The role of steroids, growth factors and cAMP stimulators on the gap junction activity in cumulus oocyte complexes in the rat

By

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Abstract

Bidirectional communication between mammalian oocytes and their surrounding somatic cells is essential for oocyte maturation. Gap junctions promote the transfer of essential metabolites, nucleotides, amino acids and ions from cumulus cells to the oocyte that are crucial for oocyte growth and development. However, the range of factors present in the microenvironment of the developing antral follicle, which modulate gap junction activity of the cumulus-oocyte complexes (COCs), is largely unknown.

The primary objective of this study was to determine the effects of various steroids, growth factors and cAMP stimulators on the gap junction activity in rat COCs. The gap junction activity was measured in presence or absence of different treatments using a fluorescence dye and in the presence of milrinone, a phosphodiesterase type 3 inhibitor.

The major findings of this study were that cAMP stimulators increased the rate of dye transfer from cumulus cells to the oocyte. Under in vitro conditions it was established that neither steroids nor IGF1 by themselves were able to modulate gap junction activity in rat COCs. Furthermore, forskolin, a potent cAMP stimulator; caused a relative increase in Cx37 gene expression levels following a four hours incubation period.

The outcomes from the present study may help to provide new insights into developing suitable in vitro conditions, for the in vitro maturation of mammalian oocytes.

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List of Abbreviations

COC	Cumulus-oocyte complex
IVM	In-vitro maturation
IVF	In-vitro fertilisation
CC	Cumulus cells
MGC	Mural granulosa cells
GnRH	Gonadotrophin-releasing hormone
LH	Luteinising hormone
FSH	Follicle stimulating hormone
Cx	Connexin
GVBD	Germinal vesicle breakdown
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
AC	Adenylate cyclase
PDE	Phosphodiesterase enzyme
PBS	Phosphate buffered saline
PVA	Polyvinyl alcohol
BSA	Bovine serum albumin
BMP15	Bone morphogenetic protein 15
GDF9	Growth differentiation factor 9
IGF1	Insulin growth factor 1
E	Oestradiol
T	Testosterone
F	Forskolin
A	Androstenedione
P	Progesterone
dcAMP	Dibutryl cyclic adenosine monophosphate
Mins	Minutes

INTRODUCTION

1.1 In Vitro Maturation

I*n-vitro* maturation (IVM) is a process which involves retrieving immature cumulus-oocyte cell complexes (COC) from ovarian antral follicles and subjecting these to a maturation process before undergoing an in vitro fertilisation procedure (IVF). For IVM, the COC are usually recovered under the guidance of an ultrasound procedure from donors who are not subjected to a course of exogenous hormone treatments that normally occurs with the IVF procedure. Thereafter, the COC are matured in a defined culture medium for 24-48 hours (1). During this interval the oocytes mature, resume meiosis and the cumulus cells lose their attachments with one another and the oocyte. The resulting oocytes that have reached metaphase II after this time are then fertilised by adding sperm to the culture wells or by a process known as intracytoplasmic sperm injection (ICSI). Two to three days after fertilisation, selected embryos are transferred to the donors. While IVM has a greater laboratory-based commitment it is considered to have the potential to be more cost-effective, safer and less time consuming than current IVF procedures.

In a standard IVF treatment, the women donors are subjected to two weeks of hormone injections to stimulate maturation of multiple ovarian follicles. IVM avoids the use of multiple hormone treatments, involves a reduced level of monitoring of patients and is therefore less costly and demanding for the donor (1). It also has the potential to utilise large numbers of immature oocytes from developing antral follicles. As well as being time-consuming and expensive, a potentially serious side-effect of hormone injections in IVF is a condition known as ovarian hyperstimulation syndrome (OHSS) caused by to excessive ovarian response to exogenous hormones

(1). Furthermore, IVM potentially offers a safer treatment for women with Polycystic Ovary Syndrome (PCOS). Women with PCOS have a tendency to have a vigorous response to ovarian stimulation and are at significant risk of OHSS. A milder stimulation treatment as envisaged by IVM would be expected to reduce this risk (1). Thus, IVM is considered to be the next important addition to the portfolio of artificial reproductive technologies as it has the potential to utilise large numbers of immature oocytes from developing antral follicles.

Pregnancy rates following IVM are reported to be variable (4-54%) and generally lower than IVF (1, 2). Nevertheless, more than 300 children have been born worldwide through IVM treatment (2, 3). With IVF, delivery rates (i.e. of live-born), up to 59% have been achieved for fresh and frozen transfers following IVF (4) whereas in IVM, significantly lower delivery rates of around 13% have been achieved thus far (2).

To increase the efficacy of IVM technology, one great challenge is to understand the role that the ovarian follicular microenvironment surrounding the oocyte plays in its progress to become fully competent (5). IVM differs to natural *in vivo* oocyte maturation in two ways: Firstly, COC are collected from antral follicles that include oocytes that have not completed capacitation as oocytes only acquire meiotic and developmental competence during the final stages of folliculogenesis (5). Thus, premature recovery of COC from immature antral follicles hinders the progression of oocytes to acquiring meiotic and developmental competence (5). Secondly, removing COC from their follicular microenvironment results in loss of the natural meiotic inhibiting factors which can result in spontaneous meiotic maturation of the oocyte *in vitro* before competence has been acquired (5). The loss of these meiotic inhibitory factors leads to premature breakdown of gap junctions in COC, in

turn causing loss of beneficial cumulus cell metabolites, such as ions, amino acids and nucleotides needed for oocytes in their progress to acquiring meiotic competence (6).

Thus a key challenge to enhance IVM efficacy involves improving oocyte developmental competence by maintaining the integrity of the COC, delaying spontaneous nuclear maturation and providing the appropriate *in vitro* culture microenvironment to ensure that the oocyte is able to acquire meiotic competence before attempting IVF (5). Theoretically, the objective of this approach is to increase oocyte-cumulus gap-junctional communication in vitro to allow ooplasm development since gap junction mediated transport of molecules initially taken up by the granulosa cells is essential for metabolic processes crucial for oocyte growth and cytoplasmic development. The question arises as to what are the key factors present in the follicular microenvironment that are important for influencing gap- junction activity in COC.

1.2 Follicular development

1.2(i) The Mammalian Ovary

The female reproductive system includes two ovaries and a reproductive tract with uterine tubes, uterus and vagina. The ovaries are almond- sized organs in the upper pelvic cavity, one on each side of the uterus. The ovary is covered normally by a single layer of cells which constitutes the surface epithelium. The two main morphological regions of the ovary are referred to as the cortex and medulla. The cortical region encompasses most, if not all, of the growing and non-growing follicles (Figure 1) and corpora lutea separated by stromal or interstitial cells and blood capilliary vessels. The medulla incorporates all the innermost parts of the ovary and the hilum by which the ovary is attached, and through which the blood, nerves and lymphatic vessels enter (7).

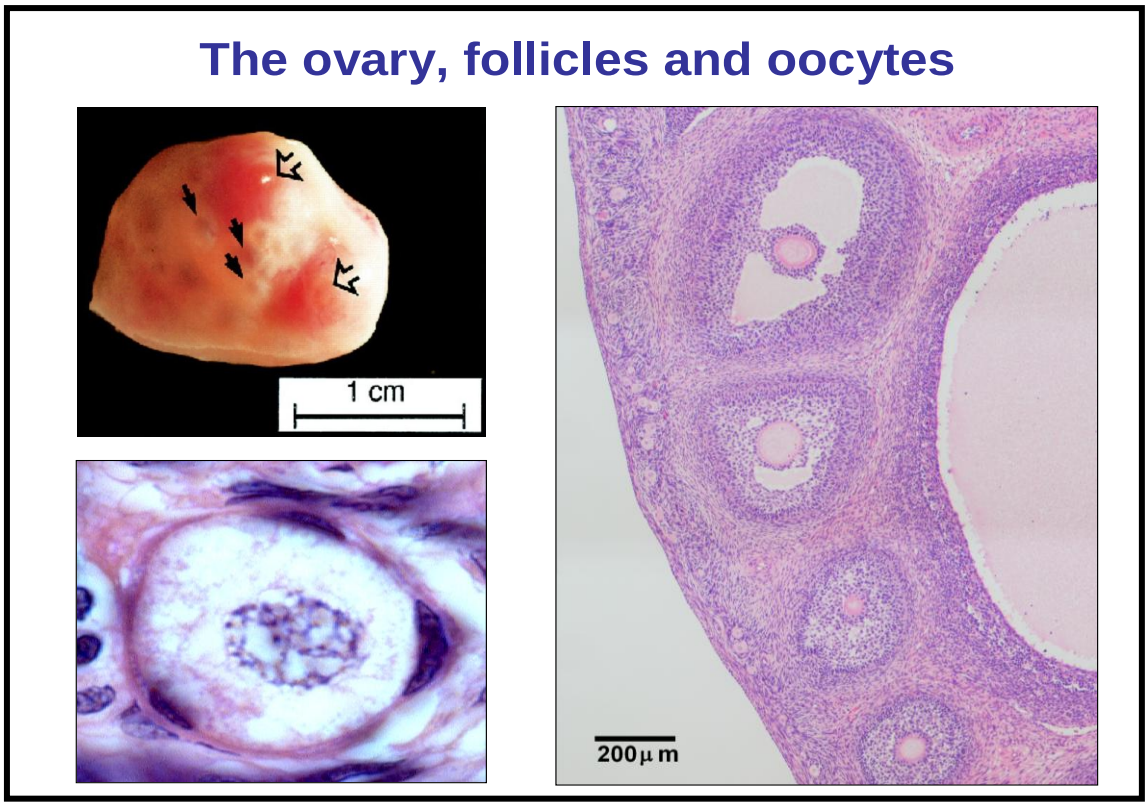

Figure 1.1:- The sheep ovary (upper left) with solid arrows indicating surface visible follicles and open arrows indicating corpus luteum, primordial follicle (lower left) showing a large oocyte and centrally-located nucleus with three darkly-stained granulosa cells around the periphery of the oocyte, and section though the ovarian cortex (right hand image) showing preantral follicles immediately below the ovarian surface epithelium and a series of growing preantral and antral follicles with the uppermost antral follicle displaying both mural granulosa cells and cumulus cells immediately adjacent to the oocyte surrounded by follicular fluid. On the extreme right hand side a large antral follicle is partly visible with the mural layer of granulosa cells and a large fluid-filled cavity (Figure supplied by Professor Ken McNatty).

1.2(ii) Development of the Oocyte: Oogenesis

In most mammals (e.g. primates, ruminants, rodents) oogenesis begins during fetal development. At birth or shortly afterwards, females will contain all the germ cells (oocytes) she will ever have during her lifetime. In human females, the number oocytes (i.e. often referred to as eggs) have been estimated to be between 2-4 million (8). Out of this vast population of eggs, only a few are destined to be ovulated with all others undergoing degeneration at some point in their development. During early fetal development (Figure 1.2), oogenesis begins with primordial germ cell formation and involves a series of transformations, from primordial germ cells to oogonia and then to oocytes (9).

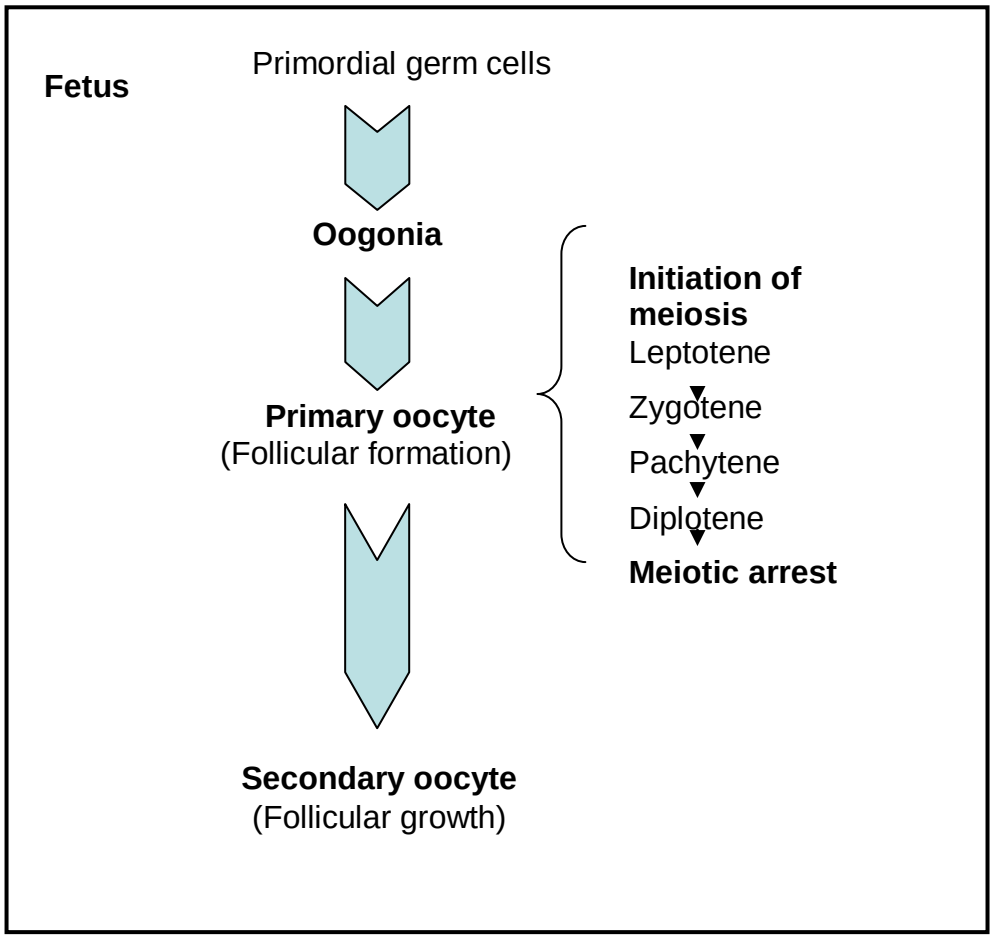

Figure 1.2:- Representation of oogenesis in mammals

Oocytes originate from embryonic stem cells which differentiate into primordial germ cells. During early fetal development in humans and ruminants, primordial germ cells differentiate further to oogonia which exhibit a high frequency of mitotic division. During this period oogonia recruit adjacent mesenchymal and surface epithelial cells which are the precursor granulosa cells (10, 115). Around the end of the first trimester, the oogonia enter meiosis, cease dividing and during preleptotene transform into primary oocytes. This preleptotene interphase marks the initiation of first meiotic prophase. At this time, the oocyte-pregranulosa cell complexes form a basement membrane that isolate them from adjacent oocyte-pregranulosa cell complexes enclosed within extensive numbers of ovigerous cords. These isolated oocyte-pregranulosa cell complexes are referred to as primordial follicles. These primary oocytes in meiotic arrest will eventually progress through leptotene followed by zygotene and pachytene stages of the first meiotic prophase (9). However, completion of this first meiotic division does not occur until shortly before ovulation during adult life. All human oocytes within ovarian follicles during the first stages of meiosis contain 46 chromosomes (9). As follicles form in fetal life, ovarian follicles begin to grow sequentially so that during the third trimester, the ovarian cortex contains oogonia entering meiosis, oocyte-somatic cell complexes within ovigerous cords, primordial follicle formation as well as follicular growth to the early stages of antral growth (10). By contrast, in rodents the sequence of events is the same, except that follicular formation and growth does not occur until the time of birth and in the first few days of post-natal life. During follicular growth, the oocyte undergoes major changes in organelle composition and function. Also, in mice for example, oocytes undergo a 300 fold increase in volume, from a diameter of 12µm to about 80 µm (9, 11).

1.2 (iii) Follicular growth

As mentioned previously, follicles form as structures known as primordial follicles. These primordial follicles each consist of a primary oocyte surrounded by a single layer of flattened cells known as granulosa cells and this entity is bounded by a continuous basement membrane (see also Figure 1.1).

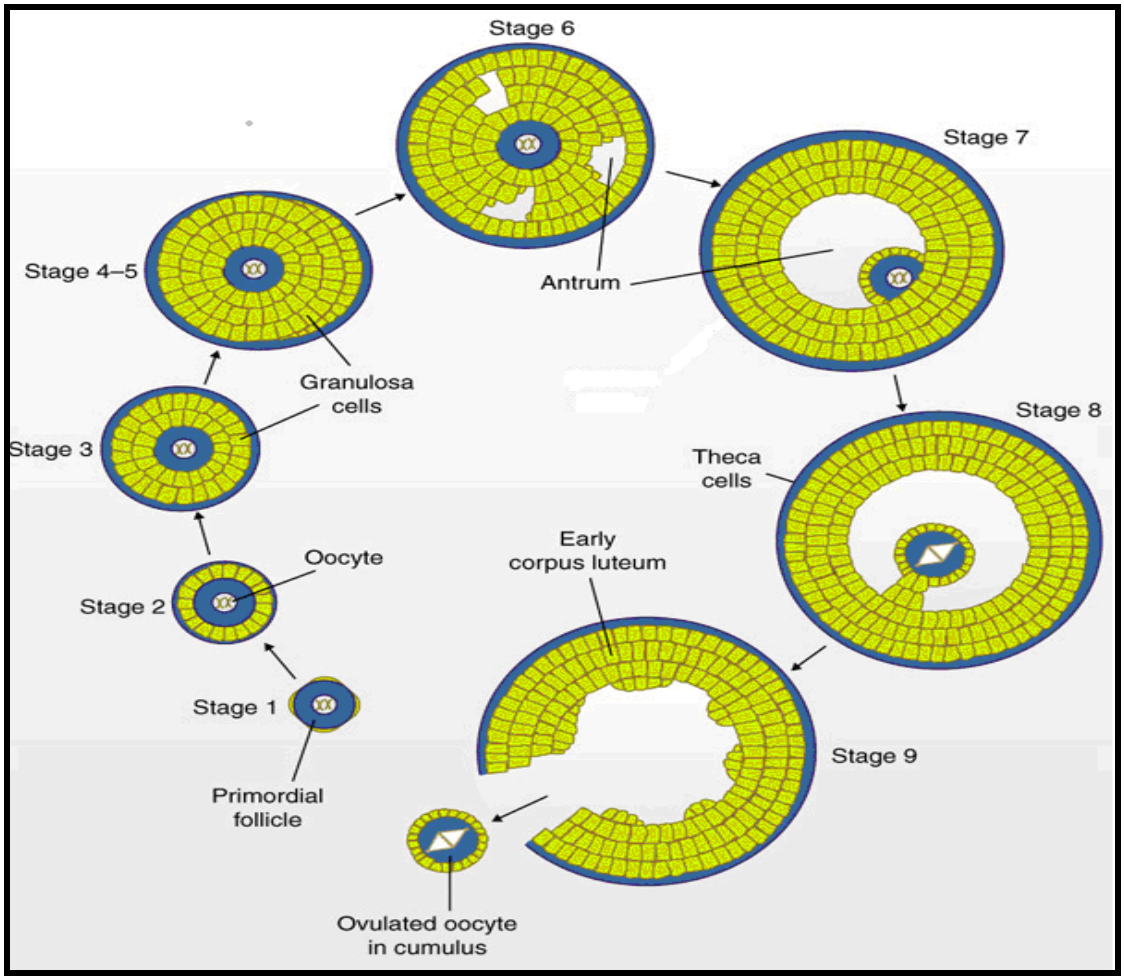

Figure 1.3:- Development of ovarian follicle in a rodent (Modified from 12).

In the rodent, the primary follicle is developmental stage 2; the preantral phases are stages 2-6 and the antral phase's stages 7-9. The blue ring immediately outside the granulosa cell layers represents the basement membrane (basement membrane surrounding primordial follicle not shown). Growing follicles also include a cell layer

immediately outside the basement membrane (not shown) and this cell-type is referred to as theca interna cells.

The growth of primordial follicles into mature ovulatory follicles is a process referred to as folliculogenesis. During folliculogenesis, ovarian follicles progress through a series of defined developmental stages that are classified as the primary, secondary (pre-antral) and tertiary (antral) growth stages (12; see Figure 1.1 and 1.3). As follicular growth is initiated, the flattened granulosa cells become cuboidal (13) and proliferate by mitosis to form multiple concentric layers around the oocyte. By the secondary follicle stage, somatic cells immediately outside the basement membrane of the follicle differentiate to form cells known as theca interna. As increasing numbers of concentric layers of granulosa cells form, proteoglycans secreted from these cells lead to pockets of fluid accumulation (Figure 1.3) (14). As a result of these secretions, an enlarged fluid-filled space, called the antral fluid, forms amongst the granulosa cells. As the antrum expands, the granulosa cell layers immediately surrounding the egg differentiate into cumulus cells (CCs) and the cells lining the follicle wall develop into the mural granulosa cell (MGC) phenotype. These events lead to the formation of a tertiary or antral follicle.

In humans, the progression of primordial follicles to the preantral and early antral stages occurs sequentially beginning in fetal life and continues throughout childhood, the menstrual cycle and even pregnancy until the primordial pool of follicles is depleted around the menopausal interval. Once follicles start to grow they continue to grow until they die (atresia) or ovulate (15). Thus although most follicles in the ovaries are present as primordial follicles, there are also numerous follicles present in the primary, preantral and antral growth stages (9).

1.2 (iv) Hormones and the regulation of ovarian follicular development

Follicular development from primordial to the secondary stage of growth is essentially gonadotrophin-independent and involves enlargement of the oocyte, granulosa and theca cell proliferation. During preantral development, granulosa and theca cells acquire receptors for and develop a responsiveness to gonadotrophins but the presence of gonadotrophins is not essential for follicular growth at this time. However, from the secondary stage onwards the follicular growth becomes increasingly dependent upon the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH) (16, 17). FSH and LH are synthesized and secreted by the anterior pituitary gland following stimulation by gonadotrophin-releasing hormone (GnRH) which is, in turn, synthesised and secreted by the hypothalamus. LH and FSH are relatively large glycoproteins composed of a common alpha but different beta subunits. The alpha subunit contains 92 amino acid residues with five disulphide bonds and two carbohydrate moieties. The FSH beta unit is composed of 111 amino acids, 6 disulphide bonds and carbohydrate moieties which are N- linked at asparagine residue (18). LH has a beta subunit of 121 amino acids. The unique beta subunit of FSH and LH endows each hormone with the ability to bind its own receptor.

The two distinct lineages of granulosa cells that arise during folliculogenesis, namely the MGC and CC, exhibit increasingly divergent responses during antral follicular growth and the preovulatory period. This is due to different functions required by these cells. The CCs become subservient to the needs of the oocyte whereas the MGC are more actively involved in steroidogenesis and endocrine signalling (19, 20). Moreover, during preovulatory development, MGC develop more

LH-receptors than the CC enabling the former to undergo luteinisation after ovulation (11, 21). Importantly during the later preantral, antral and preovulatory growth phases CC and MGCs acquire receptors and responsiveness to FSH (22).

There is always a number of developing preantral and early antral follicles in the adult ovary. However, the development of the antral follicles to the preovulatory stage is critically dependent on stimulation by FSH and LH. One key role for FSH is to stimulate granulosa cells to stimulate aromatase (P450) enzyme synthesis. Another key role is to stimulate proteoglycan synthesis to enhance fluid uptake and enlargement of the antrum. LH and thecal cells play key roles in helping granulosa cells produce the steroid oestradiol (see Figure 1.4). LH acts upon the theca cells, stimulating them to synthesize androgens such as, androstenedione and to a lesser extent testosterone. The androgens are secreted into the bloodstream but they also diffuse across into the granulosa cells where they are converted by the aromatase enzyme into oestrogen (23).

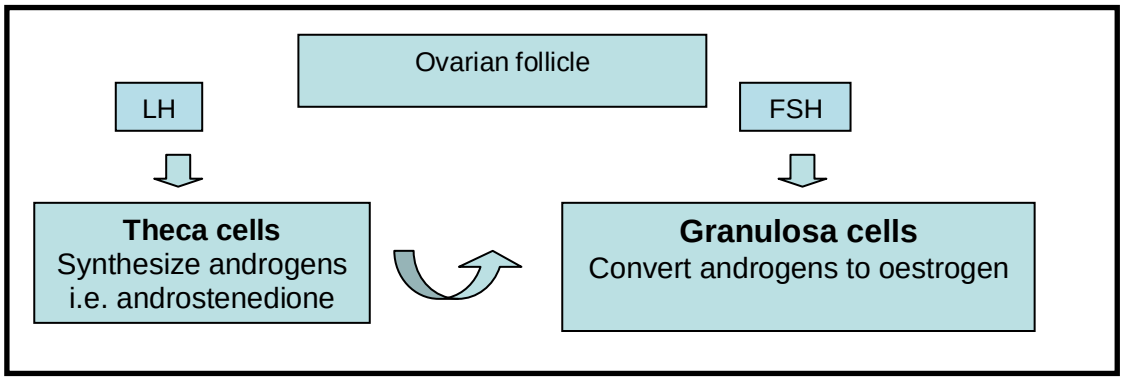

Figure 1.4:- Control of oestrogen secretion in the ovarian follicle

During the initial transition of a small antral follicle into a preovulatory follicle, the level of oestradiol continues to increase concomitant with increasing numbers of both granulosa and thecal cells and an increasing sensitivity of these cells to FSH and LH. As a result, plasma FSH concentrations decline through the negative feedback effects of oestradiol on FSH release. Consequently, with declining FSH only

the largest and most responsive follicle(s) continue to be able to respond to FSH whereas all the smaller antral follicles are unable to maintain their growth and undergo atresia. Thus, the declining FSH concentrations results in only the largest most gonadotrophin-sensitive (i.e. dominant) follicle(s) able to survive to continue growth to ovulation.

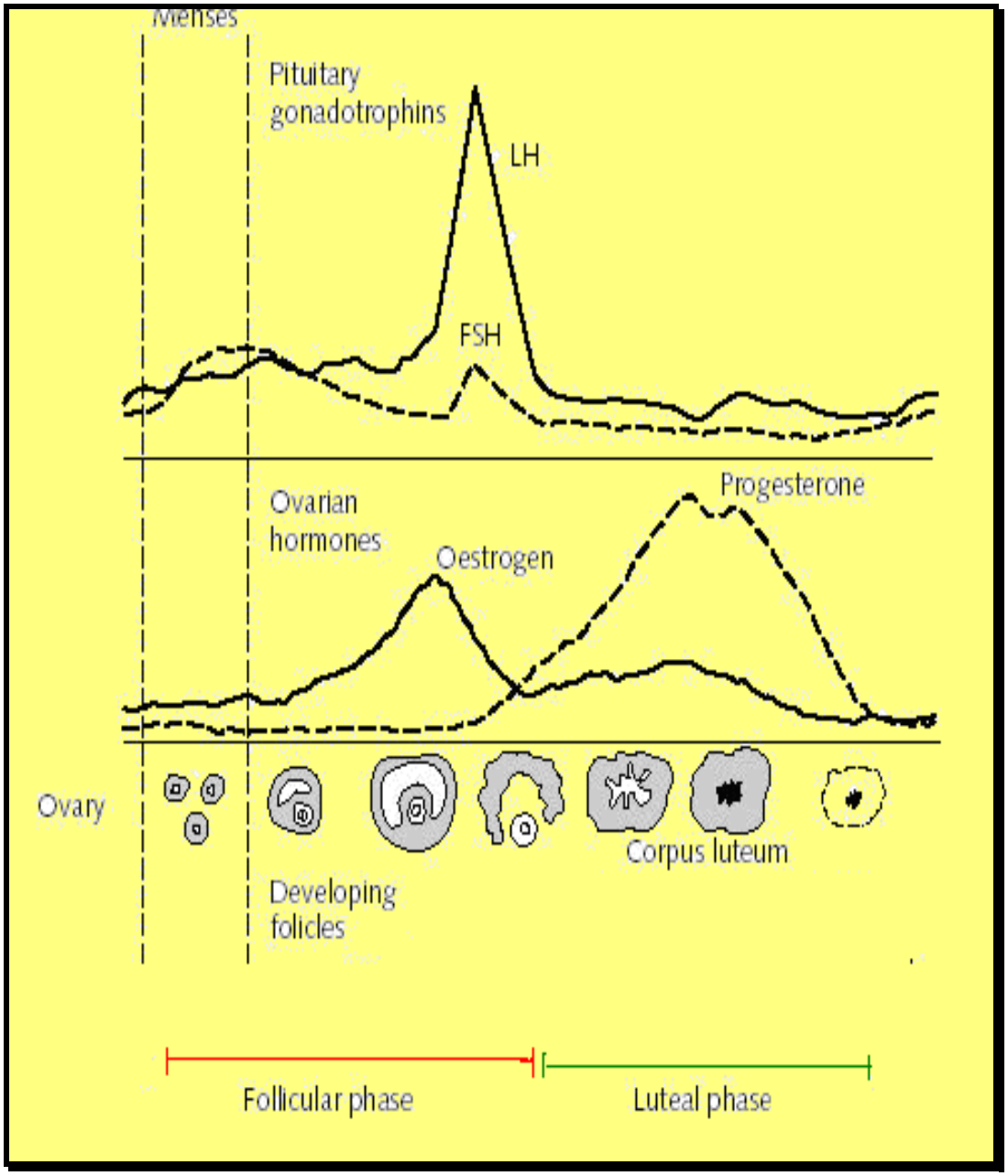

Figure 1.5:- Changes in hormonal concentrations and impact on follicular development in humans (Modified from 26)

In addition, during this critical period, granulosa cells in the dominant follicle acquire LH receptors in response to increasing responsiveness to FSH and aromatase activity. Thus, granulosa cells now begin to be stimulated not only by FSH but also by LH (24). In this way, FSH and LH have profound influences on the proportion of follicles which can either undergo ovulation or atresia (25). Eventually, the dominant follicle starts to secrete enough oestradiol to cause a significant rise in the plasma concentration of this steroid. In turn, this increase in plasma oestradiol exerts a positive-feedback effect on the pituitary release of both FSH and LH (see Figure 1.5). The net result is that, as the rate of follicular oestradiol secretion reaches a critical threshold during late follicular phase, it causes a major surge of release of LH (and FSH). As a consequence of this gonadotrophin surge, the oocytes resumes and completes its first meiotic division and also cytoplasmic maturation and an irrevocable chain of events is initiated that lead to follicular rupture, release of the matured oocyte and luteinisation of the MGC. This is followed by the formation of the corpus luteum (CL) and in humans an increase in both the plasma concentrations of progesterone and oestradiol. The CL-derived secretions of progesterone and oestradiol cause a decrease in secretion of gonadotrophins by the pituitary gland which in turn, prevents the growth of large antral follicles during the luteal phase (Figure 1.5). However, if pregnancy doesn’t occur, the corpus luteum degenerates leading to a rise in gonadotrophin secretion and the stimulation of growth of follicles beyond the small antral stages.

1.3 Role of steroids in follicular development

As indicated in Figure 1.5 and in the previous section, ovarian follicles synthesize and secrete several steroid hormones such as progestins, androgens and oestrogens. The major precursor is cholesterol and a key intermediatory is pregnenolone for the synthesis of these steroids. Progesterone is the major progestin produced by the ovary and is synthesised mainly by the corpus luteum and also by the follicular granulosa cells shortly before ovulation in some species. Androstenedione and testosterone are the two major androgens present in ovary. Androstenedione is a biologically weak androgen and a major precursor for the more potent testosterone or for the synthesis of oestradiol. The follicle has the highest levels of androstenedione and oestradiol-17β during its final stages of growth i.e. antral follicle due to the size of its granulosa cell population and capacity for androgen aromatisation (Figure 1.5; 27).

In vitro culture of mouse pre-antral follicles in presence of testosterone showed significant increase in follicular diameter due to more granulosa cell proliferation (28). By contrast when follicles were cultured with an anti-androgen or an androgen receptor antagonist they grew at a relatively slower rate (29, 28) whereas the addition of androstenedione caused an accelerated rate of growth. Mice lacking an androgen receptor had a reduced ovulation rate. Ovulatory genes such as HAS2 were not induced and as a result the COC released at ovulation had a disorganized structure, suggesting role of androgen receptor in ovulatory process (30). Overall, it seems evident that androgens play a role in regulating follicular development.

Oestrogens act on target cells via the receptors, ERα and ERβ. Mice knockout studies suggest that knockout of these receptors lead to infertility in mice. ERβ receptor knock out mice show a partial loss of fertility due to fewer growing follicles

arrested in the early antral stage and the ovaries being smaller in size (31, 32). ERα knock out mice showed evidence of ovulation inhibition and increased proliferation of granulosa cells (33). Thus as with androgens, there is compelling evidence to suggest that oestrogen is important for normal follicular development.

The effect of steroids on mammalian oocyte maturation has also been studied. It has been postulated that although the gonadotrophin-induced meiotic response of the mammalian oocyte is not steroid-mediated (34), that some steroids may influence oocyte maturation (35). Oestradiol-17β, progesterone, androstenedione and testosterone have all been shown to inhibit meiotic maturation in mice (35), and oestradiol to inhibit spontaneous maturation of porcine oocytes (36). Progesterone has been reported to inhibit mouse and rabbit (37) oocyte maturation, but not to affect maturation of human (38) or porcine oocytes (36). It remains uncertain as to whether the very high concentrations of steroids that may be present in follicular fluid are influential in effecting the bi-directional communication systems including gap junction functions between MGC and CC or between the CC and oocyte.

1.4 Gap Junctions

In the previous section, attention was focussed on the roles of gonadotrophin and steroidogenesis during follicular development. This largely involved the MGC and thecal cells. Here the focus is on the cell networks within the follicle including that for the COC. Intercellular communication is an essential aspect of ovarian folliculogenesis (39). The granulosa cells communicate with each other and also with the oocyte via a network of gap junctions (13, 40). This network of gap junctions unites the mural and cumulus granulosa cells with the oocyte which assists in

coordinating the timing of oocyte development with that of somatic cell proliferation and differentiation.

During the early stages of follicular growth, signalling between developing oocytes and the surrounding granulosa cells is facilitated by transzonal projections from the granulosa cells that contact the oocyte surface (41). These projections from granulosa cells that traverse the zona pellucida and terminate on the oocyte cell surface are reported to be most abundant in preantral follicles (41). In rat, rabbit and sheep, the expression of gap junction genes and the presence of the proteins in granulosa cells and oocytes are detectable from the primary stage of follicular growth (McNatty & Bibby, unpublished data). It is likely that gap junctions become extremely important as follicles begin to form an antrum and the appearance of a cumulus cell phenotype becomes apparent (13). There is an increase in the size and number of gap junctions concurrent with granulosa cell proliferation and oocyte growth (42).

Gap junctions are specialized intercellular channels responsible for the transfer of low molecular weight molecules between cells with intimate contact (43; see also Figure 1.6). The gap junction channels possess unique structures as they span the plasma membranes of adjacent cells. The basic unit of the gap junction is a hexameric structure known as a connexon. Two connexons docking end-to-end within the extra cellular space results in the formation of a gap junction channel (see Figure 1.6). Each connexon is formed by the oligomerization of the structural protein subunits, connexins (Cx).

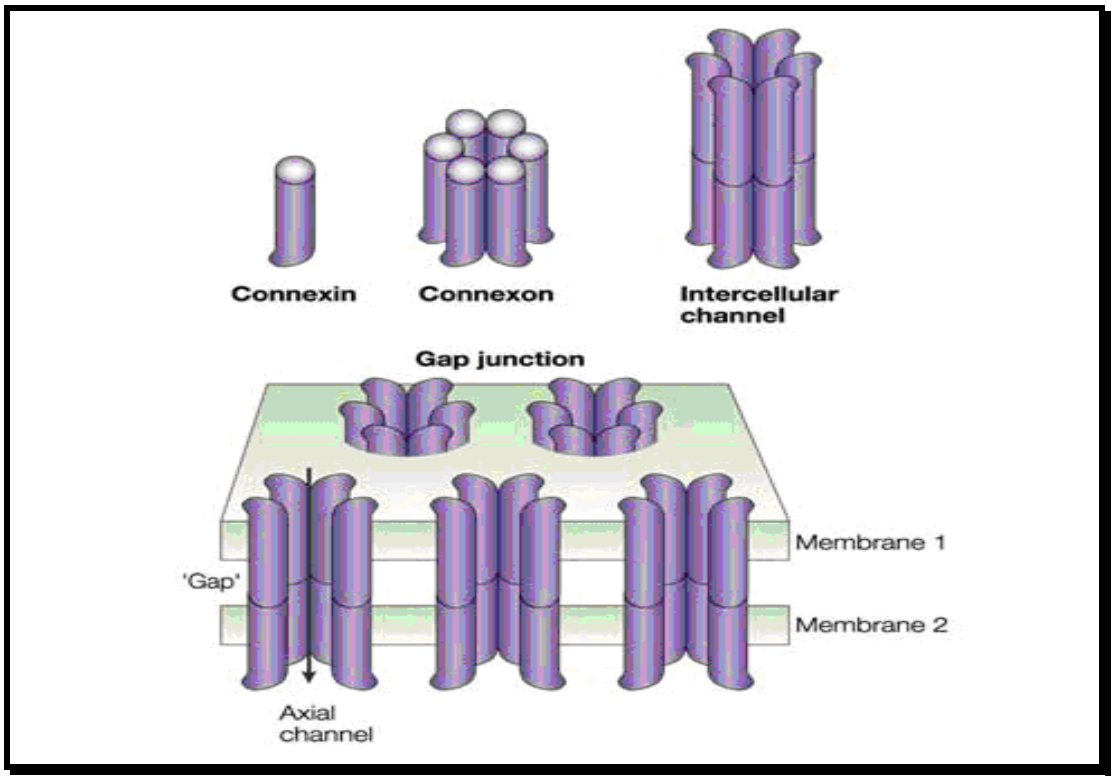

Figure 1.6:- Assembly of connexins, connexons, intercellular channel and gap junction (Adapted from 24)

Connexins comprise a family of homologous membrane proteins. All connexins consist of four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic N and C termini (39). The connexins oligomerize as a connexon, which is then transported to the plasma membrane. In turn, connexons from adjacent cells then interact to form the gap junctions: these cell-to-cell channels generally cluster in specialised regions on plasma membranes. Although there are many connexin (Cx) subtypes expressed in ovarian follicles, it is evident that two of these, Cx 43 and Cx37, are essential for ovarian follicular development (6, 45 and 46). Cx43 is expressed in the granulosa cells throughout folliculogenesis (6, 47) whereas Cx37 is found on the oocyte plama membrane within the zona pellucida (6, 39).

1.5 Importance of Gap junctions in follicular development

Ovarian folliculogenesis and the production of fertilisable oocytes are critically dependent upon gap junctions for intercellular communication. Gap junctions facilitate the transfer of low MW materials (e.g. <1000mer) between cells. Reagents such as amino acids, inorganic ions, second messengers, nucleotides and glucose metabolites are amongst the materials transferred to the growing oocyte from gap junctions (48). Gap junctions also mediate metabolic cooperativity (46). Disruption of gap junctional communication can compromise the oocyte's ability to produce energy from glucose metabolites since the granulosa/cumulus cells have a high capacity to metabolise glucose to pyruvate and to supply the oocyte with this essential metabolite (49). In addition, regulatory signals for protein phosphorylation and dephosphorylation, and signals that control meiotic maturation are regulated by granulosa/cumulus cells and these regulators rely upon gap junctional communication for their specific targeted effects (50).

The importance of gap junctional intercellular communication has been established in connexin knockout mice. Mice lacking Cx37 as a result of targeted mutation in the Cx37 gene show arrested folliculogenesis and lack mature follicles (45). Oocyte development arrests before meiotic competence is achieved and follicles fail to ovulate (45). These results demonstrate that gap junctional communication influences the acquisition of meiotic competence by the oocyte and provides evidence that gap junctions play essential roles in folliculogenesis and oogenesis.

1.6 Oocyte Maturation

In mammals, the first meiotic division is initiated during fetal life and proceeds up to the diplotene stage of the first prophase (51, 52, and 53). Oocyte growth occurs concomitantly with follicle growth, but the oocyte remains arrested at prophase I until a preovulatory surge of LH from the pituitary stimulates meiotic resumption. The fully grown immature oocyte then resumes meiosis, progressing from prophase of the first meiotic division to metaphase of the second meiotic division, where it becomes arrested again. The progression from prophase I to metaphase II is termed oocyte maturation and is a process that includes nuclear as well as cytoplasmic changes that allow the mature egg to be fertilized (53, 54). Only oocytes that have been meiotically matured are capable of being fertilized and undergo development normally (53).

The first structural change that is evident during the resumption of meiosis in oocytes is germinal vesicle breakdown (GVBD) (11, 51). GVBD is followed by chromosome condensation and spindle formation. Separation of homologous chromosomes then occurs, together with asymmetric cleavage of oocyte cytoplasm. Further progression of oocytes from metaphase II requires fertilization.

During follicular growth, oocytes remain in diplotene stage and are incompetent to resume meiosis. The acquisition of meiotic competence occurs during oocyte growth and during the transition from preantral to antral stage; oocytes become increasingly competent (55; see Figure 1.7). In humans, ruminants and rodents, oocytes acquire full meiotic competence and attain full size during the final phases of the menstrual and oestrous cycles respectively.

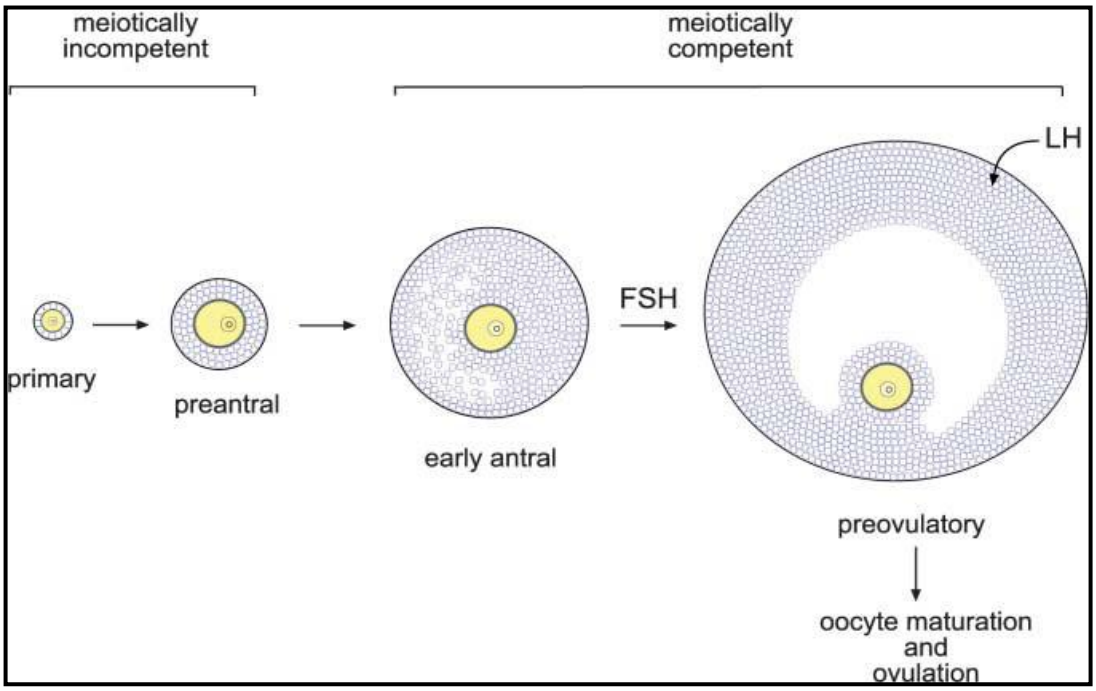

Figure 1.7:- Acquisition of meiotic competence during follicular growth (Modified from 56)

It has been observed that when meiotically-arrested oocytes are removed from the ovarian antral follicles they spontaneously resume meiosis, suggesting that the follicular microenvironment maintains the oocyte in meiotic arrest (57). In vivo, it has been well established that in preovulatory follicles, LH induces a sequence of events leading to the resumption of oocyte maturation (11, 16, and 54). Since LH receptors are located on the mural granulosa cells, but not cumulus cells or the oocyte, the mechanism by which LH stimulates oocyte maturation is indirect (58). It has been hypothesized that LH acts in either removing an inhibitory, maturation arresting substance or it could provide a positive, maturation-promoting substance to the oocyte (51, 52). Among the various explanations as to how LH triggers meiotic resumption, one is that LH causes gap junction degradation which in turn results in reduction in the transfer of meiosis-arresting substances from granulosa and cumulus cells to the oocyte. Hypoxanthine has been shown to be a factor capable of inhibiting meiotic

resumption and is present in follicular fluid of rodents at concentrations of ~2-4mM (59). In the absence of hypoxanthine, meiotically competent oocytes underwent spontaneous GVBD in vitro whereas in the presence of hypoxanthine 95% of competent oocytes were maintained in meiotic arrest (59). Cyclic adenosine 3,5 - monophosphate (cAMP) also functions as an inhibitory signal produced by granulosa and cumulus cells that is transferred to the oocyte through gap junctions (60).

Another hypothesis is that LH doesn't induce sufficient degradation of gap junctions per se but induces a positive signal from granulosa cells to the oocyte to override the inhibitory effects of cAMP, to promote meiotic resumption (52). The sterol, follicular fluid–meiosis-activating sterol (4,4-dimethyl-5a-cholesta 8,14,24-trien-3b-ol; FF-MAS), is a candidate oocyte maturation-inducing substance (52, 61). FF-MAS stimulate the resumption of meiosis in isolated oocytes of various mammalian species including mouse, rat, and human (61). Calcium also plays a role in providing a positive signal, since LH causes an increase in calcium in granulosa cells and this increased level of calcium in oocytes may cause GVBD (62, 63).

Irrespective of the mechanisms involved it is clear that the loss of gap junctional communication between granulosa and cumulus cells and the oocyte is associated with the resumption of meiosis (64, 65). The loss of gap junctions between the cumulus cells and the oocyte will inevitably lead to a reduction in the transfer of meiotic inhibitory substances from follicle cells to oocyte. Just prior to ovulation, there is a significant decrease in the number of gap junctions as well as a decrease in the extent of ionic coupling (6, 65, 66). Ionic coupling and transfer of molecules are maximal before the preovulatory surge and decreased afterwards (67). In this context, LH has been shown to decrease gap junctional permeability between follicular cells, before GVBD (68).

1.7 Regulation of Oocyte maturation

When mammalian COC are recovered from antral follicles and cultured in vitro, they spontaneously undergo meiotic maturation. As mentioned earlier, the follicular microenvironment prevents this premature maturation. It is likely that the recovery of COC from the follicular microenvironment disrupts the extent and viability of gap junctions formerly present between MGC and CC but also between the CC and between the CC and oocyte. This process of recovery of COC and separation from MGC is likely to cause a loss of cAMP synthesis and thus a reduction in delivery of this factor to oocytes.

1.7 (i) cAMP

The level of cAMP in tissues or biological fluids is regulated by two major enzymes; adenylate cyclase (AC) and phosphodiesterase (PDE) that respectively catalyze the synthesis and degradation of cAMP respectively. Adenylate cyclase catalyzes the conversion of ATP to 3', 5'-cyclic AMP (cAMP) and pyrophosphate. The resulting cAMP acts as a second messenger by interacting with and regulating other proteins such as protein kinase A (PKA) and cyclic nucleotide-gated ion channels. Cyclic AMP acts by activating the cellular type I and II PKA pathways and in turn the timing of oocyte meiotic maturation (59).

Cyclic AMP can be generated within the oocyte and/or transferred to the oocyte from the CC via gap junctions (67). The evidence suggests that the CC are the major contributor to the total cAMP content of the oocyte and thus cAMP within the oocyte is maintained at high levels through transfer from somatic cells. As mentioned earlier, high levels of cAMP have shown to prevent meiotic resumption (70, 71). The high levels of cAMP maintain PKA in an active state which causes phosphorylation

of protein substrates. These phosphorylations are inhibitory to oocyte maturation. A fall in intracellular cAMP levels signals reentry of the oocytes into meiotic progression and a decrease in cAMP levels of the oocyte occurs just before GVBD in vivo and in vitro (72, 73). Under low cAMP conditions, the oocyte phosphoproteins would be dephosphorylated and meiotic maturation would be initiated.

Therefore, it is likely that cAMP is continuously transferred to the oocytes to maintain meiotic arrest. However, during the LH surge, the resulting high cAMP levels in mural granulosa cells induces a major differentiative change in these cells resulting in luteinization and a concomitant loss of gap junctions with cumulus cells (74). In turn, the high intrafollicular gonadotrophin levels, probably of FSH, results in high CC cAMP levels leading to CC expansion due to a change in proteoglycan synthesis and an associated loss of gap junctions and therefore the uninterrupted flow of cAMP to the oocyte is compromised (6, 71).

1.7 (ii) Phosphodiesterases

Phosphodiesterases (PDE) are enzymes that degrade cAMP. They act by hydrolysing the bond between the phosphate and the 3-hydroxyl group of the ribose sugar in cAMP. Different types of PDEs control cAMP levels in the granulosa cells and oocyte and play essential roles in the control of meiosis. In mammals, PDE4 gene expression is specific for the follicular cells such as mural granulosa and cumulus cells, whereas the PDE3 gene is expressed primarily in the oocytes (75). Recently, the use of PDE inhibitors has demonstrated the essential role of PDEs in regulating meiotic arrest (75, 76). PDE inhibitors prevent cAMP degradation resulting in the accumulation of cAMP, which in turn maintains meiotic arrest. Inhibitors selective for PDE3 were most effective inhibitors of GVBD suggesting that PDE3 activity is

necessary for resumption of meiosis or oocyte maturation both in vitro and in vivo (75, 76).

1.8 Oocyte-Somatic cell interactions: role of GDF9 and BMP 15

Bi-directional communication between the oocyte and somatic cells plays an important role during folliculogenesis (Figure 1.8). Granulosa and cumulus cells assist oocyte growth and development by influencing nutrient and energy availability and promoting meiotic arrest and maturation as outlined earlier.

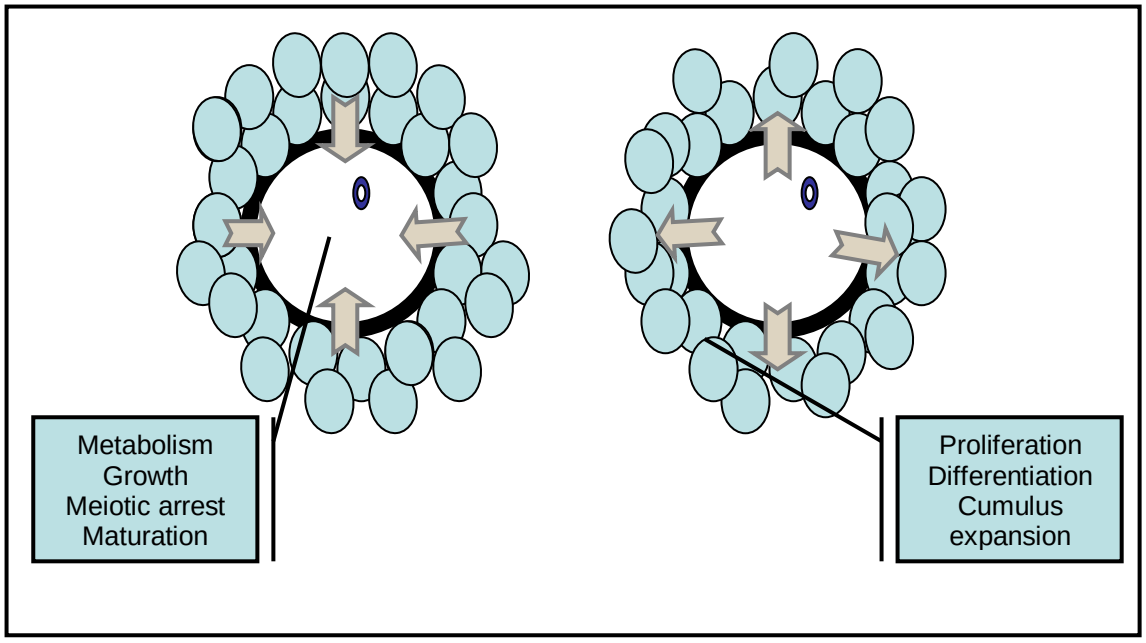

Figure 1.8:- Bi directional communication between the oocyte and its granulosa cells (adapted from 11). The Oocyte (O) secretes paracrine factors that influence adjacent somatic cell proliferation and differentiation.

In turn, the growth and differentiation of follicular somatic cells is critically dependent upon oocyte paracrine signalling. The oocyte promotes follicular growth and somatic cell differentiation via the secretion of paracrine factors such as growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (78).

GDF9 and BMP15 are members of the Transforming Growth factor β (TGF β) family of proteins. The amino acid sequence of mouse GDF9 contains four N- linked glycosylation sites, one of which is in mature region, whereas mouse BMP15 contains five N- linked glycosylation sites, two of which are in the mature region (79, 80). The GDF9 mature region is comparatively conserved as opposed to BMP15 mature region, which diverges greatly among species. Within the ovary, the oocyte is the site of GDF9 and BMP15 gene expression (81).

GDF9 and BMP15 have been found to play important roles in fertility; however the relative importance of them differs among species. GDF9 knockout mice showed cessation of follicular development and were infertile due to impaired granulosa cell proliferation (82); however, BMP15 knockout mice did not have arrested follicular growth suggesting that BMP15 doesn't play part in normal follicular development in this species (83). Naturally occurring mutations of BMP15 or GDF9 in sheep resulted in infertility due to an inhibition of normal follicular growth from the primary follicle stage (84). This suggested that BMP15 and GDF9 are both critical for follicular development and fertility in sheep. Also, it's worth noting that naturally-occuring genetic mutations in BMP15 in women also results in an absence of normal follicular development and primary ammenorhoea (85).

In addition to GDF9 being essential for follicular growth, it is also important for the development of the cumulus cell phenotype (81, 82). Recombinant GDF9 enhances the growth of preantral follicles isolated from immature rats and also promotes proliferation of granulosa cells (86) by increasing DNA synthesis. Also, as the oocyte grows, the secretion of GDF9 stimulates granulosa cells to secrete the growth factor kit-ligand. In turn, kit ligand interacts with the kit receptor on the

oocyte to promote oocyte growth until it becomes mature, where GDF9 then suppresses kit ligand expression on granulosa cells hence ceasing oocyte growth (87). These oocyte secreted factors are mitogenic promoting both MGC and CC DNA synthesis and they either indivually or collectively regulate ganulosa/cumulus cell differentiation (78). CC differ from MGC as they have very low LHR expression, high proliferation rate and can undergo expansion. GDF9 suppresses luteinizing hormone receptor expression on CCs and regulates steroidogenesis in these cells by inhibiting luteinisation (81, 86). Hence the oocyte maintains the distinct CC phenotype via the secretion of GDF9, while the MGC some distance away from the oocyte and thus exposed to a lower concentration of GDF9 are thought to be inhibited sufficiently not to luteinise during follicular growth but not enough to inhibit the expression of the LHR.

BMP15 can also inhibit progesterone synthesis by granulosa cells in vitro (88) and as progesterone is an indicator of cellular luteinisation, it shows that BMP15 and GDF9 can both act as luteinisation inhibitors during follicular development. While there is strong evidence to indicate that GDF9 is synthesized and secreted to act in a paracrine manner with the developing follicle, it remains uncertain, in rodents at least, as to whether BMP15 protein is secreted in rodents before the preovulatory LH surge (88).

After the preovulatory gonadotrophin surge, in rodents, GDF9 and BMP15 play vital roles in the preovulatory follicle by enabling CC expansion in part by up regulating the genes encoding hyaluronan synthase 2 in CC (78). In sheep, these factors have been shown to be critical for ovulation and the formation of normal functioning corpora luteum (81). Hence BMP15 and GDF9 play dominant roles in female fertility in a species dependent manner.

1.9 Mimicking the follicular microenvironment: effect of Milrinone, Forskolin, FSH and IGF1

The follicular microenvironment is crucial for the attainment of developmental competence in oocytes, and it is an utmost requirement for the in vitro maturation of oocytes to have similar follicular environment conditions. As discussed earlier, the approach to delay spontaneous meiotic maturation *in vitro* allows complete cytoplasmic maturation and attainment of development competence.

In rats, selective inhibition of PDE3 blocks meiotic resumption without affecting ovulation (77). PDE inhibitors can be classified on the basis of specificity for different PDE's that are compartmentalized either within somatic cells i.e. PDE4 or the oocyte i.e. PDE3. Milrinone inhibits the PDE3 enzyme in the oocyte, rolipram inhibits PDE4 enzyme in granulosa cells and IBMX is a non-selective PDE inhibitor (75, 76). It has been reported that inhibition of PDE3 enzyme by milrinone causes a delay in oocyte meiotic maturation which was characterised by GVBD (75). Milrinone prevents cAMP degradation by PDEs causing intracellular cAMP accumulation in mammalian oocyte. On the other hand, rolipram is ineffective in preventing GVBD, instead it promotes oocyte maturation (75). It has been suggested that PDE4 is essential for normal ovulation and in PDE4 null mice, the follicles fail to reach pre-ovulatory stage due to premature luteinization (89). The lack of PDE4 causes accumulation of cAMP in granulosa cells leading to premature luteinzation and entrapment of oocytes in immature luteinized follicles (89). Also inhibition of PDE4 in granulosa cells results in activation of protein kinase II (PKAII), whose activation causes GVBD (90). These results emphasize that the selective inhibition of oocyte PDE3 by milrinone can control oocyte maturation.

As mentioned earlier, high levels of cAMP in the oocyte is important with respect to maintenance of meiotic arrest. Forskolin stimulates cAMP production by

activating adenyl cyclase very rapidly and reversibly (91). It is a plant derivative and has a diterpene structure as shown in Figure 1.9.

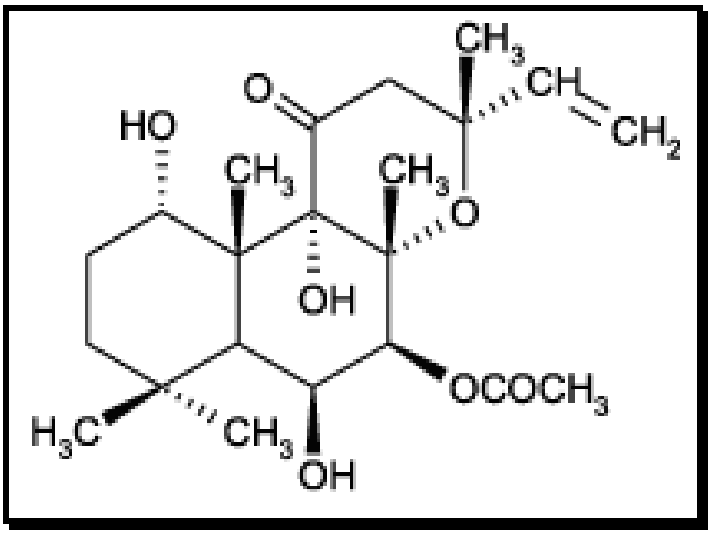

Figure 1.9:- Forskolin, a diterpene produced by *Coleus forskohlii* (Adapted from 91)

It has been demonstrated that forskolin inhibits spontaneous maturation of rat oocytes in vitro (6, 92). The effect of FSH on the oocyte is mediated through the surrounding somatic cells. It stimulates somatic cells to produce factors that are important for the completion of oocyte growth and maturation, both in vitro and in vivo. FSH is the major factor contributing towards antral follicle growth in vivo and is essential to achieve developmentally competent oocytes in vitro (93). FSH triggers granulosa cell proliferation, prevents atresia, induces synthesis of luteinizing hormone receptors on mural granulosa cells and promotes steroid expression in vivo (93). FSH causes cAMP synthesis as it activates membrane receptors and G proteins of granulosa cells which inturn activate adenyl cyclase leading to cAMP production (56). There is evidence that in defined conditions, FSH enhances the ability of the oocyte to reach the blastocyst stage after fertilization in mice and has been shown to improve embryonic development (94).

Insulin-like growth factor-1 (IGF-1) is secreted from somatic cells in response to gonadotrophins. IGF1 binds to the extracellular domain of a membrane- bound receptor 1(IGFR1) which forms a tetramer structure composed of two α subunits and two β subunits that are linked by interchain disulphide bonds (95). An important action of IGF1 is to enhance the action of FSH or LH on ovarian follicular development. IGF1 enhances FSH-induced granulosa cell proliferation and differentiation by stimulating proteoglycan production which causes antrum formation, in pre-antral rat follicles. It enhances FSH-induced aromatase activity which in turn promotes androgen production in theca cells and also enhances LHR expression (96, 97). Studies on pre-antral rat follicles cultured with IGF1 show an increased number of gap junctions between oocytes and granulosa cells, suggesting that IGF1 assists in follicular development by promoting gap junctions within the rat pre-antral follicles (98). Furthermore, the addition of IGF-1 to the culture medium increases the in vitro nuclear maturation rate in rabbits (99).

1.10 The rat oestrous cycle

The present study utilizes rats as the animal model therefore it is of value to outline the physiological events that regulate the rat oestrous cycle. The oestrous cycle of the rat is divided into four stages: pro-oestrus, oestrus, metoestrus and dioestrus. In the pro-oestrus stage the ovarian follicles develop towards the preovulatory stage and an increasing endometrial activity occurs at this stage. During the oestrous stage, the preovulatory follicles secrete high levels of oestradiol leading to ovulation and the development of the corpus luteum from the ovulated follicle starts in metoestrus cycle and release of oestrogen from the ovary is inhibited. Throughout the dioestrus phase, development of corpus luteum continues and

progesterone is released. Follicle growth increases more than 8 fold as it reaches early oestrous stage. Rats undergo regular oestrous cycles throughout the year and are poly-oestrous.

1.11 Aim of study

The primary aim of this study was to investigate the effects of various steroids, growth factors and cAMP stimulators on gap junction activity in oocyte-cumulus cell complexes. It was hypothesized that factors present within the microenvironment of the developing antral follicle such as steroids, growth factors or cAMP stimulators, influence the rate of transfer of low molecular weight compounds from the cumulus cells to the oocyte.

To address this aim, a gap junction assay was developed in which the cumulus-oocyte complexes were recovered from their follicular environment and the transfer of the fluorescent compound, Calcein-acetoxymethyl (Calcein-AM), from cumulus cells into the oocyte was used as a measure of gap junction activity. Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates through cumulus cells. Endogenous esterases within cumulus cells cleave the acetoxy methyl (AM) group producing the green fluorescent calcein, a hydrophilic strongly fluorescent compound that is well-retained in the cell cytoplasm and is non toxic to cells. In this study, the gap junction activity was assessed in COC recovered from rat antral follicle. The gap junction activity was observed within COCs obtained from sexually immature 21-25 day old Sprague-Dawley rats without any hormonal treatments. The second aim of this study was to use a PCR technique to measure Cx37 and Cx43 expression levels in oocytes and cumulus cells respectively following exposure of COC to one of the test

reagents that was shown to alter the transfer rate of Calcein from cumulus cells to the oocyte.

MATERIALS AND METHODS

2.1 Media composition:

2.1a Dissection medium

L-15 Lebowitz solution (Invitrogen; cat# 11415-114; for address of companies refer to Appendix 1) containing phenol red and L-glutamine, supplemented with 0.1% BSA (w/v: ICP Bio Ltd.), 100 IU Penstrep (Invitrogen) and 15mM Hepes buffer (Sigma-Aldrich) was used as the dissection medium. Milrinone (100µM; Sigma-Aldrich) was added to the stock dissection medium before use.

2.1b Incubation media

Phenol red-free Medium199 (M199) (Invitrogen; cat# 11150-059) containing L-glutamine, 2.2g/L Sodium bicarbonate and Earle's salts was supplemented with 0.3mg/ml Polyvinyl alcohol (PVA) and was used as incubation medium. Milrinone (100µM) was added to the stock incubation medium before use.

2.1c Wash media

The wash medium consisted of Phenol red-free M199 supplemented with 100 IU Penstrep and 0.1% BSA. Milrinone (100µM) was added to the stock wash medium before use.

2.2 Stock solutions

The phosphodiesterase inhibitor, Milrinone was dissolved in DMSO and made up in phenol red-free M199 at a concentration of 1mM and stored at -20ºC. The

fluorescent dye Calcein-AM (Invitrogen; cat# c1430), which was used to measure gap junction activity, was stored in DMSO at a concentration of 1mM at -20ºC. The cAMP stimulator, Forskolin (Sigma-Aldrich) was dissolved in absolute ethanol at a concentration of 1mM and stored at -20ºC. Insulin like Growth Factor (IGF1) (Novozymes) was made up in M199 at a concentration of 1ng/µl and stored at 4ºC. Recombinant human-Follicle Stimulating Hormone (Rh-FSH) (Prospec) was prepared at a concentration of 150mIU/µl in M199 containing 0.1% BSA, and stored at -20ºC. Dibutryl cyclic AMP (dcAMP; Sigma-Aldrich) was dissolved in M199 at a concentration of 100µM. Steroids such as oestradiol, testosterone, androstenedione and progestrone (Sigma-Aldrich) were prepared and stored at 4ºC. Stock solutions of oestradiol (100µg/ml in absolute ethanol), testosterone (100µg/ml in absolute methanol), progesterone (100µg/ml in absolute ethanol) and androstenedione (100µg/ml in absolute ethanol) were prepared and stored at 4ºC. The control solutions of absolute ethanol and methanol were also stored at 4ºC.

2.3 Microscopy

For ovarian dissections and collection of COCs, a Ziess stereo-zoom dissection microscope (475265, West Germany) was used. The intensity of fluorescence within denuded oocytes was measured using a Leica inverted fluorescence microscope (CTR4000; Albany 0751, New Zealand) fitted with a CRAIC technology fluorescence detection system. The fluorescence recordings were captured using the CRAIC technology software and saved on hard drive. The fluorescent settings on the detection system were 50 millisecond recordings over the wavelengths 300-700nm.

2.4 Ovaries

The ovaries were obtained from 21-25 day old sexually immature Sprague-Dawley rats provided by the Department of Psychology, Victoria University of Wellington. The ovaries were obtained on my behalf by Ms Norma Hudson (School of Biological Sciences) from female rats that were being culled from the breeding colony. Rats were housed in a temperature-controlled room with food and water provided *ad libitum*. Animals were sacrificed using Carbon dioxide.

2.5 Storage of ovaries before dissections

Ovaries were removed and collected into 1ml of Dissection media containing milrinone (100µM), and were stored in a 37ºC water bath until further use. COCs were isolated from the ovarian follicles within 2 hours of ovary collection.

2.6 Isolation of cumulus-oocyte complexes

Ovaries submerged in dissection media were cleaned of their surrounding fat layer using dissecting scissors and forceps under the dissecting microscope. To isolate COCs, antral follicles (~0.5-1mm diameter) visible on the ovarian surface were punctured using a 20 gauge needle to recover the COC. Approximately 60 COCs were recovered from each pair of ovaries. All COCs collected were examined under the microscope and those selected for further study were those with two or more intact layers of cumulus cells surrounding the oocyte. Selected COCs were then transferred to either a 48-well plate for performance of a gap junction assay or to a culture dish for preparation for QPCR analysis.

2.7(i) Gap Junction Assay: Effect of time

After isolation, a minimum of 10 COCs were transferred into each of six wells (T0, 60, 120, 180, 240 and 360 minutes) containing of 200µl wash media supplemented with milrinone (100µM) in a 48-well plate. Each well represented the time interval after which gap junction activity was measured. Care was taken to ensure that the amount of dissection media transferred with the COC was negligible, since phenol red is known to interfere with the fluorescence readings of calcein. To remove any excess phenol red media, this wash step was repeated twice by transferring COCs into new wells containing fresh wash media (200µl) containing milrinone (100µM). All COCs were then transferred into new wells containing 200µl incubation media supplemented with milrinone and pulsed with calcein-AM dye (1µM) for 10 minutes in a 37ºC CO2 (5%) incubator at 96% humidity. Thereafter, COCs were transferred into new wells containing fresh incubation media (200µl) supplemented with milrinone. After the respective incubation times (ie 0, 60, 120, 180, 240 and 360 minutes), COCs were washed twice in 200µl incubation media containing milrinone and were transferred to an eppendorf tube. Oocytes were then denuded of their surrounding cumulus cells by vigorous vortexing for 5 minutes. The fluorescence intensities of the denuded oocytes were then measured using the CRAIC fluorescence system.

2.7(ii) Gap Junction Assay: Effect of Treatments

The effect of different treatments on the amount of dye (Calcein) transfer from the cumulus cells to the oocyte was also examined using the gap junction assay. The various concentrations of treatments used are illustrated in Table 2.1. Appropriate

controls were used in all experiments, i.e. if a solvent was used to prepare the treatment stock solution; the solvent was added to the media in the control wells. COCs were examined at time 0 (T0) to provide a zero time reference point and after 240 min (T240) of exposure to different treatments and also for the controls.

After isolation, a minimum of 10 COCs were transferred into each of four wells (Control T0; Control T240; Treatment T0 and Treatment T240) containing 200µl of wash media supplemented with 100µM milrinone in a 48-well plate. Care was taken to ensure that the amount of dissection media transferred with the COC was minimal. This wash step was repeated twice. All COCs were then transferred into new wells containing 200µl of incubation media supplemented with 100µM milrinone, and pulsed with 1µM calcein-AM dye for 10 minutes by placing the plate in a 37ºC incubator gassed with 5% CO2 and held at 96% humidity. Thereafter, COCs from all wells were transferred into new wells containing either 200µl of incubation media supplemented with milrinone and the appropriate solvent (only if it was used to prepare treatment in question); or 200µl of incubation media supplemented with milrinone and the appropriate treatment (refer to Table 2.1) and returned to a 37ºC incubator gassed with 5% CO2 and held at 96% humidity. After the respective incubation times (i.e. 0 and 240 minutes), COCs were washed twice in 200µl of incubation media containing milrinone and transferred to an eppendorf tube. Oocytes were denuded of their surrounding cumulus cells by vigorous vortexing for 5 minutes. The fluorescence intensities of the denuded oocytes were measured using the CRAIC fluorescence system.

Treatment	Concentration
Oestradiol	1µg/ml
Testosterone	1µg/ml
Progesterone	1µg/ml
Androstenedione	1µg/ml
IGF1	10ng/ml
Forskolin	10^{-5}M/ml
Rh-FSH	50mIU
dcAMP	1µM
Oestradiol+ Forskolin	1µg/ml + 10^{-5}M/ml
Testosterone+ Forskolin	1µg/ml + 10^{-5}M/ml
Androstenedione + Forskolin	1µg/ml + 10^{-5}M/ml
Oestradiol+ Forskolin+ IGF1	1µg/ml + 10^{-5}M/ml+10ng/ml
Testosterone+ Forskolin+ IGF1	1µg/ml + 10^{-5}M/ml+10ng/ml

Table 2.1:- The different treatments and respective concentrations used in the gap junction assay

2.8 Quantitative PCR (QPCR)

QPCR was employed to analyse changes in the gene expression of Cx37 and Cx43 present in oocytes and cumulus cells, respectively after exposure to no treatment or forskolin. The QPCR method was validated previously by Dr Janet Crawford (School of Biological Sciences).

2.8(i) Collection of COC samples for QPCR

After isolation, approximately 30-35 COCs were selected and transferred into a clean culture dish containing 2000µl of wash media supplemented with 100µM milrinone. This wash step was repeated twice and the COCs were then transferred into

respective culture dishes (with or without treatment), with a minimum of 7-8 COCs in each dish. The culture dish with treatment contained 2000µl of incubation media supplemented with 100µM milrinone and 10^{-5} M/ml Forskolin or vehicle (i.e ethanol). The culture dishes were kept in the incubator gassed with 5% CO2 and held at 96% humidity and after the respective incubation times (i.e. 0 and 240 minutes), each individual COC was washed in phosphate buffered saline (PBS) supplemented with milrinone, so that one COC was present in each well of a 96-well plate. Thereafter, each individual COC in a volume of 5µl was transferred into a sterile eppendorf tube on dry ice and then stored at -80ºC until QPCR analysis.

2.8(ii) ArrayPure Nano-scale RNA extraction

Total RNA (tRNA) was extracted from COCs using reagents provided in the ArrayPure purification kit (Epicentre; cat# MPS04050) and following manufacturer's instructions.

For logistical purposes, RNA was extracted from 10 samples at a time. Samples were thawed, vortexed and briefly centrifuged. A pre-mix solution of 10µl of Proteinase K solution (5µg/µl) in 300µl of Nano-scale Lysis solution was prepared on ice. To each sample, 30µl of this premix solution was added and the samples were mixed thoroughly by vortexing for 1 minute followed by incubation at 67ºC in a water bath for 15 minutes. The lysed samples were then placed on ice for 5 minutes, after which 18µl of MPC Protein Precipitation Reagent was added and samples were vortexed briefly. The precipitate was pelleted by centrifugation for 7 minutes at 4ºC at 12000*g* and the supernatant was transferred to clean eppendorf tube. The tRNA was precipitated by the addition of 50µl of absolute isopropanol (Sigma-Aldrich) and then centrifugation for 5 minutes at 4ºC at 12000*g*. All of the residual isopropanol was

removed using a pipette and the pellet was air-dried for 5 minutes. Meanwhile, a pre-mix solution of 5µl of RNase-free DNase I in 200µl of 1 x DNase Buffer was prepared on ice and 20µl was added to each tRNA pellet for resuspension. Samples were then vortexed for 1 minute followed by incubation at 37 ºC in a water bath for 30 minutes to ensure all contaminating DNA is removed. After incubation, 20µl of 2x Nano-scale lysis solution and 20µl MPC Protein Precipitation Reagent were added to the samples and were mixed by vortexing briefly after the addition of each reagent. The precipitate was pelleted by centrifugation for 5 minutes at 4ºC at 12000*g*. On ice, the supernatant containing the tRNA was transferred into sterile eppendorf tubes. The tRNA was precipitated by addition of 50µl isopropanol and centrifuged for 5 minutes at 4ºC at 12000 x g. The isopropanol was aspirated and the pellet was washed by the addition of 50µl of 70% ethanol and centrifuged at 12000 x g for 3 minutes. All the residual ethanol was removed using a pipette and the pellet was air dried for 5 minutes. The purified tRNA was resuspended in 10µl Ultra-Pure distilled water. All samples were then incubated at 56ºC for 2 minutes, vortexed for 1 minute and stored at -80ºC.

2.8(iii) Reverse Transcription PCR

Complementary DNA (cDNA) was synthesised using the reagents provided in the SuperScript VILO kit (Invitrogen cat # 11754-250) and following manufacturer's instructions.

This kit consisted of a 10X SuperScript Enzyme mix and a 5X VILO Reaction mix. The 10X SuperScript Enzyme mix comprised an RNaseOUT Recombinant Ribonuclease inhibitor, a proprietary helper protein and SuperScript III RT enzyme,

which had reduced RNase activity and an increased thermal stability. The 5X VILO Reaction mix contained random primers, $MgCl_2$ and dNTPs in a buffer.

For logistical purposes, Reverse Transcription PCR was performed for 20 tRNA samples at a time. The tRNA samples were thawed, vortexed and briefly centrifuged. A pre-mix solution of 4µl of 5X VILO Reaction mix, 2µl of 10X SuperScript enzyme mix and 4µl of Ultra Pure distilled water were prepared for each set of samples. To each Corbett 0.2ml PCR tube (Corbett Research) on ice, an aliquot of 10µl of this pre-mix solution was added to 10µl of tRNA for a total volume of 20µl. Care was taken to ensure that the RNA was well mixed with the premix reagents by vortexing all tubes briefly. Samples were then placed in a Rotor-Gene 6000 Rotary Analyser (Corbett Research; model# RG6000) under the following conditions: 25ºC for 10 minutes, 42ºC for 120 minutes and the reaction was terminated by 85ºC for 5 minutes. The cDNA samples were stored at -20ºC until further analysis using QPCR.

2.8(iv) Quantitative PCR

QPCR reactions were carried out using Brilliant Multiplex QPCR Master mix (Stratagene; cat # 600553) as it provided all the buffer components in a preoptimized solution. It also contained SureStart *Taq* DNA polymerase with hot start capability and 5mM of $MgCl_2$.

Specific forward and reverse primers, and Taqman probes were designed for each gene of interest (i.e. Cx37 and Cx43) and the housekeeping gene (RPL19) using the computer package 'Beacon Designer' (premier Biosoft International, Palo Alto, CA, USA) by Dr Janet Crawford (School of Biological Sciences) (Refer to Table 2.2, 2.3).

Gene	Primer sequence (5'-3')	Molecular Weight (µg/µmole)	%GC
Cx37-Forward	GACGGTCGTCTTCGCATTCG	6423	60
Cx37-Reverse	ACGCCACTGGCCATAGAGG	6128.8	63
Cx43-Forward	AGACTGCGGATCTCCAAAATACG	7415.6	47
Cx43-Reverse	CTTGTTGTAATTGCGGCACGAG	7138.4	50
RPL19-Forward	GACCCCAATGAAACCAACGAAATC	7683.8	45
RPL19-Reverse	GGAATGGACAGTCACAGGCTTC	7141.4	54

Table2.2:- Sequences of forward and reverse primers specific for Cx37, Cx43 and RPL19 genes

Gene	Probe sequence (5'-3')
Cx37	[6FAM] CCTCCAGCACACTCTTACACAGCACACTG [BHQ1]
Cx43	[HEX] CCTCACCAACGGCTCCACTCTCGC [BHQ1]
RPL19	[Cy5] CCAATGCCAACTCTCGTCAACAGATCAGGAAG [BHQ3]

Table2.3:- Taqman probes contained a fluorophore located at the 5' end and a quencher (Black Hole Quencher; BHQ) on the 3' end.

For logistical purposes, QPCR was performed for 30 samples at a time and were repeated once to give two technical replicates. All samples were in duplicate, and a positive control (rat calibrator) and negative control (no template control) were included in each run.

The cDNA samples were thawed, vortexed and centrifuged briefly. In a single reaction, 12.5µl of 2x Brilliant Multiplex QPCR mastermix, primers and probes at the appropriate concentrations (refer to Table 2.4); 1.5µl of cDNA and Ultra-Pure distilled water to adjust the final volume to 25µl was added into Corbett 0.1mL strip tubes (Corbett Research).

Primer/Probe	Final Concentration (nM)
Cx37-Forward	300
Cx37-Reverse	100
Cx37-Probe	50
Cx43-Forward	100
Cx43-Reverse	100
Cx43-Probe	50
RPL19-Forward	200
RPL19-Reverse	200
RPL19-Probe	50

Table2.4:- Concentration of primers and probes required for QPCR

The tubes were sealed with strip caps (Corbett Research) and subjected to the following conditions: 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute in a Rotor-gene 6000 rotary analyser (Corbett Research; model# RG6000).

The PCR program generated amplification plots and provided CT values representing gene expression due to different treatments. The CT value refers to the first cycle at which it is possible to distinguish the amplification generated fluorescence as being above the background signal. From the CT value, dCT; ddCT and $2^{(-ddCT)}$ values were calculated. The $2^{(-ddCT)}$ values were subjected to statistical analysis and results were analysed.

2.9 Statistical Analyses

In the gap junction assay, the fluorescence intensity within each oocyte was measured using a fluorescence spectrophotometer. The fluorescent readings of individual oocytes measured for each treatment were averaged to calculate the mean fluorescent intensity. One way analysis of variance (ANOVA) and the Dunnett test was performed using SPSS 16.0 on the data obtained for testing the effect of incubation time relative to time 0. The unpaired student's t test on non-transformed data was performed using the statistics program SPSS 16.0 when comparing the control versus treatment groups at times 0 and after 240 mins for the COCs treated with E; T; IGF1; F; T+F; E+F; E+F+IGF1; T+F+IGF1; P; A; A+F; dcAMP and FSH. For other treatment comparisons at 0 and 240 mins, the Levene's test for homogeneity of variances revealed that the data for forskolin; dcAMP; FSH; T+F; E+F+IGF1 and A+F treatment groups had significantly different variances. Consequently, the data obtained from these treatment groups were Ln transformed to equalise the variances before undertaking the unpaired student's t tests. For the comparisons of control, E, F, E+F and also control, T,F,T+F at times 0 and at 240 mins, ANOVA on non-transformed data followed by the Bonferroni post-hoc test was performed. In this thesis, all the data are represented as bar graphs showing arithmetic means and SEM to show the comparisons between the control and treatment groups at time 0 and at 240 mins.

The CT values obtained from QPCR of samples (COC) treated with forskolin or control (non forskolin treated), for each gene separately (i.e. Cx43 and Cx37) were calculated. The Cx43 mRNA results were corrected using the housekeeping RPL19 gene values whereas the Cx37 gene results were not corrected by RPL19.The justification for this is referred to elsewhere (i.e. in the Discussion). The relative level of gene expression in individual COCs at 0 and 240 min of incubation for Cx37 and Cx43 were each averaged to calculate the means for the forskolin treated and control

groups. Thereafter, the unpaired student's t test was performed using SPSS 16.0 to compare gene expression in the control versus forskolin treatment group at 0 and 240 mins and the data is represented as bar graphs.

RESULTS

3.1 Quality of oocytes and cytoplasmic characteristics

The impact of oocyte cytoplasm characteristics on fluorescence intensity is shown in Figure 3.1. The oocytes without any colour were first visualised under the inverted microscope prior to activation of the fluorescence system. The fluorescence intensity values were then assessed. Examples of these are shown in Fig 3.1 where the intensity values are highlighted in red for each respective oocyte. All denuded oocytes were from control group, without any treatments and fluorescence was measured at time 0.

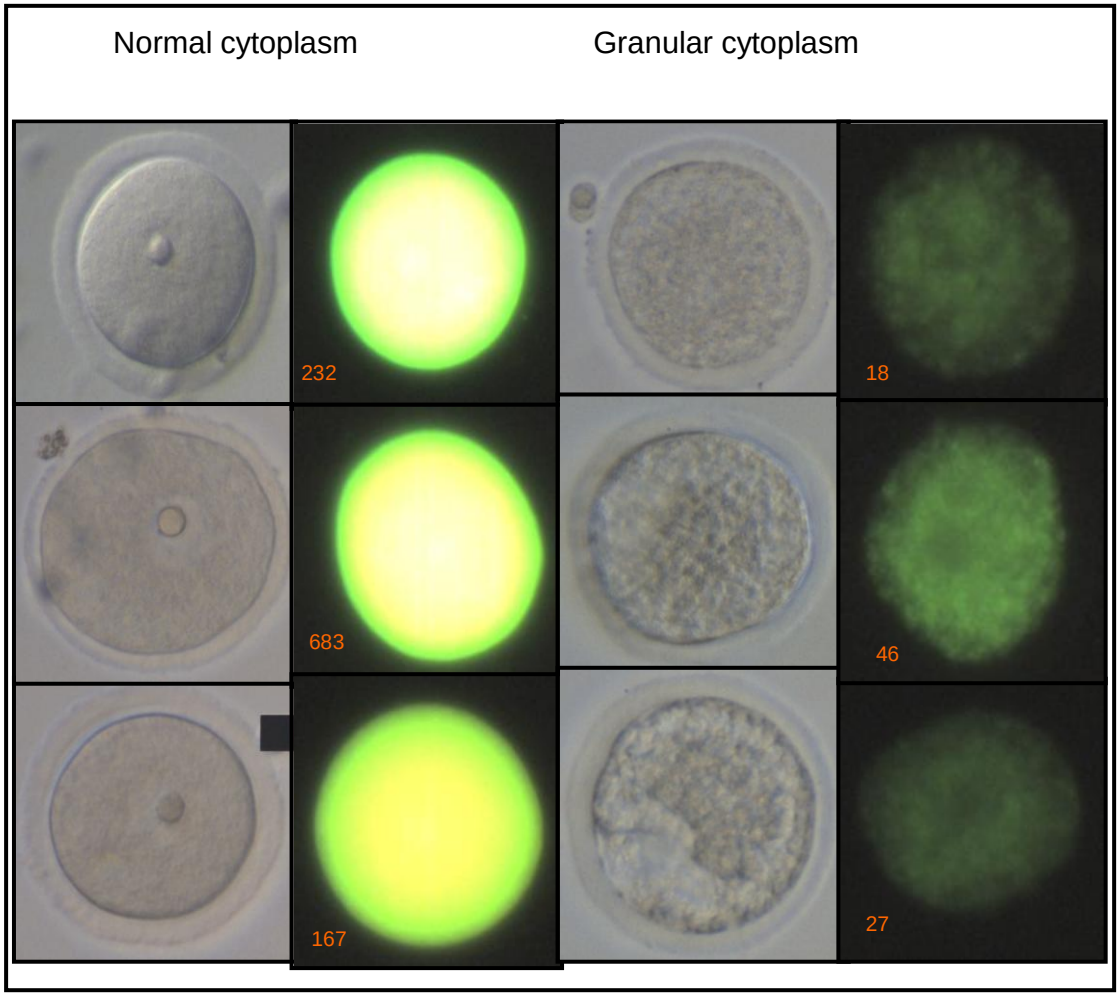

Figure 3.1:- Impact of oocyte cytoplasm characteristics on fluorescence intensity

3.2 Effect of time on calcein dye transfer through gap junctions in rat COC

The aim of this experiment with rat COC was to determine the effect of time of incubation on the rate of calcein dye transfer from the cumulus cells to the oocyte.

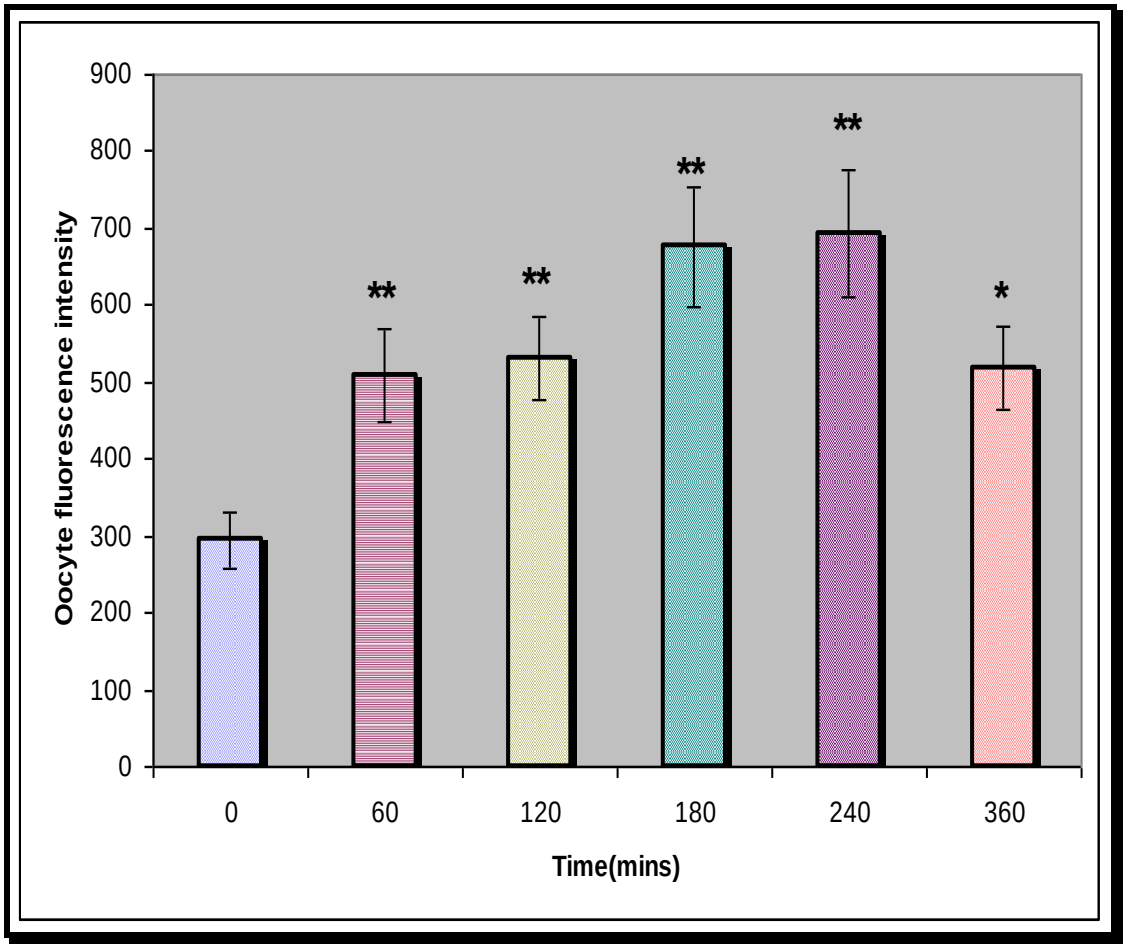

Figure 3.2:-Effect of incubation time on the relative fluorescence of calcein in denuded oocytes after exposing COC to a 10 min pulse of calcein-AM prior to incubation in calcein-free media. At each time point, the mean ± sem values were generated from 7 replicate experiments with 6-10 oocytes used in each replicate experiment. Means marked with asterisks are significantly different from 0 time period (*$p<0.05$; **$p<0.01$).

The relative mean fluorescence values in denuded oocytes at 60, 120, 180, 240 and 360 mins were significantly different from those at time 0 ($p<0.01$). The maximum dye transfer was observed at 180 to 240 mins. Thereafter, at 360 mins the relative level of fluorescence had declined by ~30% compared to that at 240 mins. For all subsequent experiments where the effects of steroids, IGF1, forskolin, dibutyryl cAMP and FSH were examined an incubation time of 240 mins for the COCs was selected.

3.3 Effect of Steroids on gap junction activity

Steroids are present in high concentrations in follicular fluid and are also important secretory products of the follicle. Since COC are exposed to high steroid concentrations their effects on gap junction communication as assessed by calcein dye transfer was examined. The major steroids known to be present in follicular fluid are oestradiol, testosterone, androstendione and progesterone. The results of the effects of these steroids when added individually to COC are summarised in Figures 3.3(i), 3.3(ii), 3.3(iii), 3.3(iv) respectively. The results show that the mean values after 240min of incubation for both control and treatment groups were significantly higher than the corresponding values at time 0, and this effect was observed for all steroid treatments ($p<0.05$). No significant difference was observed for the mean fluorescence values between control and treatment groups at time 0 or after 240 min incubation, and this was observed for all steroid treatments ($p>0.05$). These results suggest that steroids do not affect gap junction activity in rat COCs.

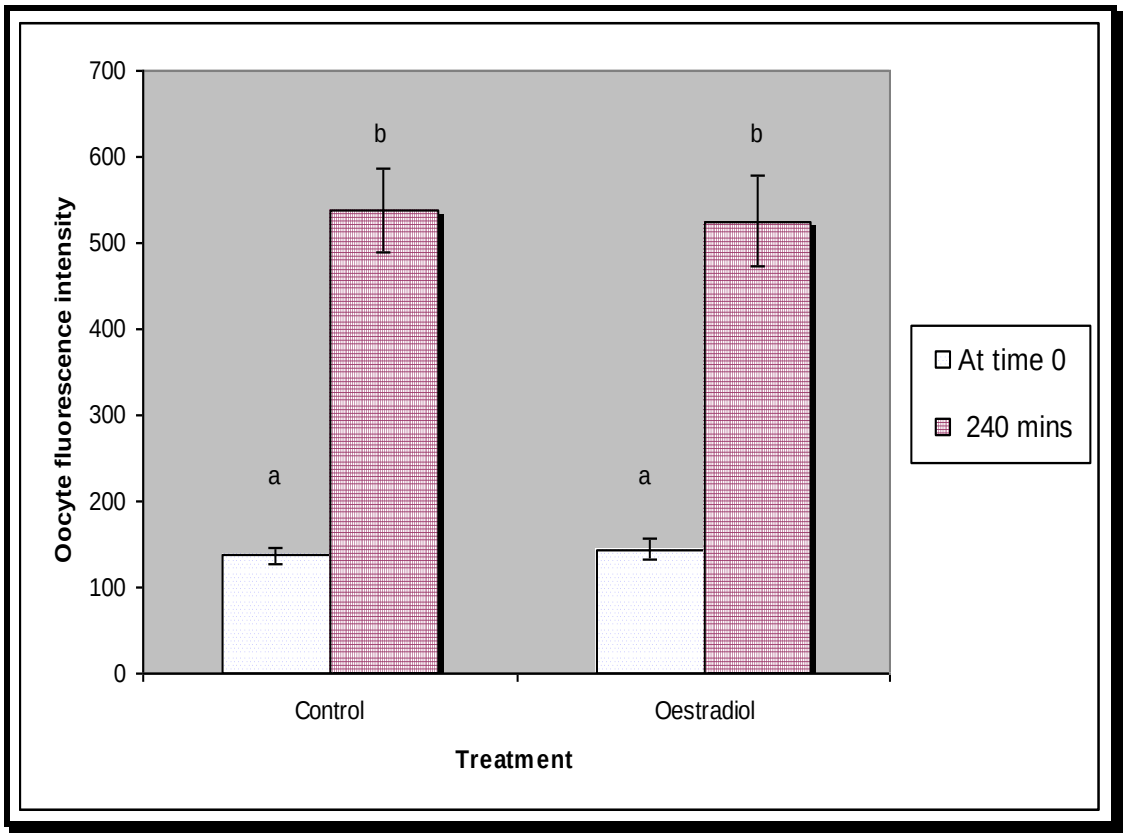

Figure 3.3(i):- Effect of oestradiol and no treatment on fluorescent intensity of calcein in denuded oocytes at 0 and 240 min. For each treatment and time, the mean± sem values were generated from 6 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

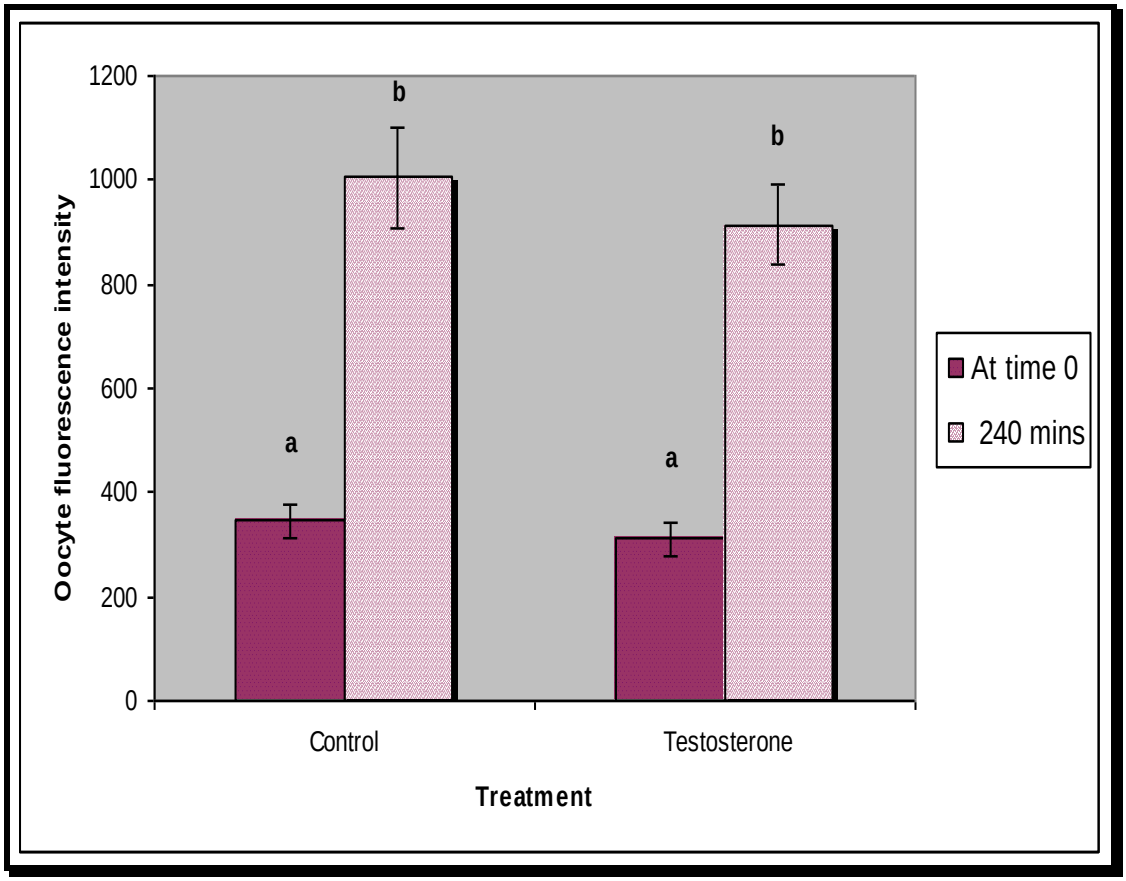

Figure 3.3(ii):- Effect of testosterone and no treatment on fluorescent intensity of calcein in denuded oocytes at 0 and 240 min. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

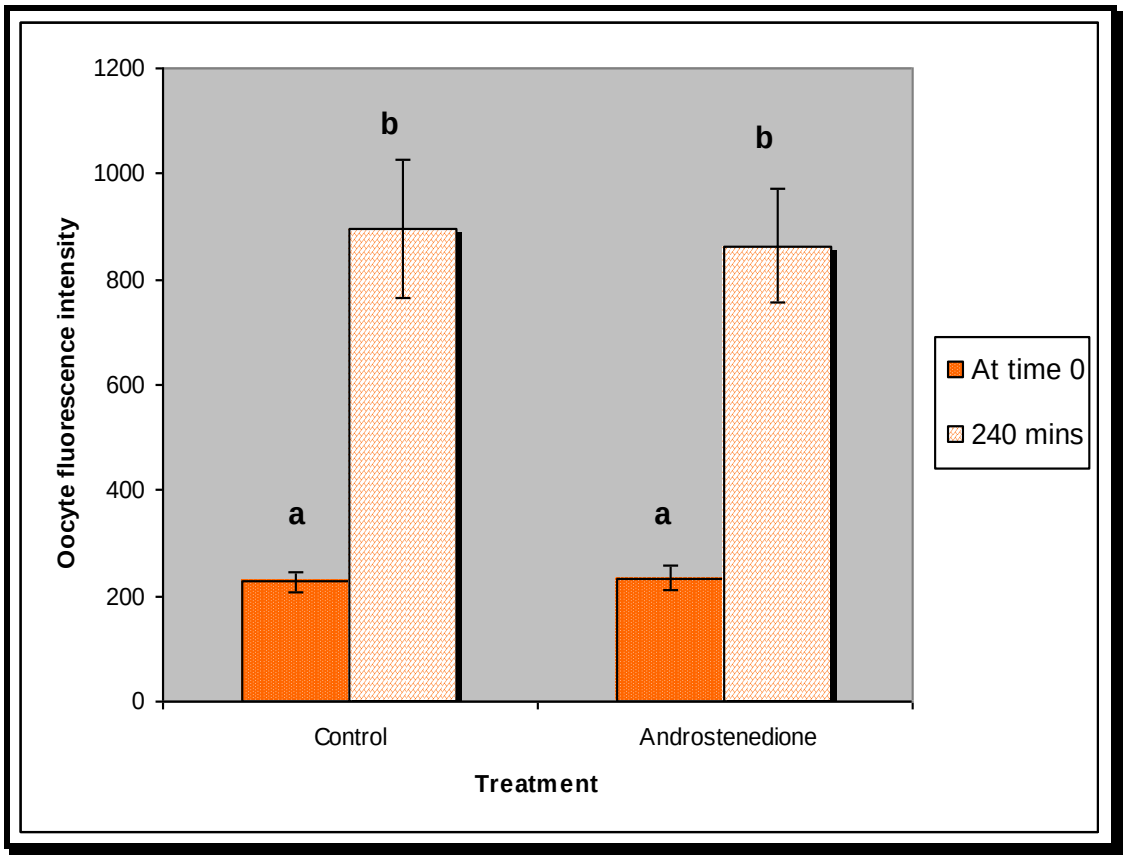

Figure 3.3(iii):- Androstenedione does not affect calcein transfer from cumulus cells to the oocyte in vitro. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

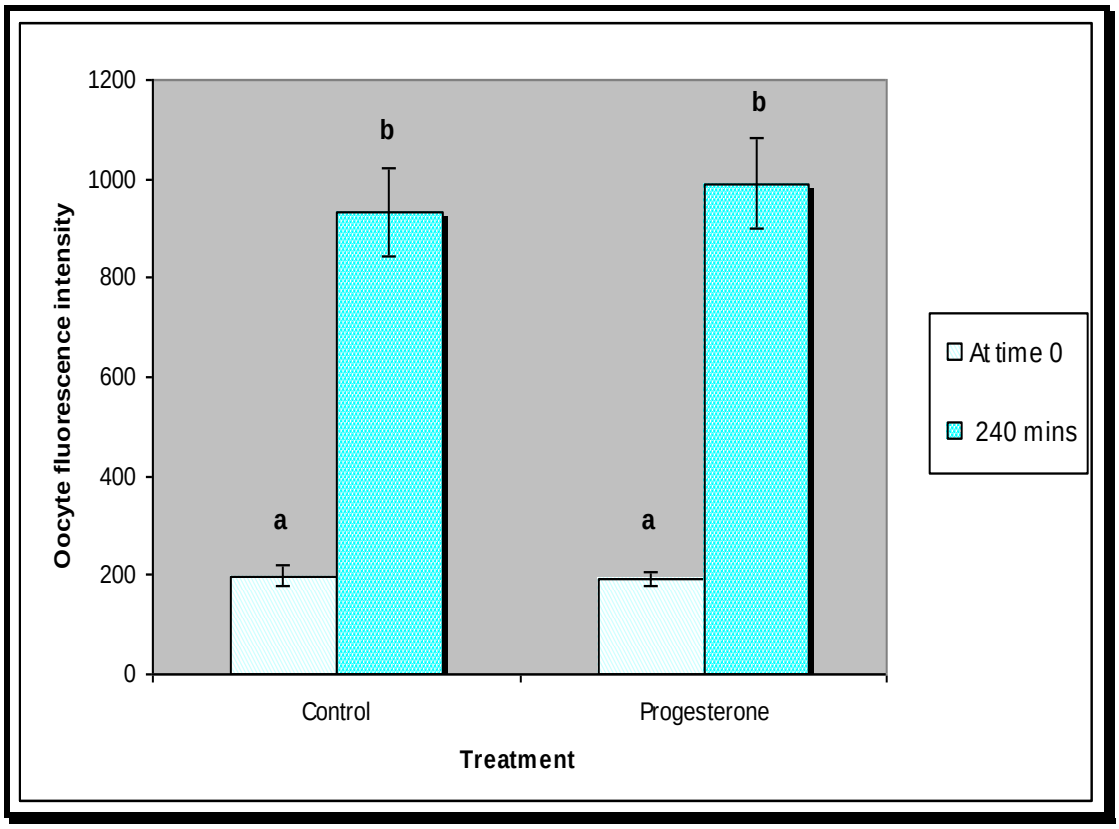

Figure 3.3(iv):- Progesterone does not affect calcein transfer from cumulus cells to the oocyte in vitro. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

3.4 Effect of IGF 1 on gap junction activity in rat COCs

IGF1 plays an important role in follicular development by influencing the effects of FSH on granulosa cells in mammals. The effects of IGF1 on gap junction activity are not known and this was the basis for the present study. As shown in Figure 3.4, treatment of COCs with IGF1 alone had no effect on calcein transfer from cumulus cells to the oocyte as there was no significant difference in the mean fluorescence values after four hours of incubation between control and IGF1 treated COCs ($p>0.05$).

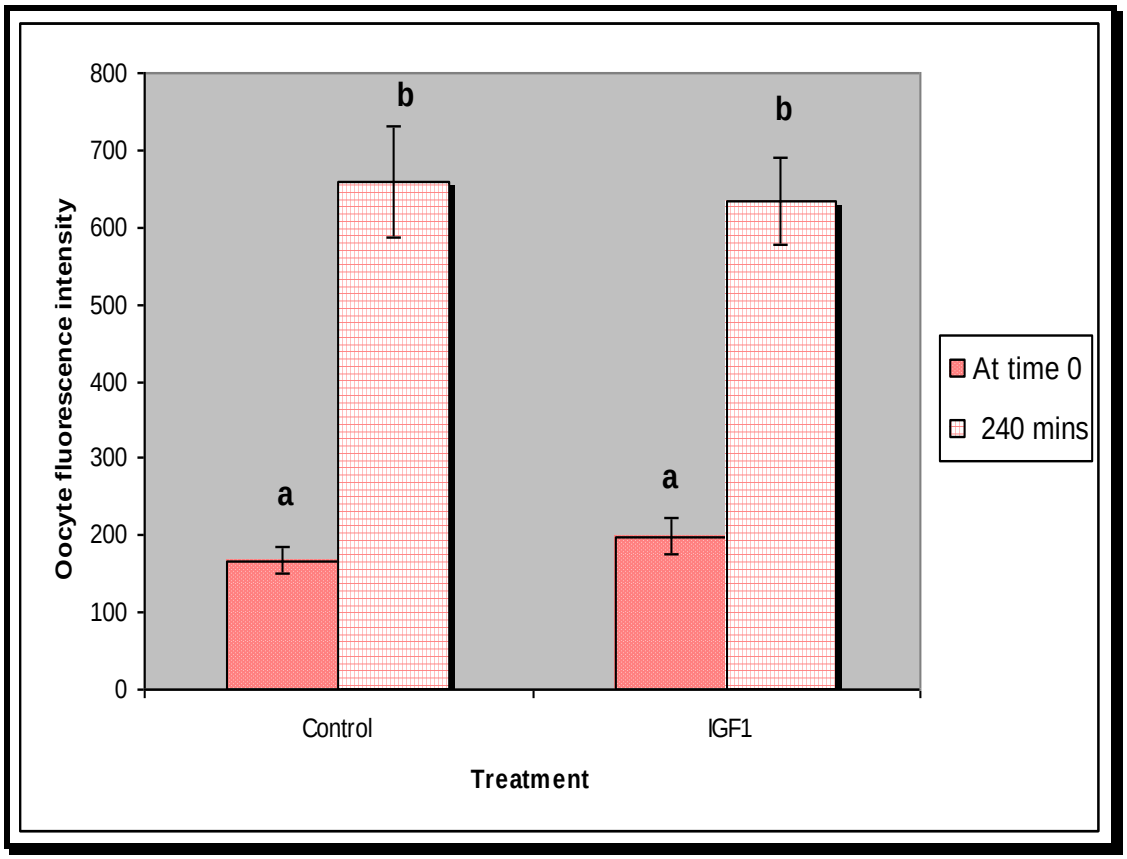

Figure 3.4:- IGF1 has no effect on calcein transfer from cumulus cells to the oocyte in vitro. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$). No difference in mean fluorescence intensity was observed between the IGF1 and control treatments at 0 or 240 min.

3.5 Effect of Forskolin on gap junction activity in rat COCs

High intracellular levels of cAMP in the oocyte are known to be important with respect to maintenance of meiotic arrest in vivo. In this and other experiments, the effect of stimulators of cAMP synthesis in COC was tested for their abilities to enhance calcein dye transfer from cumulus cells to the oocyte. In this set of experiments the effect of forskolin on gap junction communication in vitro was

investigated. The results (Figure 3.5) indicate that treatment of COCs with forskolin increased gap junction activity since there was a significant difference in the mean fluorescence values after four hours of incubation between control and forskolin treated COCs ($p<0.05$). No significant difference was observed for the mean fluorescence values at time 0 between control and forskolin ($p>0.05$).

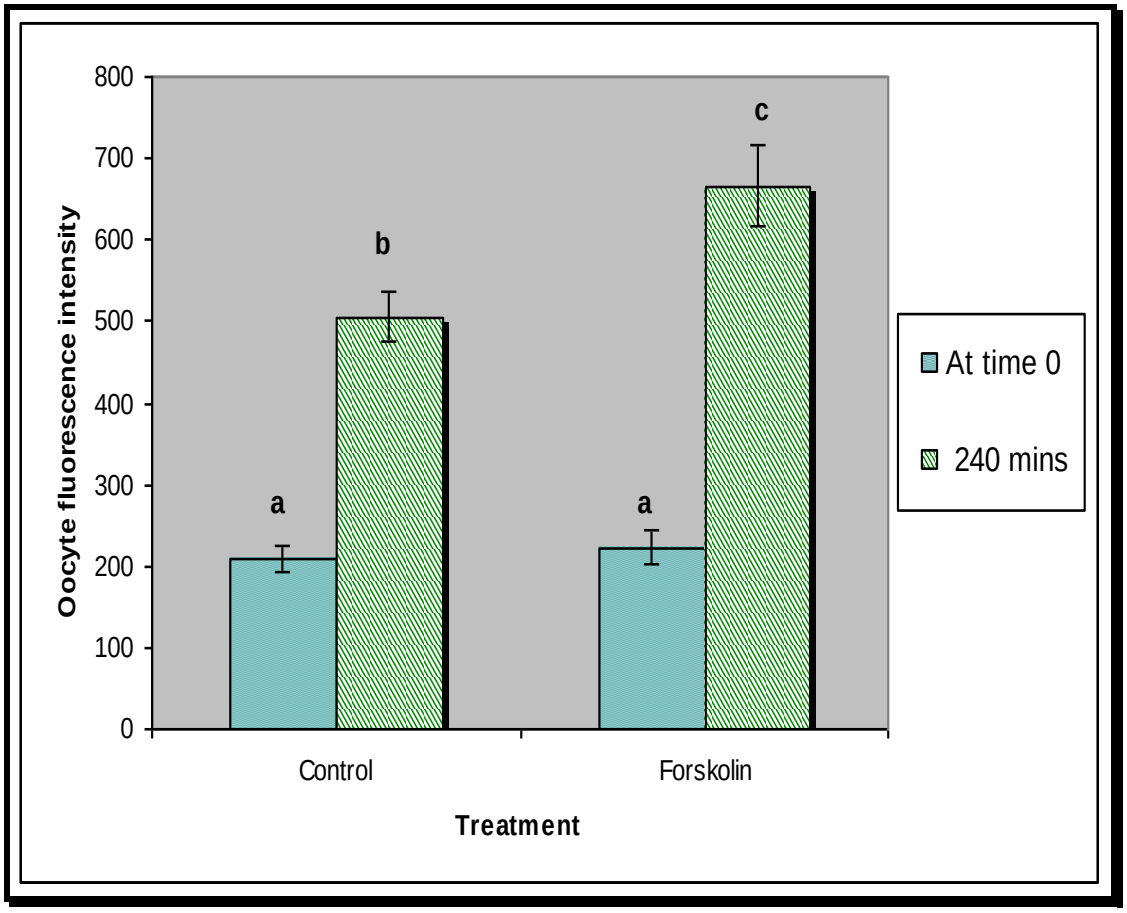

Figure 3.5:- Forskolin causes an increase in fluorescence intensity in denuded oocytes after 240 min incubation in vitro following a 10 min pulse of calcein followed by incubation of the COC in calcein free media for 0 and 240 min. For each treatment and time, the mean± sem values were generated from 7 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

3.6 Effect of other cAMP stimulators

In addition to forskolin, other factors likely to increase intracellular cAMP concentrations, such as dcAMP and FSH were tested for their abilities to enhance calcein dye transfer from cumulus cells to oocytes. The results from these experiments are summarised in Figures 3.6(i) and 3.6(ii).

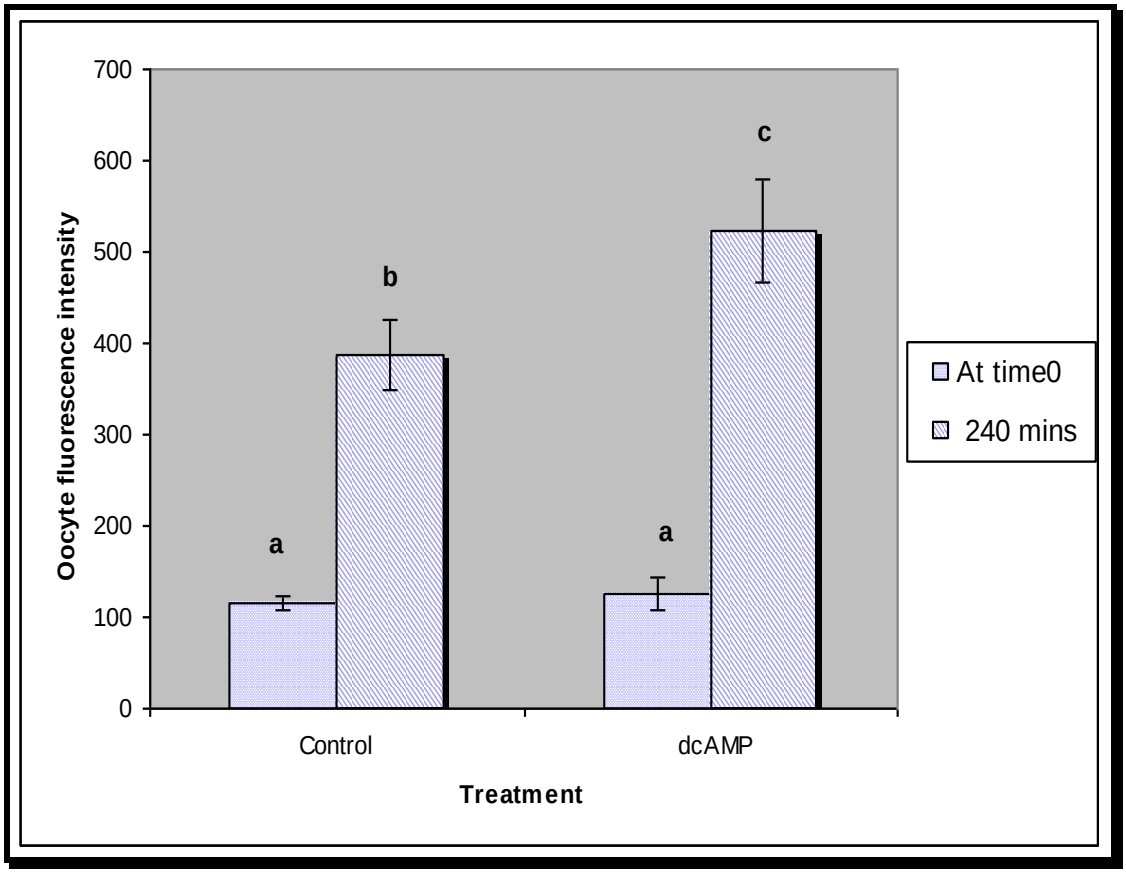

Figure 3.6(i):-Dibutyryl cAMP causes an increase in calcein transfer from cumulus cells to the oocyte in vitro. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

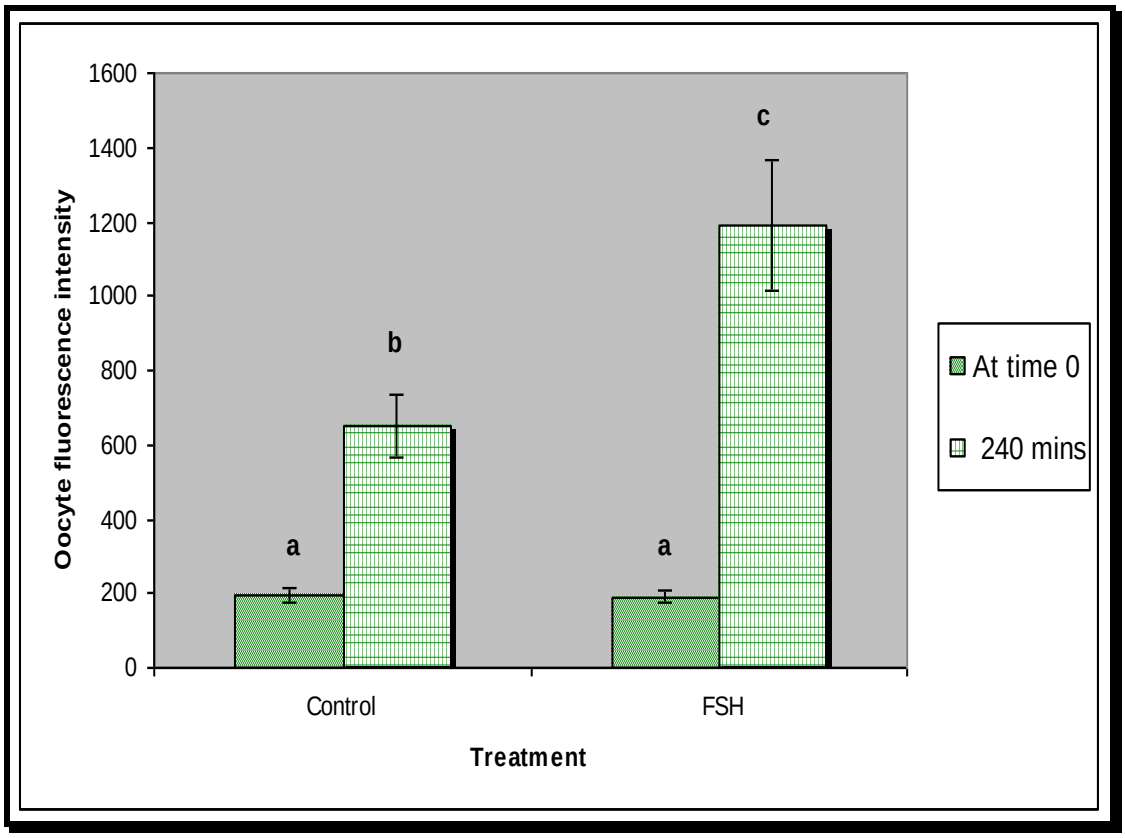

Figure 3.6(ii):- FSH causes an increase in calcein transfer from cumulus cells to the oocyte in vitro. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

Thus as with forskolin, both dibuyryl cAMP and FSH treatments to COCs increased the level of fluorescent dye present in oocytes after 240 min *in vitro.*

3.7 Effects of various treatment combinations

COCs within developing follicles are exposed to mixtures of steroid, IGF1 and cAMP stimulators. In the following experiments, rat COCs were treated with different combinations of steroids, IGF1 and forskolin to test their effects on gap junction activity. In all the following experiments i.e. testosterone + forskolin; testosterone + forskolin + IGF1; oestradiol + forskolin; oestradiol + forskolin+ IGF1, there were significant difference between control and treatments after 240 min (Figures 3.7 i-iv; all $p<0.05$) but no differences noted at time 0. The only exception to this was that no difference was observed for androstenedione + forskolin compared to the untreated controls after 240 min.

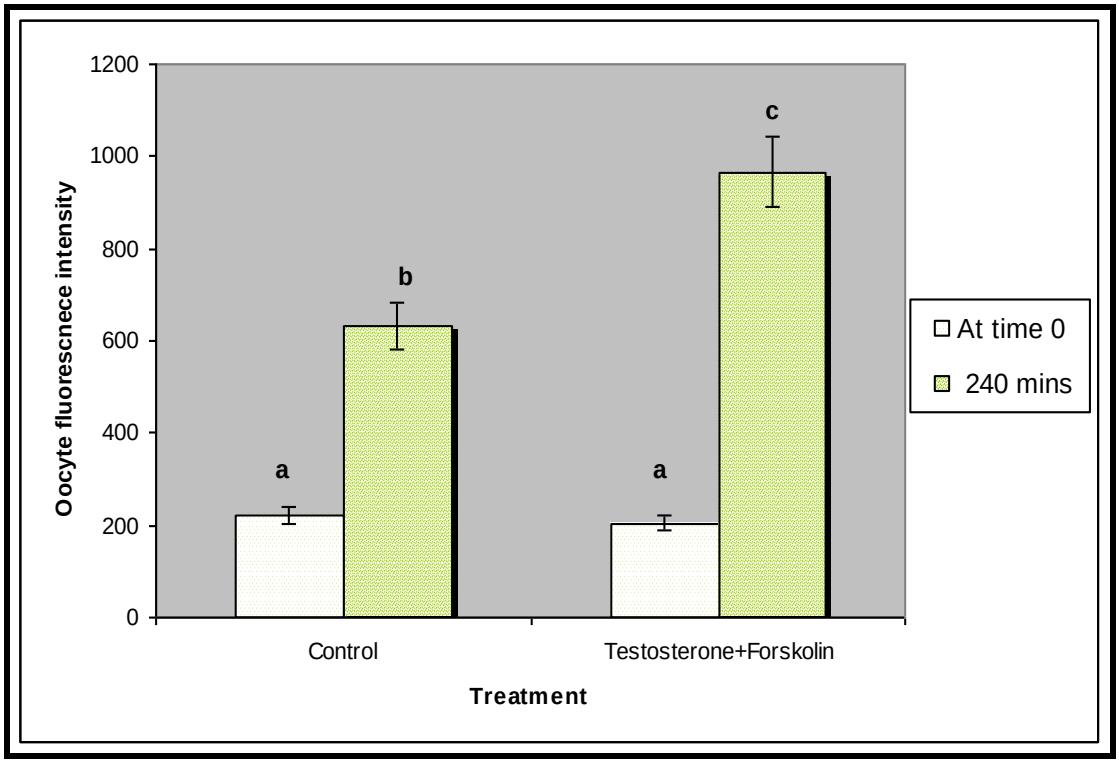

Figure 3.7(i):- Effect of testosterone + forskolin or no treatment on fluorescent intensity in denuded oocytes at 0 or 240 min. For each treatment and time, the mean± sem values were generated from 6 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

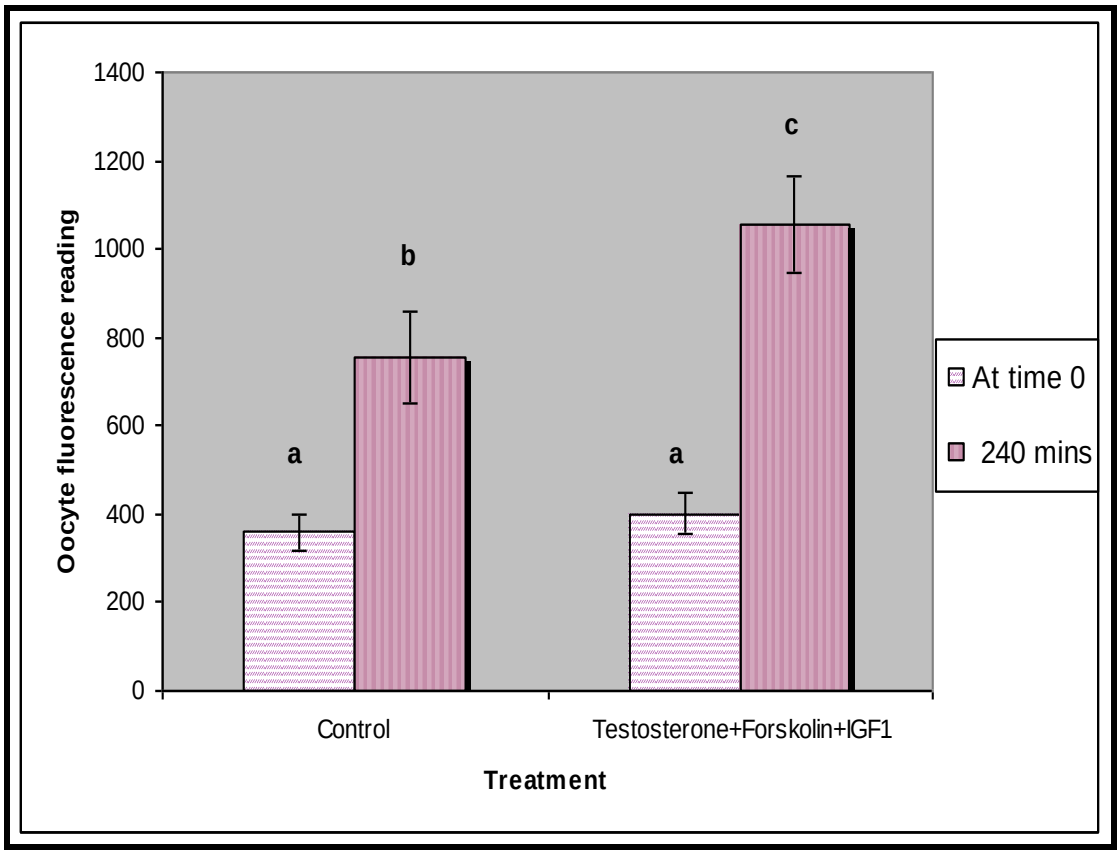

Figure 3.7(ii):- Effect of testosterone + forskolin+ IGF1 on fluorescence intensity in denuded oocytes at 0 and 240 min. For each treatment and time, the mean± sem values were generated from 6 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

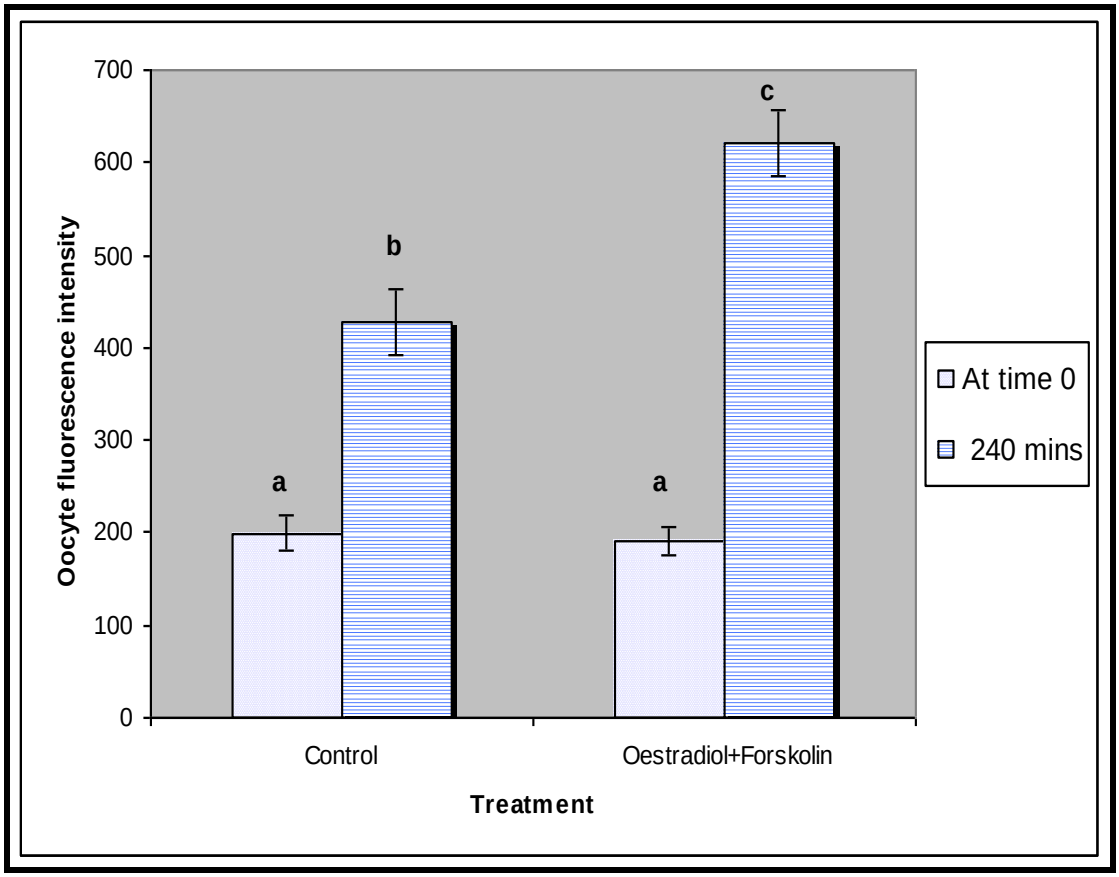

Figure 3.7(iii):-Effect of oestradiol + forskolin or no treatment on fluorescence intensity in denuded oocytes at 0 or 240 min. For each treatment and time, the mean± sem values were generated from 6 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

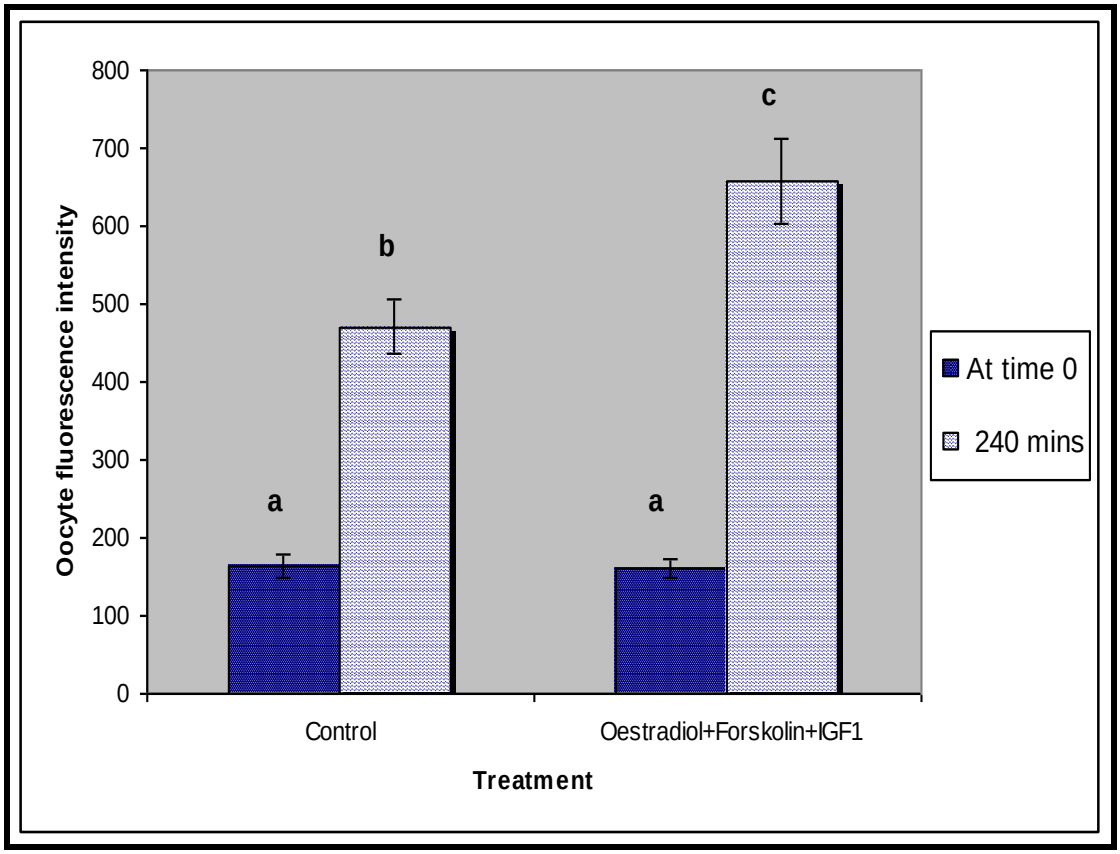

Figure 3.7(iv):- Effect of oestradiol + Forskolin + IGF1 and no treatment on the fluorescent intensity in denuded oocytes at 0 and 240 min. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

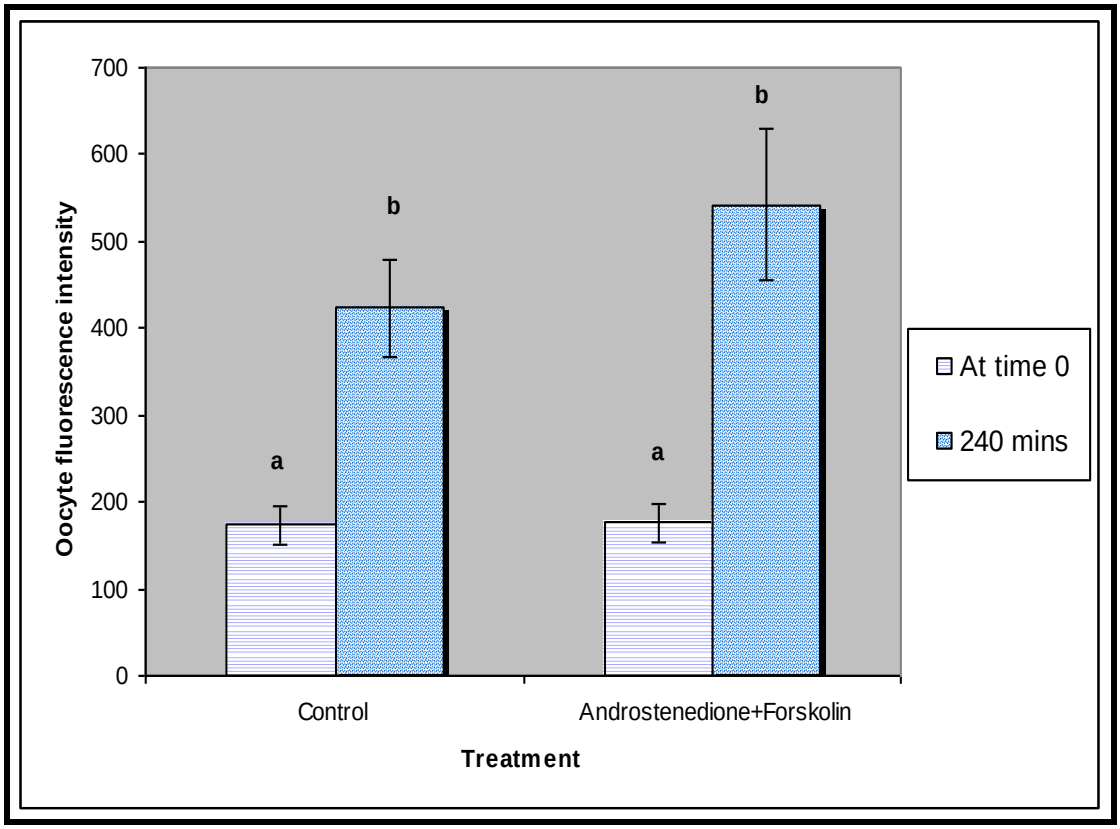

Figure 3.7(v):- Effect of androstenedione + forskolin and no treatment on the fluorescent intensity in denuded oocytes at 0 and 240 min. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

3.8 Effect of various combination of treatments on COCs in the same experiments

In these set of experiments, the effect of treatments on gap junction activity of COCs was compared by treating the COCs from the same rat with different combinations of treatments earlier used. The aims were to test whether any of the combinations added to or negated any of the effects observed with forskolin alone. The combinations tested in the same experiments were: no treatment, testosterone, forskolin and, testosterone + forskolin and; no treatment, oestradiol, forskolin and, oestradiol +

forskolin. These results are summarised in Figures 3.8(i) and (ii). The results from the testosterone and forskolin combinations show that the forskolin treatments enhanced dye transfer relative to the controls and that testosterone did not enhance the effects of forskolin when both were added to the incubation media. Testosterone alone did not enhance fluorescence transfer relative to the untreated control (see Figure 3.7(i)). Likewise, the results from the Oestradiol and forskolin combinations show that the forskolin treatments enhanced dye transfer relative to the controls and that oestradiol did not enhance significantly the effects of forskolin.

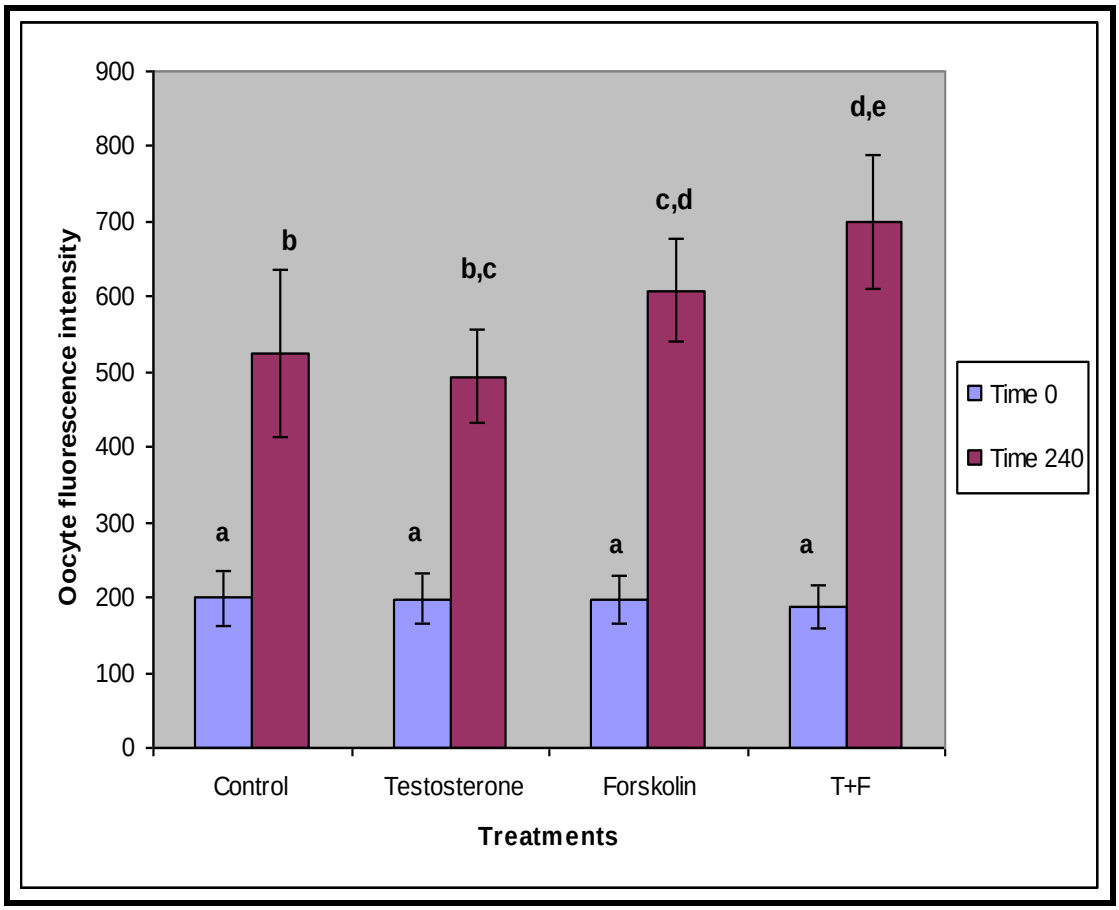

Figure 3.8(i):- Effects of no treatment, testosterone, forskolin and testosterone + forskolin (T + F) on the fluorescence intensities in denuded oocytes at 0 and 240 min. Bars represent mean+sem fluorescence values for control and treated COCs at 0 time period and after four hours of incubation. For each treatment and time, the mean± sem

values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

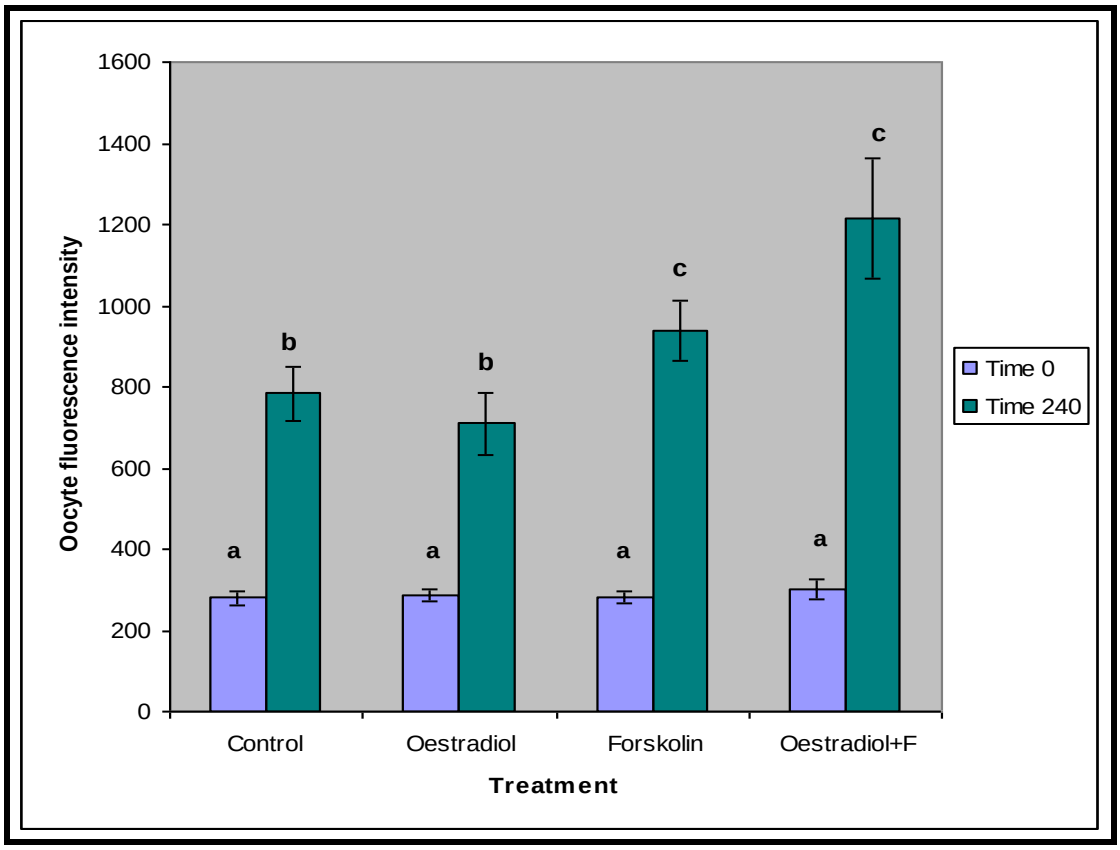

Figure 3.8(ii):- Effects of no treatment, oestradiol, forskolin and oestradiol + forskolin (Oestradiol + F) on the fluorescence intensities in denuded oocytes at 0 and 240 min. Bars represent mean+sem fluorescence values for control and treated COCs at 0 time period and after four hours of incubation. For each treatment and time, the mean± sem values were generated from 5 replicate experiments with 6-10 oocytes in each replicate experiment. Means with different alphabetical superscripts are significantly different ($p<0.05$).

3.9 QPCR results

The effects of forskolin on Cx37 and Cx42 gene expression levels in rat COC were examined. For both Cx 37 and Cx43, no differences in relative levels of gene expression were noted at time 0 between forskolin treated and control groups. As shown in Figure 3.9(i), there was a significant difference in the relative gene expression levels of Cx37 between control and forskolin treated groups after 240mins ($p<0.05$). However, no significant differences were observed in the relative gene expression levels of Cx43 between control and forskolin treated groups after 240mins (Figure 3.9(ii); $p>0.05$).

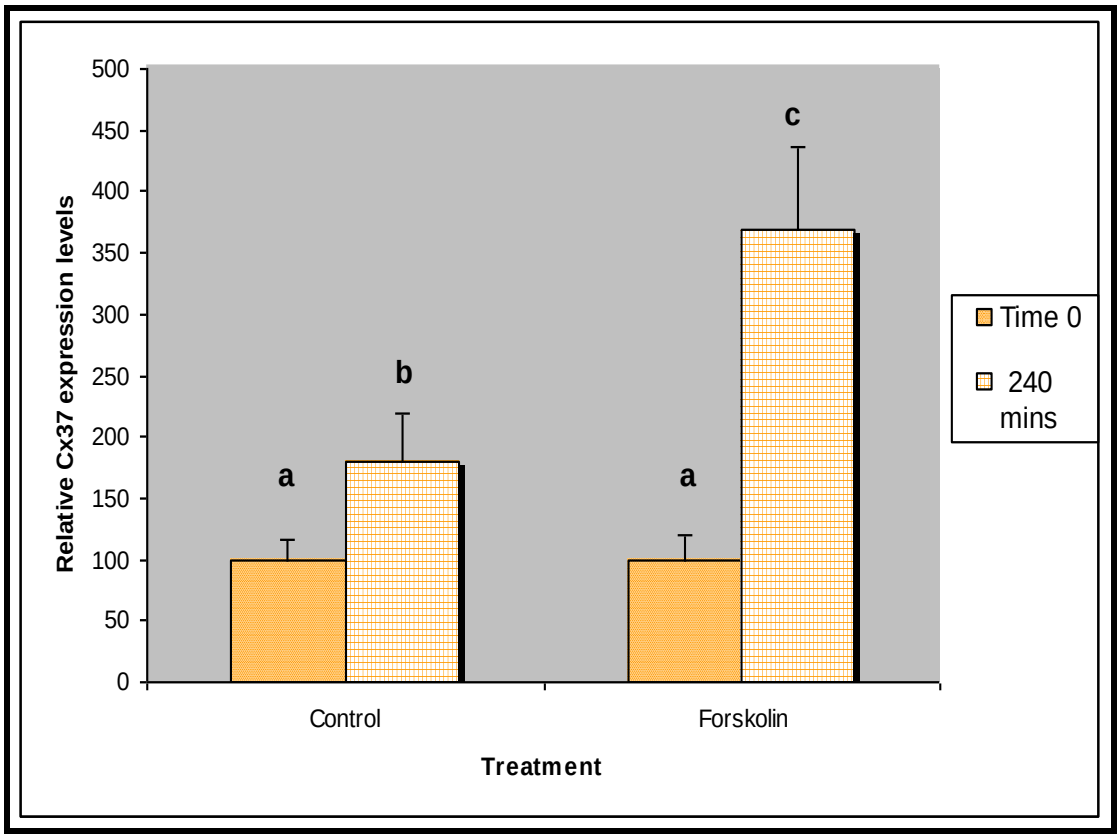

Figure 3.9(i):- Effect of no treatment and forskolin on the relative Cx37 gene expression in COC at 0 and 240mins. The bar graphs represent the mean± sem values for each treatment and means with different alphabetical superscripts are significantly different ($p<0.05$).

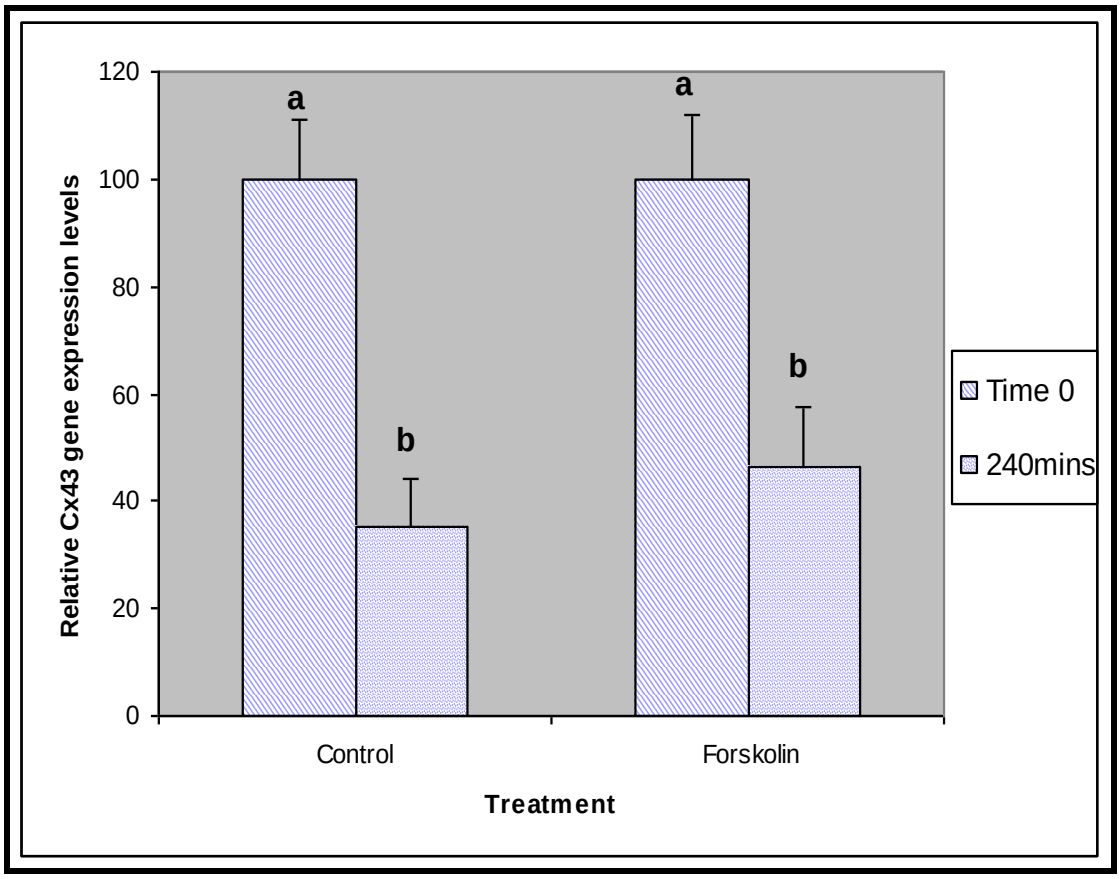

Figure 3.9(ii):- Effect of no treatment and forskolin on the relative Cx43 gene expression in COC at 0 and 240mins. Values are corrected against RPL19.The bar graphs represent the mean± sem values for each treatment and means with different alphabetical superscripts are significantly different ($p<0.05$).

DISCUSSION

The major aims in this thesis, investigating COCs recovered from antral follicles, were: to 1) establish an optimal time in vitro for maximising the transfer of calcein dye from the cumulus cells to the oocyte while maintaining the oocyte in meiotic arrest and; 2) determine the effects of steroids, growth factors and cAMP stimulators on the level of fluorescent dye in the oocyte as a measure of gap junction activity in rat COC.

The application of the Calcein fluorescent dye transfer technique to measure gap junction activity was first reported by Thomas et al, in bovine COC (6). This report validated the finding that the water-soluble and cleaved form of Calcein was unable to exit cells across the cell wall but could only move from one cell to another via gap-junction. This technique has recently been validated for application in porcine COC (100) but has not to our knowledge been investigated in other species.

To carry out the assay, only oocytes with at least two layers of intact cumulus cells around the oocyte were selected. During this study it was noted that the visible characteristics of the oocyte cytoplasm influences the level of fluorescence intensity measured Fig 3.1. Some oocytes had comparatively lower fluorescence intensities than oocytes with visibly normal cytoplasm. These oocytes with low fluorescence readings had a characteristic “granular” cytoplasm. This trend was observed in all oocytes characterised as granular, regardless of what treatment were they subjected to and the time after which they were examined. The granular and normal (agranular) cytoplasm’s were variable over all the experiments. Despite differences in fluorescence being noted based on granulosa characteristics, the fluorescence readings of such oocytes were included in all further analyses. Furthermore, no significant

difference between rats in COC readings was observed therefore the results were pooled across the experiments.

Following the exposure of COC to a 10 min exposure of Calcein, followed by incubation of the COC in Calcein-free media, it was evident that dye transfer from cumulus cells to the oocyte increased over a 180-240 min interval (see Figure 3.2) in the absence of any hormones or growth factors. This suggested that the initial isolation of COC from its follicular environment caused a disruption of gap junction activity. This may have been due to the stress imposed on the oocyte as a result of removing the COC from its gap junction network with the mural granulosa cells. If this notion is correct, then the evidence from this study showed that the COC gap junction activity can recover over time *in vitro*. After 240 min, a decrease in the gap junction activity was observed despite the presence of the PDE inhibitor milrinone. Thus, even though the COC were placed into a nutrient rich culture media, this alone was not sufficient to sustain the COC gap junction communication. An alternative explanation might be that the optimal time for maintenance of Calcein dye at maximum intensity in the oocyte is influenced by the exposure time and/or concentration of Calcein. These parameters were not tested in the present study.

In antral fluid, follicular-derived steroids are present at variable but relatively high concentrations during folliculogenesis. There is evidence that steroids influence follicular growth including granulosa cell proliferation and differentiation (101, 102). The hormonal microenvironment *in vivo* is often used as an indicator of the maturation potential of an individual follicle to develop to ovulation (103). Within this follicular microenvironment, COCs are exposed to relatively high concentrations of androgens i.e. testosterone and androstenedione. These androgens are also the major substrates for oestradiol synthesis by the granulosa cells. Our study suggests

that steroids alone do not have any effect on COC gap junction activity *in vitro*. None of the steroids tested i.e. oestradiol, testosterone, androstenedione and progesterone (See Fig 3.3 i-iv) caused any change in gap junction activity of rat COCs. In previous studies, it has been shown that the treatment of sexually immature rats with oestrogen implants leads to an increase in Cx43 protein expression (104). Burghardt et al 1982 demonstrated that exogenous oestradiol promotes gap junction growth indirectly in rat granulosa cells. They showed that the development of gap junctions occur at a basal rate in the absence of steroids, however exogenous oestradiol prevented atresia and promoted follicular growth during which an increase in gap junctions was observed. Therefore, it seems that oestradiol didn't directly cause an increase in gap junction activity in granulosa cells but as follicular growth was sustained the basal rate of gap junction activity continued over a more extended period (105). The findings from that study are not inconsistent with our results since we didn't observe any direct effect of oestradiol on gap junction activity in rat COC under *in vitro* conditions. Moreover, any indirect measures of the effects of steroids on sustaining follicular growth were beyond the scope of the present study. Nevertheless, it is acknowledged that steroids are indicative of the functional status of a follicle at any moment in time.

It is well known that the concentrations of pituitary and steroid hormones change throughout the menstrual or ovarian cycle and that they change in a specific sequence within the developing follicular environment which influences growth and secretory activity of granulosa cells (101). In the present study we tested the effects of steroids at a single concentration of 1 µg/ml. It has been found that the dominant oestrogen-secreting follicle contains 1µg of oestradiol per millilitre of antral fluid in many species and that the concentrations of androstenedione, testosterone and progesterone can, at times approach these levels (102). However, steroids do vary

significantly in their concentrations in follicular fluid. It is therefore possible that steroidal effects might be observed at lower concentrations but this was not investigated. Oestrogen is thought to be a key steroid and essential for cytoplasmic maturation and in the absence of oestrogen, granulosa cells fail to proliferate (102; 106). However, our study suggests that these processes are unlikely to be influenced directly by the effects of oestradiol on gap junction activity.

The present results demonstrate that IGF1 alone at a concentration of 10ng/ml was unable to show any effect on gap junction activity in rat COC. Previous studies have reported that more gap junctions both among granulosa cells or theca cells and between granulosa cells and the oocyte were observed in follicles cultured in the presence of IGF-I (98). In comparison to our findings, Zhao et al analysed the effect of IGF1 on preantral follicles however their measures of gap junction formation were qualitative rather than quantitative.

The role of forskolin, a cAMP stimulator, was examined in this study and an increase in gap junction activity was observed when compared to COCs without treatment (See Fig 3.5). In contrast with our results, studies with bovine COC showed that treatment of COCs with forskolin prolonged the maintenance of gap junction communication by preventing the loss of gap junctions (6). This study also demonstrated that the effect of forskolin in delaying the loss of gap junctions was enhanced by the presence of milrinone, a PDE3 inhibitor. While milrinone was also present in all our studies, we did not test the effects of time on maintaining gap junction activity nor was the effect of excluding milrinone tested. Previous studies have shown that forskolin inhibits the spontaneous maturation of rat and bovine oocytes in vitro and maintained them in meiotic arrest (6, 92). Moreover, the arresting

action of forskolin was dependent upon the presence of surrounding cumulus cells, as cumulus free oocytes underwent GVBD.

In the present study, although steroids or IGF1 alone didn't show any effect on gap junction activity, the combined effect of various steroids or IGF1 and forskolin (See Fig 3.7 i-v) caused an increase in dye transfer via gap junctions from cumulus cells to the oocyte. The only exception to this was the results of the study with androstenedione+ forskolin. In this study, the androstenedione+ forskolin values were 1.3 fold higher than the control but this did not reach statistical significance. Nevertheless, from these results it was of interest to know if whether the increases in gap junction activity were solely due to forskolin since it was the only factor that earlier demonstrated an ability to increase dye transfer or whether due to the cumulative effects of steroids or IGF1 and forskolin. The results from our studies summarised in Fig 3.8 (i), (ii) suggests that the addition of steroids did not enhance the effects of forskolin when both were added to the incubation media indicating that forskolin alone was responsible for the increase in gap junction activity. While the effects of forskolin+IGF1 were not compared to those of forskolin alone, it seems likely that the significant effects of the former were due to forskolin alone.

Other cAMP stimulators or analogues such as FSH and dcAMP also caused significant increases in the amount of dye transfer (See Fig 3.6(i); 3.6(ii)). It has been well established that FSH drives antral follicle growth *in vivo* and that the presence of this hormone is essential to obtain developmentally competent oocytes in vitro (93). Previously, Burghardt et al 1982 reported that FSH causes gap junction amplification directly in rat granulosa cells. FSH amplified both the number of junctions and the amount of junctional membranes at all follicle sizes that were examined (105). In contrast, studies on porcine COC using the Calcein dye technique published by Bagg

et al 2009 were unable to demonstrate that exposure of COC to cAMP from either small or medium to large follicles increased gap junction *in vitro* over the first 6 h period. Significantly, at that time no significant difference was noted in the intra-oocyte cAMP concentrations between the cAMP and non-cAMP treated groups (110). In that study there was no evidence that a PDE inhibitor was used. Perhaps the lack of effect on gap junction activity was the result of insufficient elevation of intra-COC concentrations of cAMP.

Our results with FSH or dbcAMP are consistent with those of forskolin and suggest that any agents that elevate cAMP intraoocyte levels are likely to cause an increase in dye or reagent transfer from cumulus cells to the oocyte by increasing gap junction activity (105, 107). Importantly, we only tested gap junction communication in COCs after four hours of incubation and did not investigate any other effects of cAMP modulating agents after this time period. With respect to exploring the improved efficacies for IVM, it will be important to investigate the effects of longer term exposure of COC to cAMP stimulators.

FSH stimulates cAMP by first binding to membrane receptors and activating G proteins in cumulus cells, which in turn activates adenylate cyclase activity leading to an increased synthesis of cAMP (108). Forskolin has also demonstrated its ability to increase intraoocyte cAMP levels, since it very rapidly activates the catalytic subunit of adenylate cyclase (91). It has been reported that an increase in intraoocyte cAMP levels causes increased intercellular gap junction communication most likely as a result of increased connexin RNA or protein levels (109). A previous study has demonstrated that cAMP can also increase gap junctional permeability by increasing assembly of Cx43 to plasma membrane gap junctional plaques (110).

It is well known that cAMP plays a crucial role in mammalian oocyte maturation. High levels of cAMP within the oocyte maintain oocyte meiotic arrest, whereas a fall in cAMP enables resumption of meiosis. Both forskolin and dcAMP display an inhibitory effect on oocyte maturation and this inhibitory effect is cAMP mediated (92, 111). In our study, it is suggested that forskolin, FSH and dcAMP stimulated cAMP production in the cumulus cells which is then transferred to the oocyte. This is consistent with previous results showing that higher levels of cAMP are present in oocytes derived from forskolin-stimulated COCs compared to similarly stimulated cumulus-free oocytes (112). It has been suggested that *in vivo* GVBD caused after the LH surge is cAMP mediated i.e. LH causes a rise in follicular cAMP in mural granulosa cells that luteinise leading to a progressive breakdown of gap junction communication across the ganulosa cell-cumulus cell syncytium (60). In the present *in vitro* studies the intra-oocyte cAMP levels that were generated as a result of forskolin, FSH and dcAMP treatments were unlikely during a 4 hour interval to have been high enough to interfere with intercellular communication as compared with the entire follicle *in vivo* where the cAMP produced by the whole follicle over a longer time interval is much greater.

In order to explain how cAMP modulating agents lead to an increase in gap junction activity, one possibility is that elevated cAMP levels restores or maintains pre-existing gap junction connections *in vitro*. Another possibility could be that elevated cAMP levels induce connexin gene expression and in turn this then leads to an increase in gap junction protein.

In the present study, the relative mRNA levels of Cx37 and Cx43 in COC after 0 and 240 min of incubation were examined. From our QPCR results (refer to Fig 3.9(i); (ii)) it can be observed that in both control and treatment groups, the relative

levels of Cx37 mRNA increased over 240min of incubation. Moreover, treatment of COCs with forskolin caused a significant increase in Cx37 gene expression when compared with COCs without any treatment (control) at 240 min. In contrast, the relative Cx43 mRNA levels decreased after 240 min incubation and no difference was observed in gene expression levels of Cx43 when compared with the control group. Since Cx43 is present in cumulus cells and in order to prevent any variation caused by the number of cumulus cells attached to each oocyte examined, the data for Cx43 was corrected against the relative expression levels of the housekeeping gene (RPL19). Furthermore, since Cx37 levels were analysed in one individual oocyte per experiment, it isn't likely to have caused variation and in turn we didn't correct our Cx37 results with RPL19. A key issue to address here is the possibility that there may be some variation introduced by the housekeeping gene itself since it is present in both cumulus cells and the oocyte. However we selected for similar sized COCs and treated them with the same treatment i.e. forskolin, thereby it is unlikely to influence the results observed with Cx43.

It seems clear from these results that the elevated cAMP levels caused by forskolin increase the levels of Cx37 gene expression. However, further studies with respect to measures of Cx43 are required in order to examine changes when corrected with more localised housekeeping genes i.e. genes only present in cumulus cells and not within the oocyte.

Gap junction communication involves a sequence of events including connexin synthesis (transcription and translation), trafficking to plasma membrane and connexon assembly. It is likely that each event in this sequence is regulated by phosphorylation steps involving the cAMP mediated cascades. Therefore we propose that cAMP may lead to elevated or maintained connexin mRNA levels to thereby

promote gap junctional permeability. It has previously been reported that the upregulation of junctional communication can be induced by cAMP which facilitated an increase in gap junctional plaques recognized by freeze fracture analysis (113).

With respect to IVM, the inclusion of a specific PDE3 inhibitor such as milrinone and cAMP modulating agents in media are both likely to be important for enhancing the developmental potential of oocytes collected as COC from antral follicles not considered to have reached the preovulatory stage of maturation. We propose that the use of milrinone with forskolin or FSH or dcAMP will elevate intra-oocyte cAMP, inhibit the resumption of meiosis and importantly promote gap junction communication thereby prolonging the opportunity for exchange of essential metabolites from the cumulus cells to the oocyte to advance cytoplasmic maturation in oocytes.

Important future directions include examining the effects of the oocyte-secreted factors such as GDF9 and BMP15. As outlined earlier, both GDF9 and BMP15 have essential regulatory roles in ovarian follicular development (78). Studies have shown that when COCs are treated with exogenous GDF9 and BMP15, an improved oocyte developmental potential is observed (114). Such treatments were shown to cause a 50% increase in developmental competence as well as improved embryo quality. It has also been demonstrated that the addition of GDF9 in IVM media increases the embryo production efficiency in mice (114). Furthermore, analysing the effects of cAMP stimulators at various concentrations for longer time intervals together with further information on connexin 37 and 43 mRNA and protein synthesis as well as other culture/incubation media supplements would provide new insights into improving the applications of IVM in artificial reproductive technologies.

CONCLUSION

In this study, we determined the effects of various factors on the gap junction activity in rat COCs. Isolation of rat COC from their follicular microenvironment appears to impair gap junction activity within COCs as assessed by the measurement of dye transfer however this activity was recovered over time in vitro. Under *in vitro* conditions all cAMP stimulators i.e. forskolin, FSH, dcAMP together with milrinone caused an increase in gap junction activity between cumulus cells and the oocyte. Steroids and IGF1 alone didn't show any effect on gap junction activity. Furthermore, forskolin caused an increase in the relative Cx37 gene expression levels after a four hour incubation period.

Together the findings from this study suggest that cAMP stimulators are important in terms of maintenance of gap junction permeability between cumulus cells and the oocyte in rats and in order to develop an in vitro maturation culture medium, it is vital to incorporate agents that elevate intraoocyte cAMP levels.

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APPENDICES

Appendix1:- Company addresses

Company	**Address**
Invitrogen	Penrose Auckland 1006, New Zealand
Sigma-Aldrich	Auckland 1030, New Zealand
Novozymes	Bagsvaerd 2880, Denmark
Prospec	Rehovot 76103, Israel
Epicentre	Madison, WI153713, USA
Corbett Research	Mortlake NSW 2137, Australia
Stratagene	La Jolla 92037, CA