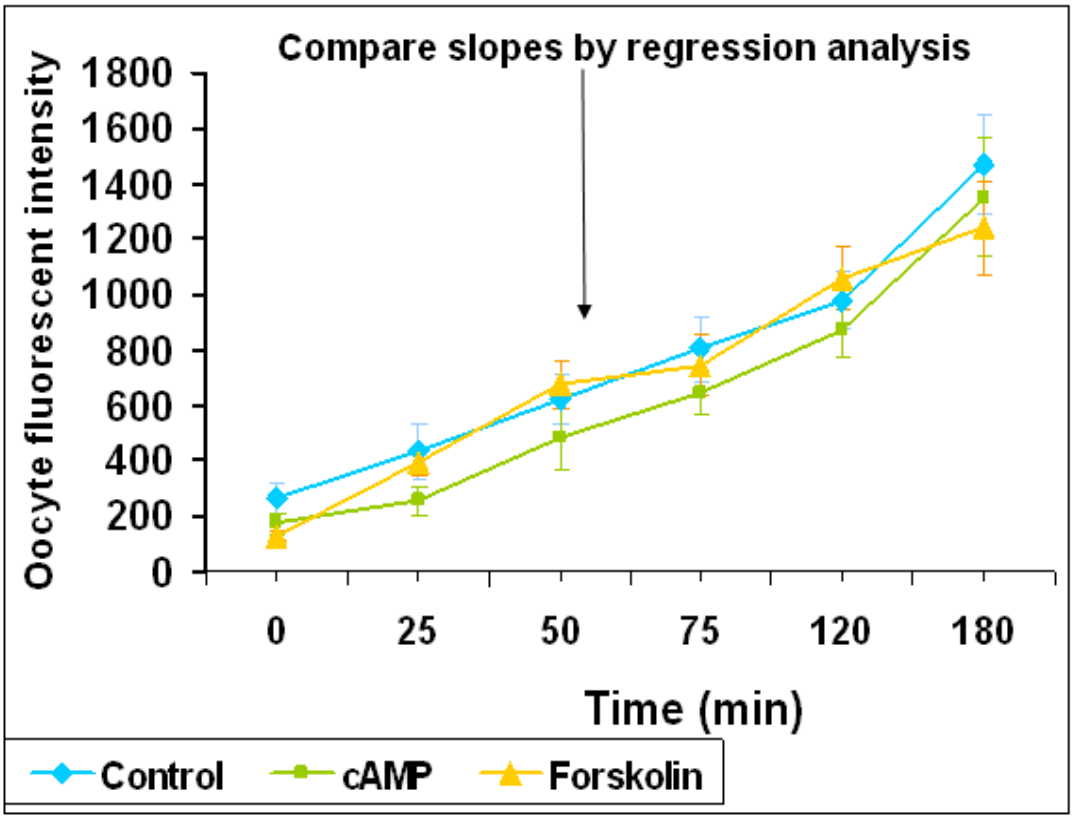


Figure 27b Effect of cAMP and forskolin on the gap junction activity of rat COC at various time points

Values for cAMP and forskolin results at 0, 25, 50, 75, 120, 180 minute are represented as a line graph. Five independent experiments were performed for control and four and ten experiments were performed for cAMP and forskolin treatments respectively using 6-10 COC per individual experiments at each time points. The results were obtained by comparing the slopes of lines with regression analysis, showing that the slope of the cAMP and forskolin results are statistically not different ($p<0.05$) from the slope of the control result.

3.9 Effect of growth differentiation factor 9 (GDF9) plus bone morphogenetic protein 15 (BMP15) on fluorescence dye transfer between cumulus cells and oocytes

The oocyte-derived growth factors GDF9 and BMP15 play an important role in ovarian follicular development. In this experiment, the combined effect of GDF9 and BMP15 on the gap junction activity in the rat COC was examined. The reason for adding both reagents together to the COC was that in previous studies, McNatty et al., (2005) reported that BMP15 + GDF9, together but not alone, caused a significant increase in thymidine incorporation and steroid secretion by rat granulosa cells. In the present study, the COC were pulsed with 1μM Calcein-AM for 10 minute. Thereafter, the COC were incubated in the presence of GDF9 + BMP15 for 180 minute in a Calcein-AM free media. Fluorescence intensity of the denuded oocytes was read at 0 and 180 minute using the microscope spectrophotometer (CRAIC fluorescence technology) (see Method E). The results represented in Figure 28 show that the fluorescence value after GDF9 + BMP15 treatment was not significantly different from the control results at either 0 or 180 minute. However, in both the control and treated groups, the fluorescence values at 180 minute was significantly higher than at 0 minute ($P<0.05$). This result suggests that the oocyte-derived growth factors GDF9 + BMP15 have not influenced the gap junction activity in the rat COC.

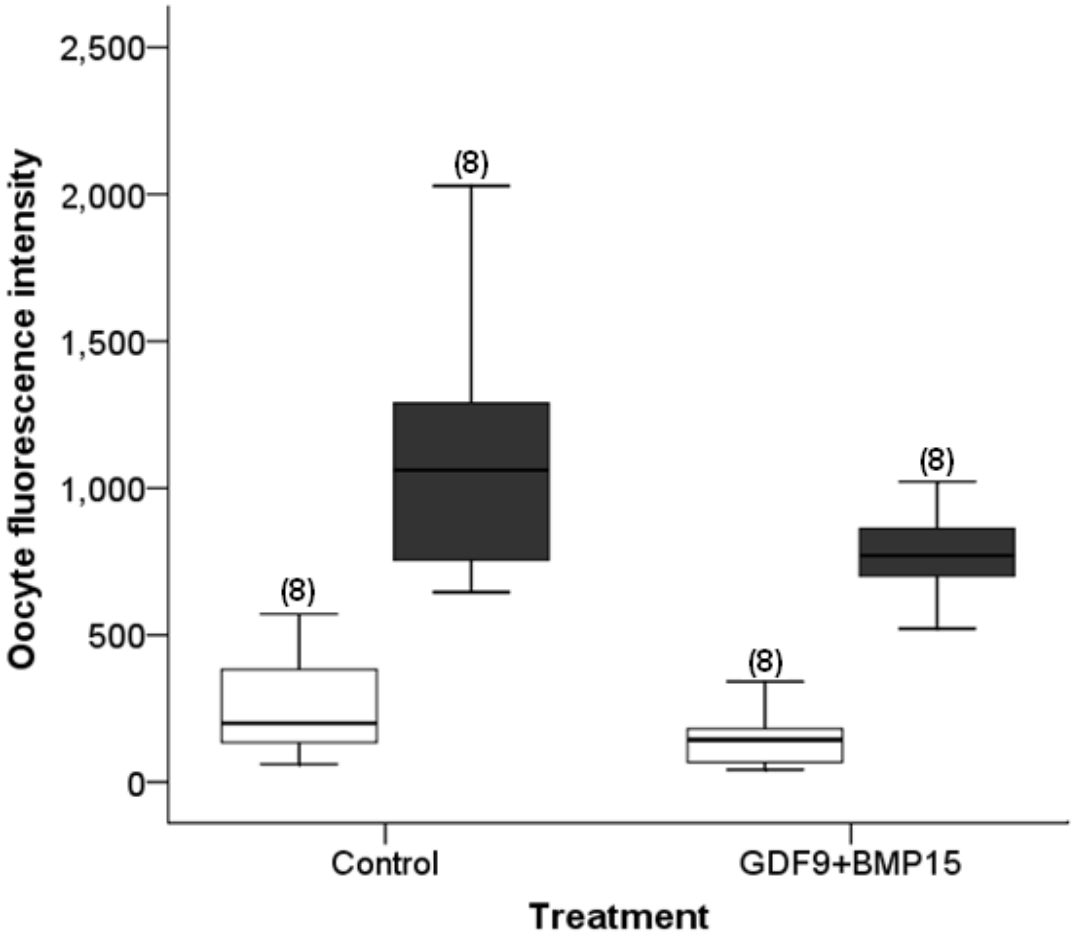


Figure 28: Effect of GDF9 + BMP15 or no treatment (Control) on gap junction activity of the rat COC

Values are shown as Box and Whisker plots at 0 (white) or 180 minute (black). Values in the brackets refer to the number of independent experiments performed using 6 - 10 COC per individual experiment.

3.10 Effect of insulin-like growth factor-1 (IGF-1)

IGF-1 has been shown to enhance the effects of FSH during mammalian follicular development. Therefore, in this study the effect of IGF-1 on the gap junction activity of the rat COC was examined. Rat COC were pulsed with Calcein-AM for 10 minute followed by a 3 hour incubation in the presence of low (1.7ng/ml) or high concentrations (10ng/ml) of IGF-1. The fluorescence intensity of denuded oocytes was read at 0 and 180 minute (see Method E). The results show that at both low and high concentrations of IGF-1, the 0 and 180 minute readings were not significantly different from those of the control group (Figure 29). The values at 180 minute for the control and the IGF-1 treatments were significantly higher than the 0 minute values ($P<0.01$). Overall, this result demonstrates that at both low and high concentrations IGF-1 alone had no effect on the gap junction activity of the rat COC.

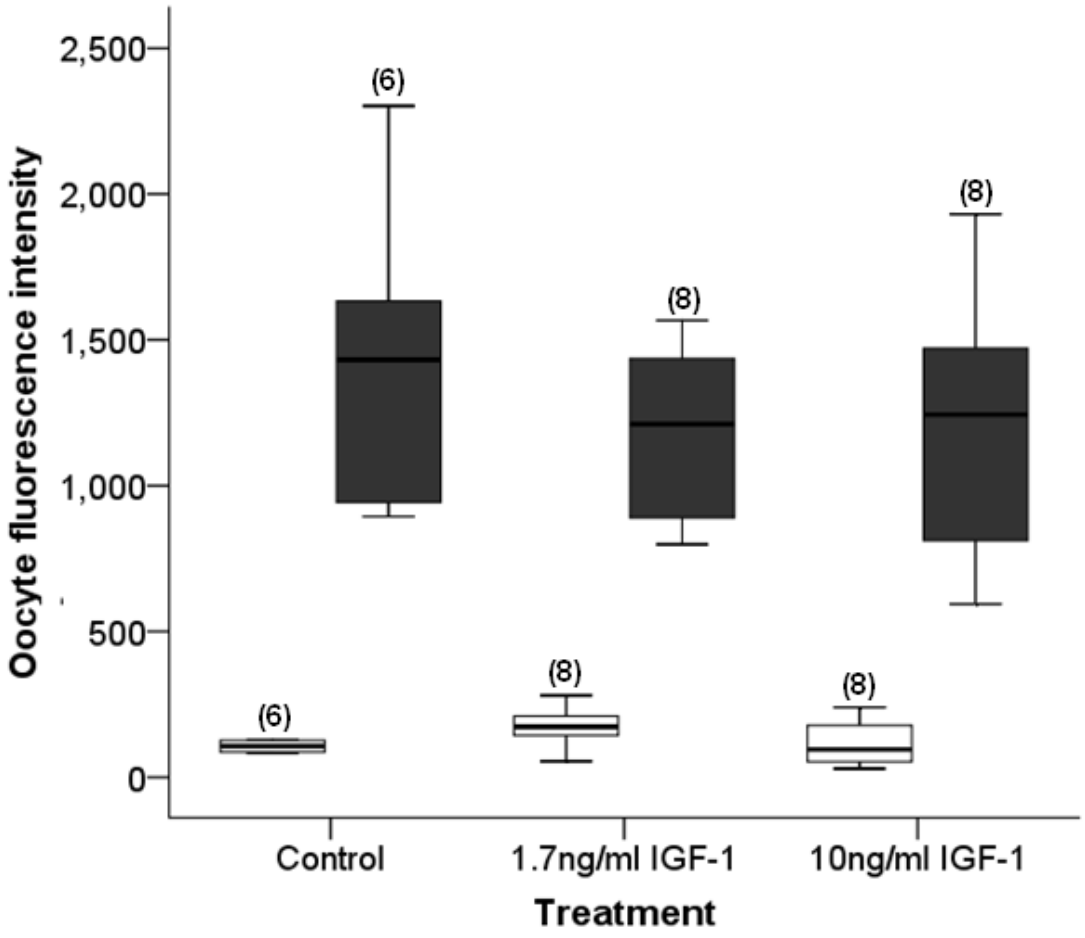


Figure 29: Effect of IGF-1 the gap junction activity of rat COC

Results are presented as Box and Whisker plots at 0 minute (white) and 180 minute (black). The values in the brackets refer to the number of independent experiments performed using 6 - 15 COC per individual experiment.

3.11 Effect of oestradiol 17β

Oestradiol 17β is an important secretory product of the follicle during granulosa cell proliferation and differentiation. Oestradiol concentrations may also be present at very high concentrations in follicular fluid. Therefore, in this study the effects of oestradiol 17β on gap junction activity of rat COC were examined. Rat COC were pulsed with Calcein-AM for 10 minute followed by 3 hour incubation in Calcein-AM free media in the presence of 13.8μM oestradiol 17β. The fluorescence intensity of the denuded oocytes was read at 0 and 180 minute following the Calcein-AM pulse (see Method E). Results of this study showed that the fluorescence values for the oestradiol 17β treatment was not statistically significant ($P<0.05$) from that of the control at 0 or 180 minute (see Figure 30). The fluorescence values at 180 minute were significantly higher than those at 0 minute for both the control and oestradiol 17β treatment ($P<0.05$). These results suggest that at the concentration 13.8μM of oestradiol 17β that was tested, there was no effect on gap junction activity in the rat COC.

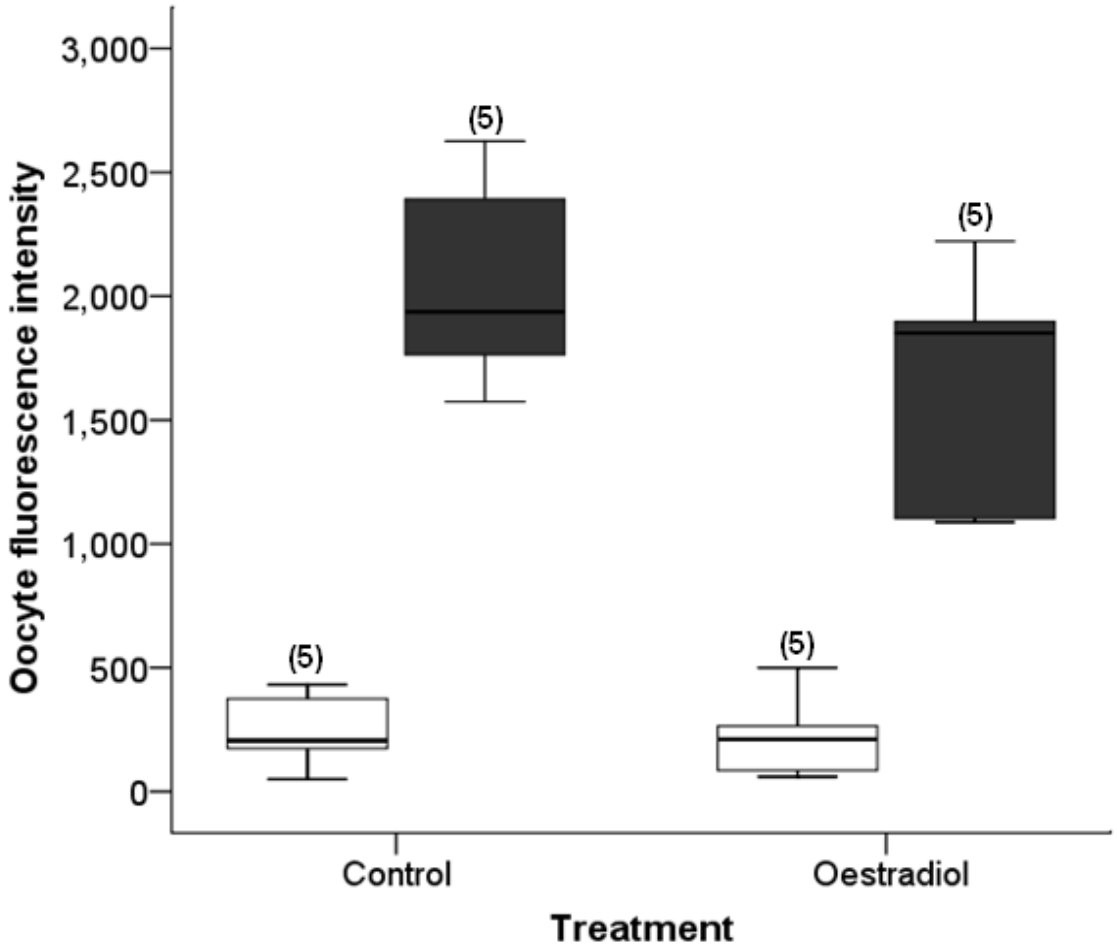


Figure 30: Effect of oestradiol 17β or no treatment (Control) on gap junction activity in rat COC

Values are represented by Box and Whisker plots. The fluorescence values at 0 minute are shown in white and at 180 minute in black. Values in the brackets refer to the number of independent experiments done with 6 - 15 COC per individual experiment.

3.12 Effect of testosterone, testosterone + forskolin, testosterone + forskolin + IGF-1 on gap junction activity

Testosterone is a significant secretary product of the ovarian follicles and concentrations of this steroid are relatively high in the follicles before they produce large amounts of oestradiol (Carson et al., 1981). In this study, the effects of the testosterone alone and the combined effects of testosterone + forskolin and also testosterone + forskolin + IGF-1 were examined on gap junction activity. For this study, COC from rat follicles were exposed to the Calcein-AM dye for 10 minute followed by a 3 hour incubation in the presence of any one of the following treatments at a time [testosterone (13.8µM), testosterone + forskolin (100µM) and testosterone + forskolin + IGF-1 (0.00131µM)]. The fluorescence intensity of the denuded oocytes was read at 0 minute (i.e. immediately after 10 minute exposure to Calcein) or after 180 minute in Calcein free media (see Method E).

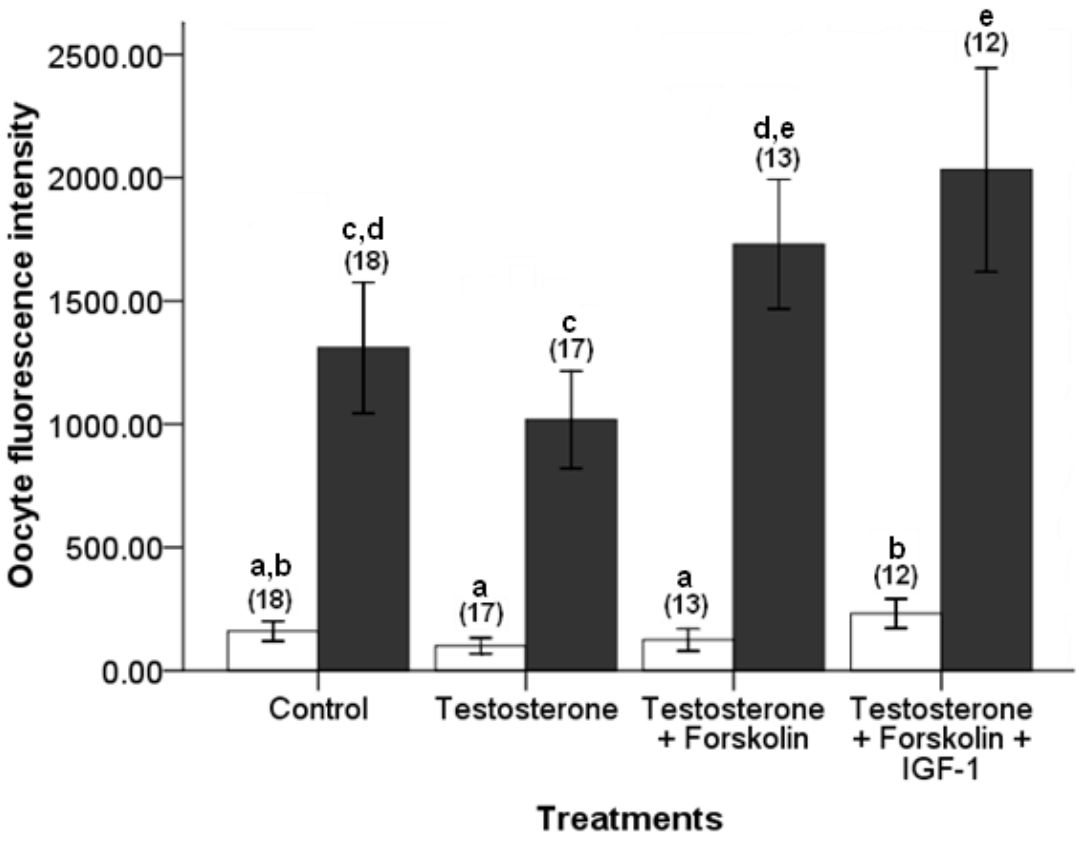


Figure 31: Effect of testosterone alone, combined effects of testosterone + forskolin and combined effects of testosterone + forskolin + IGF 1 on the gap junction activity of the rat COC

Values in the brackets refer to the number of independent experiments performed using 10 - 20 COC per individual experiment. Values are the mean ± s.e.m. In Figure 31, the bars which do not share a common alphabetical letter are significantly different from one another at 0 minute ($p<0.05$). Also at 180 minute, bars not sharing a common alphabetical letter are statistically different from one another ($p<0.05$) as calculated by the ANOVA and Post hoc Tukey test.

Figure 31 shows that the 180 minute values in the control and different treatments were statistically significant ($p<0.01$) higher than the respective values at 0 minute. When comparisons were made between the treatments, the results show at time 0 minute, that the mean value of the combined treatment of testosterone + forskolin + IGF-1 was significantly different from value of testosterone treatment alone and the combined treatment of testosterone + forskolin ($p<0.05$). At 180 minute, the value of testosterone treatment was significant low than the value of combined treatment of testosterone + forskolin and testosterone + forskolin + IGF-1 ($p<0.05$). Further, the result showed that the mean combined treatment of testosterone + forskolin + IGF-1 was significantly higher than the control results ($p<0.05$) (see Figure 31). Therefore this shows that testosterone alone and that the combined treatment of testosterone + forskolin were unable to modulate the gap junction activity of the rat COC at time 0 and 180 minute and the combined treatment of testosterone + forskolin + IGF-1 has enhanced the gap junction activity of the rat COC at time 180 minute ($P <0.05$).

CHAPTER 4 DISCUSSION

Bidirectional communication between mammalian oocytes and surrounding follicular somatic cells (cumulus-cells) is essential to achieve oocyte development competence. The oocyte secretes factors (oocyte-secretory factors; OSF) that regulate metabolic activity in adjacent somatic cells and molecules transferred from the surrounding cumulus-cells support oocyte development by providing them with nutrient and energy sources such as protein substrate like amino-acids and pyruvate by delivering them through specialized cell adhesion molecules known as the gap junction (Eppig, 1991). Thus the gap junctions are essential for mammalian follicular development. The gap junctions develop as the follicle grows through the primary, secondary and tertiary (antral) follicular stages (Mitchell and Burghardt, 1986). Many different ovarian factors are thought to modulate the gap junction activity within mammalian follicles.

In this study, a method was developed to assess the gap junction activity within the rat cumulus-oocyte complex (COC). As part of this study, the effect of different incubation media and pH were examined. For assessing and optimising this assay, effects and roles of some of growth factors, steroids and chemicals on the gap junction activity of the rat COC were examined.

In the present study, the effect of different incubation media on the gap junction activity of the rat COC was examined to identify an appropriate medium in which the rat COC could be incubated in the presence of different treatments. When COC were incubated in the presence of incubation medium A, composed of glutamax, sodium pyruvate and M199 and incubation medium C, composed of polyvinyl alcohol and phosphate buffer saline the transfer of calcein dye from the surrounding cumulus cells into the oocyte was impaired. However when incubation medium B, composed of M199 and polyvinyl alcohol was used there was an increase in calcein dye transfer from the surrounding cumulus cells into the oocyte compared to that observed with medium A and

C. This shows that incubation of rat COC in the presence of relatively simple medium like PBS or M199 was adequate for assessing the gap junction activity in rat COC. In these studies, the COC were also incubated in phenol red-free medium containing bicarbonate buffer. Therefore, medium B was chosen as the preferred medium for assessing the gap junction activity in the rat COC. These COC remained viable under the *in vitro* conditions as assessed in culture under the inverted microscope.

An important issue to address in this study on rat COC was the duration of the Calcein-AM pulse time and the removal of the calcein solution into a medium without the dye, a preferred optimal time to accurately assess the transfer of dye from the cumulus cells into the oocyte in an *in vitro* conditions. Calcein dye has previously been used to monitor dye transfer from cumulus cells to the oocyte in bovine COC (Thomas et al., 2004). In the published study, a 1µM of calcein was pulsed for 15 minute followed by an incubation period of 25 minute without calcein to monitor dye transfer. In the present study, it was evident that the COC pulsed with 1µM of Calcein-AM for 10 or 15 minute was satisfactory to obtain the results. After examining the effect of a 5 minute Calcein-AM exposure, it was concluded that this was too short time for sufficient amount of calcein dye to enter the rat cumulus cells (data not shown). In present studies, a 10 minute pulse was chosen for most studies to minimise background fluorescence levels from the oocyte at removal of the dye. However, after substituting the calcein medium with a calcein free medium, it was clear that the dye transfer over the first 30 minute was low and / or variable. It was evident that the dye transfer increased steadily over a 180 minute interval in the absence of any hormones or growth factors in the medium. These findings suggest that upon isolation, the COC gap junctions may have become non-functional or lost due to the trauma of being isolated from the other follicular cells. It is not known whether the gap junction function in rats is more labile than in cows or whether the same phenomenon is true in bovine COC. In the Thomas et al., (2004) study, the effect of time following the removal of the dye was not investigated in detail. This has implications for *in vitro* oocyte

maturation procedures and further work is needed to explore these issues. In the present study the gap junction assessment was either monitored sequentially on different COC over time by measuring the calcein dye concentration in the denuded oocyte or comparisons were made by assessing dye concentrations in the oocyte immediately after removal from the dye to those COC present in calcein free medium some 180 minute later.

In order to manipulate the transfer of calcein dye from the surrounding cumulus cells into the oocyte, the effects of various concentrations of the gap junction blockers, 1-octanol and carbenoxolone were investigated on the rat COC. When the rat COC were incubated in the presence of 8mM and 4mM 1-octanol concentration, the cumulus cell contacts with the oocyte broke down and calcein dye was not able to enter the oocyte. This shows that at higher concentration (8mM and 4mM), 1-octanol was toxic to the rat COC (see Figure 25). However, when the rat COC were treated with 2mM concentration of 1-octanol, the rate of calcein dye transfer from the surrounding cumulus cells into the oocyte increased at 0 minute and then decreased after 50 minute of incubation in calcein free medium. This effect of 1-octanol on the gap junction uncoupling could be mediated by initial acidification within the rat COC followed by alkalinisation. This effect of 1-octanol could be compared with the effect of pH where at pH 6.5 (acidic) when there was an initial increase in calcein uptake by the oocyte at 0 minute whereas at pH 8.5 (alkaline), there was a decrease in calcein transfer from the surrounding cumulus cells into the oocyte after 180 minute. Therefore, one interpretation of this result is that 1-octanol causes an initial acidic shift that uncouples the gap junction within rat COC followed by alkalinisation. These results are further supported by an early study done by Pappas et al., (1996), which showed that 1-octanol (1mM) causes a biphasic change in pH of the hippocampal astrocytes which contain connexin (Cx43) mediated by an initial acidification that led to uncoupling of the gap junctions followed by a rapid increase in pH. In contrast to our findings for follicle enclosed oocytes, FitzHarris and Baltz, (2005) reported that 1-octanol (gap junction

inhibitor) eliminated the pHi regulatory machinery and thus impeded the recovery of the oocyte from an induced alkalosis. The reason for this discrepancy is not known.

Using scrape loading dye transfer assay, Sela-Abramovich et al., (2006) have shown that the incubation of the rat follicle enclosed oocytes in the presence of 100µM concentration of carbenoxolone caused a decrease in number of gap junctions within the rat ovarian follicle. In the present study, the effect of 100µM carbenoxolone on the gap junction assay of the rat COC was examined. However, we were unable to demonstrate that 100µM carbenoxolone could block calcein transfer from cumulus cells to the oocyte even after 180 minute of treatment (data of carbenoxolone experiments are not shown). In contrast, Thomas et al., (2004) observed the effects within 60 minute in bovine COC. They showed that 30µM carbenoxolone was able to block the transfer of calcein from the surrounding cumulus cells into the oocyte. In the present study, there was insufficient time to explore a wide range of concentrations of carbenoxolone. Therefore, it remains to be established whether carbenoxolone is an effective blocker of the gap junction activity in an isolated rat COC under the *in vitro* conditions employed in the present studies.

Granulosa cells of mammalian antral follicles possess the pH (pHi) regulatory machinery that maintains the oocyte pH at 7.2 or 7.3 units. The pH regulatory machinery in antral follicles possess the $HCO3^-/Cl^-$ exchanger (AE) that corrects alkalosis by exporting the $HCO3^-$ in exchange for extracellular Cl^-, and Na^+/H^+ exchanger (NHE) that extrude proton and corrects acidosis (Alper, 1994; Orlowski and Grinstein, 1997; 2004; Roos and Boron 1981; Romero et al., 2004). In the present study, effects of different pH on the gap junction activity of the rat COC were observed. When the rat COC were incubated in the presence of media pH 6.5, Calcein-AM dye uptake was enhanced at 0 minute in comparison to that observed when the pH was 7.2. In contrast, a media pH of 8.5 decreased calcein dye transfer from cumulus cells into the oocyte over a 180 minute interval relative to that when COC were incubated in the presence of pH 7.2. This shows that the transfer of small molecules into the oocytes is influenced by the pH of the external environment. The

second messenger, cAMP produced by cumulus cells and transferred to the oocyte via gap junctions, maintains the oocyte in a meiotic arrested state [Germinal Vesicle (GV) stage] (Figure 19a) (Lindner et al., 1974; Dekel and Beers, 1978; Dekel et al., 1981). Mammalian oocytes, when isolated from their follicular environment,8r will eventually resume meiotic maturation; the timing of this resumption is species dependent (Pincus and Enzmann, 1935) and due to the phosphodiesterases (PDEs) in both the oocyte and cumulus cells degrading cAMP together with or coincident with, a loss of the gap junction activity (Dekel and Beers, 1978; 1980). To maintain isolated rat COC in a meiotic arrested state PDE inhibitors such as milrinone are often used with *in vitro* studies. Milrinone is an inhibitor inhibiting the catalytic subunit of the PDE (phosphodiesterase) enzyme and thus helps to maintain the intra oocyte levels of cAMP (see review by Tsafriri et al., 1996). In this study, the incubation of the rat COC with PDE inhibitor milrinone for 3 hours was found not to influence gap junction activity in rat COC. In the rat, GV was easy to observe in isolated oocytes under an inverted microscope. It seemed that over the 180 minute of incubation used in the present study, the COC did not really require the presence of milrinone to prevent GV breakdown. However, as a precaution milrinone was added to all incubation media when testing the effects of hormones or growth factors on gap junction activity. The result for this study with milrinone is consistent with previous findings that milrinone by itself did not influence dye transfer in the bovine cumulus cell-oocyte complexes for the first 3 hours of culture (Gilchrist et al., 2004).

The roles of dbcAMP and forskolin (a cAMP stimulator) were also examined for their effects on modulating the gap junction activity of rat COC. Neither of the stimulators was able to enhance calcein dye transfer over a 180 minute interval following a 10 minute pulse of Calcein-AM compared to incubation medium devoid of either stimulator. In contrast to our results, studies with bovine COC showed that forskolin was able to enhance the transfer of calcein dye through gap junctions. In the present study on rat COC, although forskolin by itself was unable to increase gap junction activity, as it was capable, in the

presence of IGF1 plus high concentrations of steroid (e.g. testosterone), which significantly enhance dye transfer through gap junctions over a 180 minute interval.

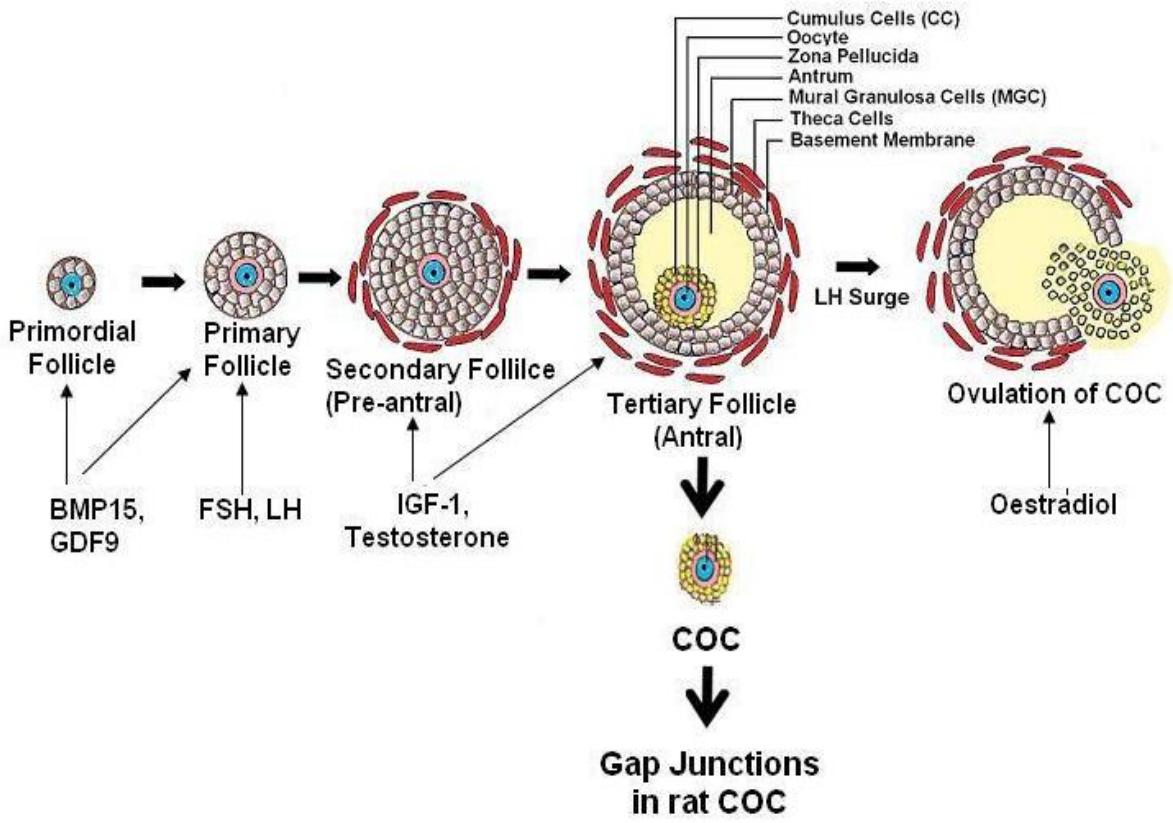


Figure 32: Effect of different growth factors and steroids on different stages of follicular development (modified from MSc thesis by Rebecca Dragovic 2006, University of Adelaide).

The above figure exhibits the effect of different oocyte derived growth factors like BMP15 and GDF9, insulin like growth factor IGF-1, hormones like FSH, LH and steroids such as testosterone and oestradiol 17β on the different stages of follicular development. In the present study, we have observed the effect of different growth factors and steroids on gap junction activity of rat COC from an antral follicle.

Oocyte derived growth factors like BMP15 and GDF9 play an important role in primary and primordial follicle development (see Figure 32). Previously McNatty et al., (2005) reported that BMP15 and GDF9 together, but not each factor separately, caused a significant increase in thymidine incorporation and steroid secretion by the rat granulosa cells. With limited amounts of these growth factors, the effects of these two growth factors together were examined on calcein dye transfer in rat COC rather than examining the roles of each growth factor together. The results from these studies shows that combined effects of GDF9 plus BMP15 did not modulate gap junction activity as assessed by dye transfer. This finding was similar to that by Gittens et al (2005), in which gene expression of GDF9 was analysed in connexion-43 deficient mice or expression of connexion-43 was analysed in GDF9 deficient mice. The results for these studies showed that the gap junction coupling between granulosa cells was not required to maintain the expression of GDF9, and growth factor GDF9 alone was not required to maintain gap junction coupling among granulosa cells.

Studies with IGF-1 have been shown to increase the number of gap junctions in preantral follicles (Zhao et al., 2001) and related *in vitro* studies suggest that this growth factor was essential for early antral follicular development (Zhao et al., 2000) (see Figure 32). Therefore, the possible role of insulin-like growth factor-1 (IGF-1) at both low (i.e. 1.7ng/ml) and high (i.e. 10ng/ml) concentrations on the gap junction activity of the rat COC was examined. The results show that IGF-1 was unable, at either low or high concentrations to modulate dye transfer rates in the rat COC. In comparison to the findings of Zhao et al., (2001) on the gap junction number in preantral follicles in this thesis, the effect of IGF-1 was observed on the dye transfer from the surrounding cumulus cells into the oocyte via gap junctions in an antral follicle. In rats, IGF-1 causes FSH-induced granulosa cells proliferation and differentiation in pre-antral rat follicles (Adashi et al., 1990). Thus one can assume that IGF-1, by itself, was unable to modulate the gap junction activity of the rat COC. In contrast to this, when we looked at the combined effect of IGF1

with testosterone and forskolin, the dye transfer via gap junctions from cumulus cells to oocyte was increased.

Oestradiol was found to increase the expression of Cx43 gap junction protein in the rat myometrium cell line (Piersanti and Lye, 1995). Oestradiol 17β effects the development of pre-ovulatory follicular development (see Figure 32). Thus here we have observed the effect of oestradiol 17β on the gap junction assay of rat COC. Results show that oestrogen 17β was unable to modulate the gap junction activity between cumulus cells and the oocyte. This could be because oestradiol exerts different effects on different cell complexes. In an ovarian follicular development, oestradiol is produced during the late follicular phase, when the follicle possesses all the steroidogenic enzyme systems and being stimulated by both FSH and LH (Carson et al., 1981). Thus, preovulatory follicles have very high intra-follicular levels of oestradiol (Hillier, 1981, McNatty, 1982). However, the present study suggests that oestradiol alone is unlikely to influence oocyte-cumulus cell gap junction activity. In the present study, the oestradiol concentration used (13.8μM) was ~ two fold higher than that normally found in human or bovine follicular fluid (7.35μM) (McNatty, et al., 1984). No data are available for concentrations in the rat follicular fluid. However, even at this high concentration oestrogen was unable to modulate gap junction activity of rat COC. Its worth noting that previous studies have shown that exogenous oestradiol inhibits the decrease in the number of gap junctions within ovarian follicles (Burghardt & Matheson, 1982). Moreover, the treatment of sexually immature rats with oestrogen implants leads to an increase in Cx43 mRNA and protein expression (Wiesen & Midgley, 1994). However by themselves, these results neither support nor refute the findings from our study. Bley et al., (1997) reported that oestradiol and FSH together increases granulosa cell proliferation rate and that this effect was further enhanced by IGF1. In future it would be worth testing the combined effects of oestradiol and growth factors such as IGF-1 and/or FSH/forskolin on the gap junction activity in rat COC *in vitro*.

Androgen mRNA and protein expression are abundantly found in rodent ovaries (Hirai et al., 1994, Tetsuka and Hillier et al., 1996). Therefore, COC are likely to be exposed to relatively high concentrations of androgen (i.e. androstedione and/or testosterone) during follicular development. These androgens are also the major substrates for oestradiol synthesis (Carson et at., 1981). Testosterone affects early follicular development (see Figure 32); thus here we have observed the effect of steroid testosterone on gap junction assay of rat COC. Results show that incubation of the rat COC with testosterone had no significant effect on the transfer rate of calcein dye from the cumulus cells via gap junctions to the oocyte. Therefore, this result shows that testosterone by itself was unable to modulate gap junction activity of rat COC. In this study, the concentration of testosterone was physiologically about four times higher than that found in the follicular fluid (i.e 13.8 v 3.47µM) (McNatty et al., 1984) of humans. No data are available for rats. It is possible that effects might be observed at lower concentrations but this was not investigated. Testosterone is known to increase the murine follicular responsiveness to FSH by increasing the cAMP levels (Wang et al., 2001). We tested the combined effect of testosterone and forskolin (as a cAMP stimulator) on the gap junction activity of the rat COC. The results show that forskolin together with testosterone were unable to modulate the transfer of calcein dye to oocytes from cumulus cells via gap junctions. In comparison to that, the combined treatment of IGF-1, forskolin and testosterone caused a significant increase in transfer of dye via gap junctions from the cumulus cells to the oocytes compared to testosterone plus forskolin and testosterone treatment alone. These results were consistent with other studies that testosterone, FSH and IGF-1 all enhance the aspects of follicular development. For example, *in vitro* treatment of primate ovaries with testosterone or DHT resulted in an increase in IGF-1 mRNA and IGF-1 receptor mRNA (Vendola et al., 1999 a, b) in rodents, the combined treatment of androgen and FSH stimulates granulosa cell proliferation *in vitro* and this effect was further amplified by insulin or IGF-1 (Bley et al., 1997). Follicular development is a complex procedure

controlled by different growth factors, hormone, steroids and chemical agents. At any stage of follicular development, multiple factors are responsible for controlling development to the next stage of growth. Different factors acting on follicular cells may increase or decrease the gap junction activity of rat COC. They act by altering the transcription rate of connexion, altering connexin RNA, connexin protein levels (Traub et al, 1987, Schiller et al, 1992), connexin trafficking to and from the cell membranes (Atkinson et al, 1995; Paulson et al, 2000), modifying the conductance of the gap junction channels (Saez et al, 1986) or by altering connexin phosphorylation states (Traub et al, 1987; Saez et al, 1986).

CHAPTER 5 CONCLUSION

In conclusion, in this study we have successfully developed a method to measure the gap junction activity within the rat cumulus-oocyte complexes. This method has allowed us to determine the effects of different chemical agents, growth factors and steroids on the rate of calcein dye transfer via gap junctions from cumulus cells to oocytes in rat COC *in vitro*. Isolation of rat COC from their follicular environment appears to impair the gap junction activity within cumulus-oocyte complexes as assessed by the measurement of dye transfer but this activity was recovered over time *in vitro*. Under the *in vitro* conditions employed, dbcAMP, forskolin and milrinone previously thought to stimulate or maintain intracellular cAMP levels within COC, were all unable to modulate the dye transfer via gap junctions between rat cumulus cells and oocyte. However, minor changes in pH were shown to influence the gap junction activity whereas the oocyte-derived growth factors GDF9 + BMP 15, IGF-1, or steroids such as testosterone or oestradiol 17β, by themselves, were unable to modulate the dye transfer via gap junctions. However, important findings were that testosterone and forskolin together or testosterone, forskolin and IGF-1 together significantly enhanced dye transfer via gap junctions between cumulus cells and the oocyte. Thus, it seems that the coordinated actions of more than one factor are responsible for regulating the gap junction activity during ovarian follicular development. In future, further optimization of the gap junction assay is likely to assist in improving *in vitro* maturation of oocyte's quality for subsequent IVF procedures.

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APPENDIX A STAINING PROTOCOL

1.0 Orcein dye

To prepare the orcein dye, 0.5g orcein powder was dissolved into 22.5ml acetic acid. The mixture was than stirred with gentle heat for 1hour. The mixture was then added to 27.5ml dH_2O to make a final volume of 50ml. Finally the mixture was filtered twice using a filter paper before it was used for staining the cells.

2.0 Clearing solution

The clearing solution was composed of one part glycerol, three parts dH_20 and two parts acetic acid.

3.0 Oocyte fixative

The oocyte fixative was prepared by adding three parts ethanol and one part acetic acid.

4.0 Fixing the oocyte on the slide

The denuded oocytes were then transferred into another well containing ~ 400µl of L-15 dissection medium supplemented with the 100µM milrinone. A few drops of oocyte fixative were added to the medium until the medium turned yellow. The plate was sealed using parafilm and stored at 4ºC for 24 hours after which the oocytes were mounted on an ethanol washed slide using a finely drawn mouth pipette. A minimum amount of medium was transferred during this process with excess medium being removed using a mouth pipette, making sure that the oocytes did not dry out. Vaseline was applied to the two edges of a cover slip which was placed on the slide, making sure that the vaseline side was down. The cover slip was gently pressed down gently, while observing the oocytes under the dissection microscope. This technique allowed the oocyte to bulge but not rupture. A small quantity of the fixative was drawn from the side of the cover slip free of vaseline to

confirm that the oocyte had not dried out. A glued side of the cover slip was sealed with clear nail polish. Labelled slide was place in a coplin jar containing ethanol acetic acid fixative solution for 18 hour.

5.0 Staining the slide

After removing the slide from the coplin jar. In order to stain the slide, stain was passed under the cover slip along its unsealed edge, using a syringe. The slide was then kept flat on a bench for 1-2 hours. Clearing solution was used to de-stained the slide in the same way as the staining. Once the slide was dry, the open edges of the cover slip were sealed using clear nail polish. The meiosis status of the oocytes was observed using DMI 4000 inverted microscope at 400x magnification.

APPENDIX B

Treatments	Stock conc.	Conc. in use	Company	Country
Milrinone	1mM	100µM	Sigma/Aldrich	New Zealand
Forskolin	10mM	100µM	Sigma/Aldrich	New Zealand
cAMP	10mM	100µM	Sigma/Aldrich	New Zealand
17-β Oestradiol	3.65mM	2.94µM	Sigma/Aldrich	New Zealand
BMP15	5×10^{-6}mM	5×10^{-7}mM	Ag research	New Zealand
GDF9	5×10^{-5}mM	5×10^{-6}mM	Ag research	New Zealand
Testosterone	3.47mM	1.388µM	Sigma/Aldrich	New Zealand
IGF-I	0.131mM	0.000225µM, 0.00131µM	Novazymes	Australia
Carbenoxolone	0.3mM	100µM	Invitrogen	New Zealand
Hepes Buffer	1000mM	15mM	Sigma/Aldrich	New Zealand
PVA	0.3mg/ml	15mg/50ml	Sigma/Aldrich	New Zealand
Sodium Pyruvate	0.23mM	1.14mM	Sigma/Aldrich	New Zealand
Penstrep	10,000IU	100IU/ml	Invitrogen	New Zealand
Glutamax	200mM	2mM	Invitrogen	New Zealand
Calcein-AM	10mM	1µM	Invitrogen	New Zealand
BSA	4mg/ml	0.1%, 0.4%	IOCP	New Zealand
1-Octanol	2000mM	1mM,2mM, 4mM,8mM	Sigma/Aldrich	New Zealand

Media	Company	Country
M199	Invitrogen	New Zealand
Lebowitz (L15)	Invitrogen	New Zealand
PBS	Sigma	New Zealand
Serum	Ag Research	New Zealand