OOCYTE-FOLLICLE INTERACTIONS

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Declaration

I declare that the studies undertaken for this thesis were devised and conducted by me. Where other sources have been used, they are acknowledged by reference.

No part of this work has been previously submitted or accepted for any other award.

Adam Marsh
December 2011

Abstract

The ovarian follicle is an individual functional unit that provides the optimal environment for the oocyte within to develop. This thesis outlines the research in the field of ovarian follicular dynamics that has already been established, and further develops these findings to explore in greater detail the relationship between the oocyte and its environment, both in an *in vitro* and *in vivo* setting, using a variety of species.

The first major research area involved studying the role of oocyte-secreted factors, which was examined using a series of dose response experiments. These were performed using an ovine granulosa cell culture model, and elucidated a possible role for a collaborative action of BMP15 and GDF9 in the promotion of oestradiol synthesis, while inhibiting production of progesterone in this species. This finding was then further investigated using an ovine *in vivo* immune-neutralisation study, the endocrine and histological results of which confirmed these findings in a proportion of these animals, although this study was limited by the animals appearing to have been in seasonal anoestrus.

The second major topic that was investigated was based around the ovarian microenvironment, in terms of angiogenesis and hypoxia. Again, ovine granulosa cell cultures were used, in this instance to examine the effect of hypoxic conditions on steroid hormone production. These experiments indicated that somatic cell steroid hormone production is likely to be compromised by a hypoxic environment, and therefore that the provision of oxygen through a local blood supply may be a vital requirement for these cells.

To investigate the relevance of studying ovarian blood supply and physiology in a clinical setting, perfusion studies were carried out based on a series of bovine phantom experiments, which were used to study the effect of varying flow rate on the parameters routinely measured using this technology. The routine clinical ultrasonographic methods of ovarian assessment such as 4D View™, SonoAVC™ and VOCAL were also examined, based on bovine phantom experiments, revealing possible weaknesses in the data provided by ultrasound that are increasingly relied upon in the clinical setting.

Finally, a clinical trial was carried out to try and encompass all of the findings of the *in vitro* and *in vivo* work, in order to place these theories into context in a human IVF setting. This work was unfortunately limited severely by a lack of patient numbers, but some interesting results were observed with regard to oocyte developmental potential relationships with follicular fluid and somatic cell factors, as well as ultrasound measures of peri-follicular blood supply.

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Abbreviations

2D	Two Dimensional
3D	Three Dimensional
AFC	Antral Follicle Counting
ALK	Activin-like Kinase
AMH	Anti-Müllerian Hormone
ART	Assisted Reproductive Technology
BMP	Bone Morphogenetic Factor
BTC	Betacellulin
cDNA	Complementary Deoxyribonucleic Acid
CEEF	Cumulus Expansion Enabling Factor
COC	Cumulus-oocyte Complex
COX	Cylcooxygenase
CV	Coefficient of Variation
d(V)	Diameter by Volume
DAB	Diaminobenzidine
DARS	Donkey Anti-rabbit Serum
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DO	Denuded Oocyte
DOB	Date of Birth
DON	6-diaxo-5-oxo-L-norleucine
DTT	Dithiothreitol
E2	Oestradiol
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
FDX	Ferredoxin
FGF	Fibroblast Growth Factor
FI	Flow Index
FSH	Follicle-stimulating Hormone
GAST	Gondaotrophin-releasing Hormone Against Stimulation Test
GC	Granulosa Cell
GDF	Growth Differentiation Factor
Grat.	Graticule
GREM	Gremlin
h	Hours
H&E	Hematoxylin & Eosin
HAS	Hyaluronan Synthase
hCG	Human Chorionic Gonadotrophin
HD	High Density

HIF	Hypoxia-inducible Factor
HRE	Hypoxia-responsive Element
HRP	Horseradish Peroxidase
ICSI	Intracytoplasmic Sperm Injection
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IMS	Industrial Methylated Spirit
INHBA	Inhibin A
IRAS	Integrated Research Application System
IU	International Units
IV	Intravenous
IVF	*In Vitro* Fertilisation
k	Thousand
KitL	Kit Ligand
KLH	Keyhole Limpet Hemocyanin
L&R	Left & Right
LD	Low Density
LDCC	Low Density Cobalt Chloride
LDSA	Low Density Sodium Azide
LH	Luteinizing Hormone
LIF	Leukaemia Inhibitory Factor
LO & RO	Left & Right Ovaries
m-d	Mean Diameter
M-MLV	Moloney Murine Leukaemia Virus
MII	Metaphase II
min	Minutes
mRNA	Messenger Ribonucleic Acid
n	Number
NIDDK	National Institute of Diabetes + Digestive + Kidney Diseases
NRS	Normal Rabbit Serum
NSB	Non-specific Binding
NT	No Treatment
OCM	Oocyte-conditioned Medium
OOX	Oocytectomized Complex
OSF	Oocyte-secreted Factor
P4	Progesterone
PAPP	Pregnancy-associated Plasma Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PI	Pulsatility Index
POEM	Pre-implantation Oocyte and Embryo Markers
POI	Primary Ovarian Insufficiency

PTGS	Prostaglandin-endoperoxidase Synthase
QC	Quality Control
RCF	Relative Centrifugal Force
RI	Resistance Index
ROS	Reactive Oxygen Species
rpm	Revolutons Per Minute
S-D	Systolic-diastolic Ratio
SAPU	Scottish Antibody Production Unit
SD	Standard Deviation
SEM	Standard Error of Means
SET	Single Embryo Transfer
TC	Total Count
TGF	Transforming Growth Factor
TMB	Tetramethylbenzidine
TNFAIP	Tumour Necrosis Fector Alpha-induced Protein
Tx	Treatment
UMP	Uridine Monophosphate
UOP	Utero-ovarian Plexus
V	Volume
v/v	Volume by Volume
VEGF	Vascular Endothelial Growth Factor
VFI	Vascular Flow Index
VI	Vascular Index
VOCAL	Virtual Organ Computer-Aided Analysis
VOI	Volume of Interest
w/v	Weight by Volume
β-ME	Beta-mercaptoethanol

There are in fact two things, science and opinion; the former begets knowledge, the latter ignorance.
Hippocrates (460 BC - 377 BC)

Chapter 1 – Literature Review

1.1. Introduction

In the field of reproductive medicine there is increasing awareness that oocyte quality is a major limiting factor in female fertility, probably as a result of the increasing age of first conception for mothers, resulting in poorer conception rates. The current lack of understanding of the physiological mechanisms controlling ovarian follicle development is an obstacle that needs to be overcome if the techniques associated with assisted reproduction are to improve in efficacy (Campbell *et al.*, 2003). Two of the major hurdles that need to be overcome in the near future are solving the problems of the lack of objective and reliable oocyte developmental competence predictors, and the poor success of *in vitro* maturation (Li *et al.*, 2008a; Gilchrist *et al.*, 2008). The oocyte developmental potential, and hence the chance of later obtaining a pregnancy, is determined by the quality of the oocyte. Oocytes gain developmental competence [defined as ability to mature, become fertilised, and give rise to normal fertile offspring (Duranthon and Renard, 2001)] gradually through the process of folliculogenesis, as the oocyte increases in size and its surrounding somatic cells differentiate (Eppig *et al.*, 2004). Morphological parameters are routinely used for oocyte selection during *in vitro* fertilisation, based on the visual appearance of the cytoplasm, polar body and cumulus cells according to the opinion of the embryologist. The basis this selection method is highly subjective and therefore inaccurate (Balaban *et al.*, 1998; Nicholas *et al.*, 2005; Wang and Sun, 2007). Only a third of IVF cycles lead to a pregnancy, and around 8 out of 10 transferred embryos fail to implant (Bromer and Seli, 2008). As a result, the discovery of objective and non-invasive molecular markers capable of predicting oocyte developmental potential is a key area of research (Li *et al.*, 2008a).

There is an extremely diverse range of factors that affect oocyte competence during folliculogenesis, doubtlessly not all of which are known or fully understood. For example, the size of the follicle of origin (Lonergan *et al.*, 1994; Rosen *et al.*, 2008), the health status of the follicle (Hagemann, 1999), the extent of hormonal stimulation (Sirard *et al.*, 2006) and also the bi-directional communication between the oocyte and cumulus cells (Krishner, 2004; Gilchrist *et al.*, 2008) have all been studied as potential candidate targets in research. All of these factors contributing to oocyte competence need to be further

researched and understood so that they may be used in the drive to improve efficacy of clinical *in vitro* fertilisation. By examining the molecular and cellular pathways involved in the follicular setting, it is hoped that the underlying mechanisms can be revealed and the knowledge integrated in order to develop a model of the processes determining oocyte quality.

1.2. The Reproductive Cycle

In order to fully comprehend the complex process of folliculogenesis, it is necessary to understand the basis for the reproductive cycle. This topic could, in itself, fill an entire thesis, however for the purposes of this research, the reproductive cycle and the endocrinology are summarised.

1.2.1. Reproductive hormones

Gonadal function in the female is primarily under the control of the hypothalamic-pituitary axis, forming a dynamic relationship between the ovary and the brain (figure 1.1). The sequence of interrelated endocrine events involves the hypothalamus producing gonadotrophin-releasing hormone (GnRH), the pituitary gland secreting follicle-stimulating hormone (FSH) and luteinising hormone (LH), antral follicles of the ovary producing oestradiol and inhibin and the corpus luteum secreting progesterone (Bartlewski *et al.*, 2011).

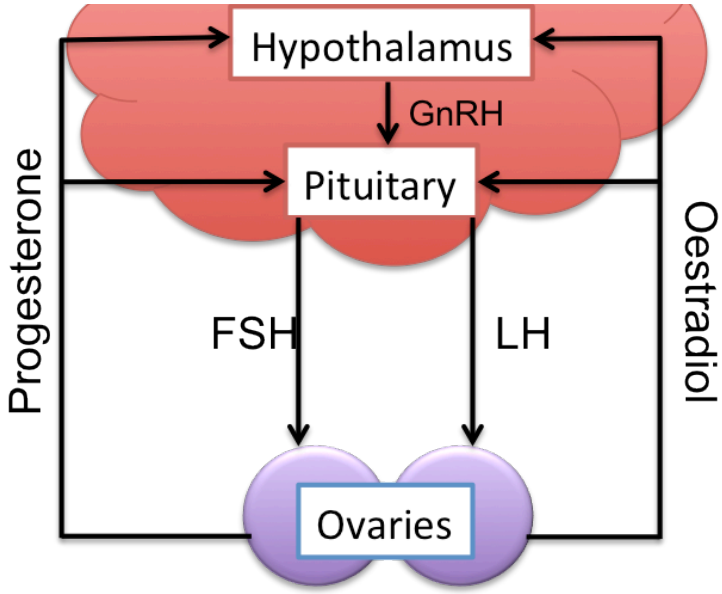

Figure 1.1. The hypothalamic-pituitary-ovarian axis

Development of the follicle, maturation of the oocyte, follicular steroidogenesis, ovulation and formation of the corpus luteum are all under the regulation of pituitary gonadotrophins, and the regulation of gonadotrophins is controlled by a range of internal factors such as ovarian steroid hormones, neuromodulators and uterine products, and external factors such as photoperiod and nutrition.

Oestradiol, a steroid hormone produced by granulosa cells in the ovary, inhibits FSH and LH secretion when at low circulatory levels. At higher levels this negative feedback switches to positive feedback, and this results in a surge in both FSH and LH production. In addition to oestradiol, when at high levels, for example in the luteal phase of the cycle, progesterone also acts to suppress FSH and LH. Progesterone is also able to block the positive feedback effects of high oestradiol levels, thereby preventing the surge of FSH and LH (Adams *et al.*, 2008).

1.2.2. The oestrous cycle

In the bovine and ovine species, which are at the core of research models for human reproductive physiology, the reproductive cycle is called the oestrous cycle. During the oestrous cycle, which lasts for an average of 21 days in cattle (Grunert *et al.*, 1973), and 17 days in sheep (Mellin and Busch, 1976), follicular development can be observed to occur in a wave-like pattern, under the control of hormonal regulation. Following ovulation, a new follicular wave commences. This begins with a number of follicles being recruited from the pool of quiescent follicles within the ovary, the mechanism of which is not yet understood. Once this follicular growth is initiated, development continues until the follicle reaches one of two fates; ovulation or atresia. In a monovulatory species such as the cow, only a tiny fraction, less than 0.1%, of these follicles will ovulate (Webb *et al.*, 2003). The rest will become atretic and regress.

In cattle, the cycle is characterised by two or three follicular waves, each with around three to five developing follicles, growing to more than 4mm in diameter (figure 1.2). These follicles then continue to grow to reach 6-8mm, whereby a mechanism of dominance is employed, which allows for the selection of just one follicle to become dominant over its subordinates and inhibiting their development (Webb *et al.*, 2003).

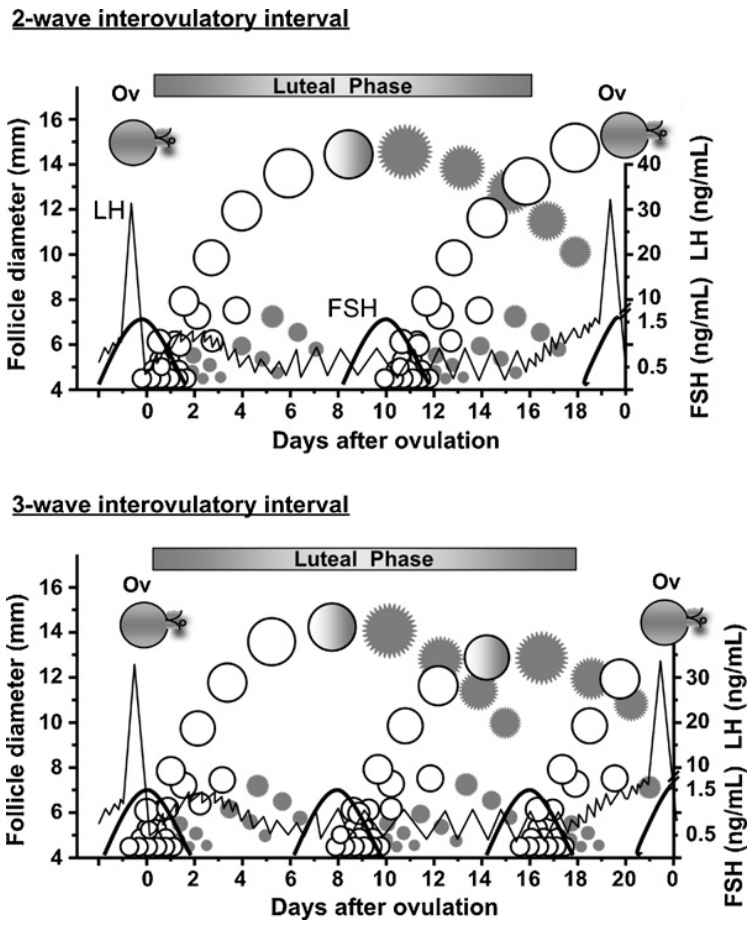

Figure 1.2. Dynamics of bovine ovarian follicular development and gonadotrophin secretion during two- and three- wave oestrous cycles (Adams *et al.*, 2008)

Dominant and subordinate follicles are open (viable) and shaded (atretric) circles. Each new wave of follicles is preceded by an FSH surge (thick line) and an ovulation is preceded by an LH surge (thin line). The LH surge is caused by high LH pulse frequency due to decreased progesterone concentrations (Adams *et al.*, 2008).

Only when progesterone concentrations are low enough, will this dominant follicle ovulate, however if a corpus luteum is present, the level of progesterone will prevent the process of ovulation, and this dominant follicle undergoes atresia. A new follicular wave will then commence.

Formation of the corpus luteum (CL) occurs as a result of a series of morphological and biochemical changes in the theca and granulosa cells of the preovulatory follicle during luteinisation, after the LH

surge. The CL becomes highly vascularised, and blood flow in the tissue increases with volume and with the production of progesterone (Berisha and Schams, 2005).

1.2.3. The menstrual cycle

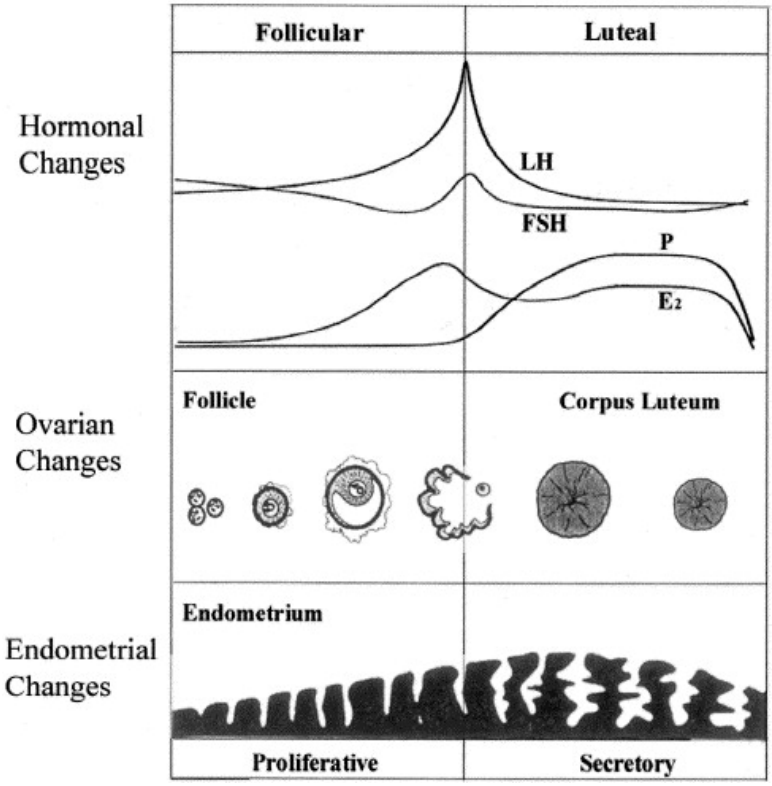

Figure 1.3. The menstrual cycle (Petroff *et al.*, 2001)

Higher primates do not present with 'oestrus' as such, and therefore their reproductive cycle is not called an oestrous cycle. The characteristic periodic shedding of the endometrial lining of the uterus distinguishes these species from others, in the process of menstruation, hence the cycle is called the menstrual cycle (figure 1.3). Although both the oestrous cycle and the menstrual cycle reflect cyclical changes in the ovary and reproductive organs, the days at which these cycles begin differs; day 1 of the oestrous cycle is considered to be the first day of oestrus, and in humans the first day of the cycle coincides with the initiation of menstrual flow.

Another difference between the oestrous and menstrual cycles is the follicular to luteal phase ratio. The menstrual cycle usually consists of a follicular phase length equal to that of the luteal phase, whereby

luteolysis is followed by menstruation and then the initiation of a new cycle. The follicular phase of the oestrous cycle is considerably shorter than that of the menstrual cycle, as follicular growth takes place in the luteal phase of the previous cycle. No menstruation takes place following, however there are usually changes in reproductive behaviour around this time.

1.3. Follicular Development

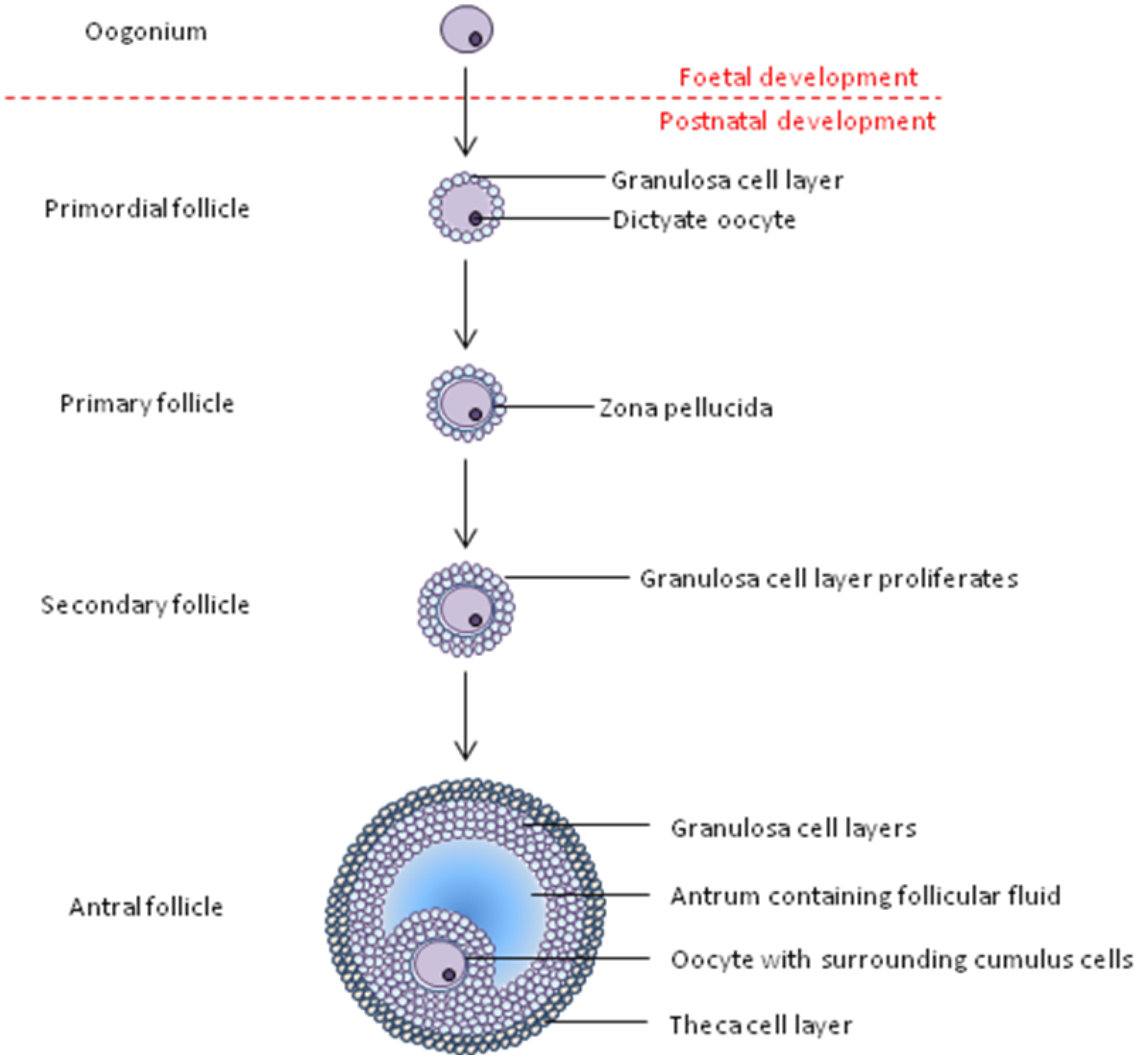

Figure 1.4. Schematic diagram of follicular development in the mammalian ovary

1.3.1. Preantral follicle development

Primordial follicles in the ovary consist of a single oocyte, which is surrounded by a flattened or cuboidal granulosa cells (McNatty *et al.*, 1999). The pool of around 100,000-250,000 ovarian primordial follicles is established during foetal development (Gougeon, 1996; Juengel *et al.*, 2002b), and once selected for development they leave the resting pool to become part of the growing population, by a mechanism that is not yet fully understood (Webb *et al.*, 2003). Once growth has commenced, follicular growth continues until the follicle reaches its final fate of either atresia or ovulation (figure 1.4).

It is thought that anti-Müllerian hormone (AMH) is one of the growth factors responsible for maintaining the primordial follicles in the dormant state (Durlinger *et al.*, 2002), and that the granulosa cell-produced kit ligand is likely to be the main factor involved with primordial follicle activation (Parrott and Skinner, 1999), the effects of which may be enhanced by leukemia inhibitory factor (LIF) (Nilsson and Skinner, 2002).

Bone morphogenetic protein 7 (BMP7) is also believed to be a requirement for the transition in the rat (Lee *et al.*, 2001), and in other species additional members of the transforming growth factor β (TGFβ) superfamily such as growth differentiation factor 9 (GDF9), activins and inhibins, as well as other growth factors including basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) may also be needed (McNatty *et al.*, 1999; Knight and Glister, 2001; Smitz and Corvindt, 2002; reviewed in Webb *at al.*, 2003). It is believed that gonadotrophins are unlikely to be involved in follicular growth initiation (Campbell *et al.*, 2000; Fortune *et al.*, 2000).

As well as the growth factors associated with the primordial to primary follicle transition, a number of gene studies have revealed three oocyte-specific genes that are also thought to be needed; the newborn ovary homeobox-encoding gene (Nobox) (Pangas *et al.*, 2004b), *Sohlhl I* and *Lhx8* (Pangas *et al.*, 2006) (reviewed in Rodigues *et al.*, 2008). However, almost all of the studies concerning oocyte specific genes have been based on polyovulatory rodents, and so further study is needed to examine the roles of these genes in different species.

The mechanism behind activation of primordial follicles has not yet been fully elucidated, however follicular growth itself can be divided into two distinct stages (Braw-Tal and Yossefi, 1997). The first begins with granulosa cell differentiation to alter their shape from squamous to cuboidal, and the second

consists of granulosa cell proliferation, as well as oocyte growth. The stimulus for this process is not known, however it seems that the oocyte is the major driving force, producing factors that act by both autocrine and paracrine mechanisms (Eppig, 2001). In addition, follicle-stimulating hormone (FSH) can accelerate preantral follicle development (Campbell *et al.*, 2000; Gutierrez *et al.*, 2000), indicating a gonadotrophic role at an early stage of folliculogenesis.

Following the formation of a complete layer of cuboidal granulosa cells around the enlarging oocyte, the primordial follicle becomes a primary follicle (Hirshfield, 1991). Although small preantral follicles are responsive to FSH, the hormone is not an absolute requirement for granulosa cell proliferation and follicular growth to occur (McGee *et al.*, 1997). However, as the follicle progresses to the next stage of development, the granulosa cells become responsive to FSH, as they acquire receptors; a process regulated by a range of locally produced growth factors including insulin-like growth factor-I (IGF-I) (Zhou *et al.*, 1997; Gutierrez *et al.*, 2000). IGF-I promotes granulosa cell proliferation and stimulates the luteinising hormone (LH) receptors of granulosa and theca cells, marking the timepoint at which these cells become receptive to LH (Magoffin and Weitsman, 1994; Richards, 2001).

1.3.2. Antrum formation

In the very early stages of development, follicles of 250μm become responsive, but not dependant on gonadotrophin stimulation. As folliculogenesis proceeds the follicles enter a critical phase in which they become completely dependant on FSH for survival, from around 2-4mm in diameter in cattle, and 1-2mm in sheep (Campbell *et al.*, 1995; Webb *et al.*, 1999a; Markstrom *et al.*, 2002). In addition, locally-produced factors such as IGFs are believed to play a role by enhancing the effect of FSH (Webb *et al.*, 1999b; 2003). IGF-II mRNA expression has been found in thecal cells of follicles at the time of antrum formation, as well as type 1 IGF receptor and some IGF binding proteins (Armstrong *et al.*, 2000). Several other growth factors, such as members of the TGFβ superfamily, are also believed to be present in the early antral stage. The roles of these factors are not fully understood, however they are probably involved in follicular differentiation by enhancing gonadotrophin action (Campbell and Baird, 2001; Souza *et al.*, 2002).

Formation of the follicular antrum occurs in cattle and sheep when the follicle reaches a diameter of around 200-400μm (Turnbull *et al.*, 1977). Antrum formation is accompanied by a divide in the granulosa cell population into two primary groups, with a switch from proliferation to differentiation (Webb and Campbell, 2007). One group become the cumulus cells, closely associated with the oocyte to form the cumulus-oocyte complex. Their cytoplasmic projections penetrate through the oocyte zona pellucida, forming the gap junctions required for oocyte-cumulus cell communication (Albertini *et al.*, 2001) (figure 1.5). The second group is composed of the steroidogenic 'mural' granulosa cells which line the follicular wall, with the cells nearest to the antrum being termed periantral granulosa cells (Eppig, 2001). These two cell types become not only phenotypically distinct from one another, but also functionally different. The cumulus cells, unlike mural granulosa cells, have a high rate of proliferation, a low rate of steroidogenesis and barely any LH receptor expression. They also have the ability to secrete hyaluronic acid, and consequently undergo expansion (Salustri *et al.*, 1990; Li *et al.*, 2000). mRNA expression changes significantly in the two cell groups, most notably that of the steroidogenic enzymes such as P450scc, P450c17 and P450arom (Bao *et al.*, 1997).

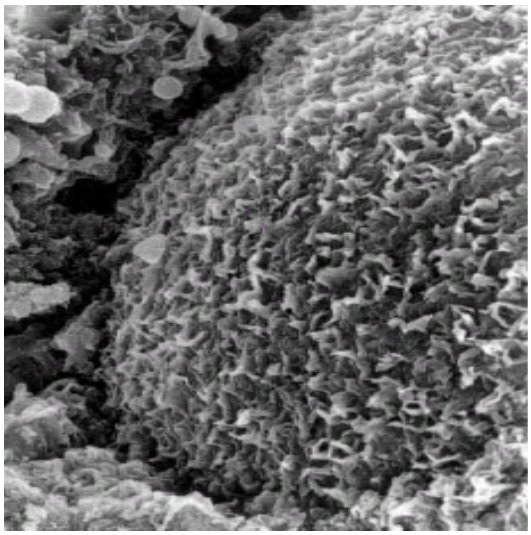

Figure 1.5. Scanning electron microscopy image of a human secondary oocyte after zona pellucida removal (x10,000) (Makabe et al., 2006).

The oolemma surface is covered with microvilli, to which the cumulus cell projections become associated, through the zona pellucida. The gap junctions, which span from the oocyte to its surrounding granulosa cells, are intercellular membrane channels, through which small molecules such as pyruvate, amino acids and signalling molecules can pass (Kidder and Mhawi, 2002; Yeo *et al.*, 2009). These molecules are a

means of communication between the oocyte and cumulus cells, and include ions, metabolites and amino acids needed for oocyte growth, along with the small regulatory molecules that are key for oocyte development (Simon *et al.*, 1997). The junctions themselves consists of groups of 6 connexins; protein subunits that join together to form a channel called a connexon. The connexons of adjacent cells then connect to form the gap junction (Bruzzone *et al.*, 1996).

If the oocyte-cumulus cell communication axis is disrupted, cumulus cell proliferation is reduced, and apoptosis increases considerably (Luciano *et al.*, 2000). Furthermore, the attachment of the cumulus cells to the oocyte has been shown to be crucial for meiotic maturation and fertilisation, using bovine and porcine models (Zhang *et al.*, 1995; Luciano *et al.*, 2005; Amano *et al.*, 2005).

Along with the cellular differentiation during antrum formation, there is also a major step in oocyte development, as the oocyte gains the molecular and cytoplasmic apparatus needed to fully support the process of embryo development (Sirard *et al.*, 2006). Prior to antrum formation the oocyte is arrested at diplotene stage of meiosis I, controlled by an intricate balance between intracellular factors including cAMP, purines and calmodulin (Lanuza *et al.*, 1998), and is meiotically incompetent, probably due to a lack of regulatory signal molecules required to resume meiosis (Eppig, 2001). However, the vast majority of oocytes in antral follicles are in fact meiotically competent, and have been shown to spontaneously resume meiosis if they are removed from follicles and cultured (Handel and Eppig, 1997).

Oocytes that are capable of resuming meiosis are, however, not necessarily able to complete nuclear maturation and hence progress to metaphase II. Because oocytes from small antral follicles resume meiosis but arrest at metaphase I, it seems that further development of antral follicle oocytes is needed to attain competence to continue to metaphase II (Handel and Eppig, 1997). The oocyte normally acquires the ability to resume meiosis after antrum formation is complete (Mehlmann, 2005), following a number of changes in the oocyte cytoplasm and nucleus.

1.3.3. Cumulus expansion

The oocyte is surrounded by a number of layers of cumulus cells, in the majority of mammals, which form a 'cloud' around the oocyte; the cumulus-oocyte complex (COC) (Yokoo *et al.*, 2008; Gosden and Lee, 2010). The structure of the cumulus-oocyte complex (COC) is such that it promotes separation from the follicle wall, as well as oocyte expulsion in ovulation (Yokoo *et al.*, 2008). The primary functions of the cumulus cells are fundamentally to support the development of the oocyte, in addition to production of a number of hormones and growth factors (Gosden and Lee, 2010). It therefore seems obvious why ovulation rate and overall fertility falls significantly if this process of cumulus expansion is prevented (Chen *et al.*, 1993; Fulop *et al.*, 2003), as the cumulus plays a vital role in the process of folliculogenesis.

In response to the preovulatory LH surge, the oocyte undergoes the maturation process, and the surrounding cumulus cells begin to secrete hyaluronic acid, which is bound to the cell surfaces by linker proteins (Chen *et al.*, 1996). When this becomes hydrated, spaces form between the cumulus cells and a sticky cell matrix is formed (Eppig, 2001). Hyaluronic acid, also known as hyaluronan, is synthesized by the hyaluronan synthase (HAS) enzyme, which exists in three isoforms (HAS1, HAS2 and HAS3) (Spicer and McDonald, 1998). HAS2 mRNA is expressed strongly in cumulus cells after an LH surge, and it appears to play a key role in regulation of cumulus expansion (Yokoo *et al.*, 2008). Hyaluronic acid is synthesized from glucose in the hexosamine biosynthesis pathway, a process regulated by uridine monophosphate (UMP), which inactivates hyaluronan synthase (Gutnisky *et al.*, 2007). 6-diazo-5-oxo-L-norleucine (DON) also has a major role in inhibition of hyaluronic acid synthesis (Prehm, 1983).

Cumulus expansion is not only due to synthesis of hyaluronic acid, but also its accumulation in the spaces between the cumulus cells. It remains in the extracellular matrix, stabilized by hyaluronan-binding proteins, and as a result there is a close relationship between the extent of cumulus expansion and hyaluronic acid accumulation. This relationship has been explored in pigs, where a link has been found to the oocyte maturation process (Yokoo *et al.*, 2008).

Cumulus expansion can be induced *in vitro* using various hormones, growth factors and serum supplements, depending on the species, which implicates distinct mechanistic differences by which expansion occurs in different species (Gutnisky *et al.*, 2008). For example, cumulus expansion in mice, in contrast to pigs, rats and cattle (Vanderhyden, 1993; Ralph *et al.*, 1995), requires the presence of the

oocyte (Sutton *et al.*, 2003), which is due to oocyte expression of the cyclooxygenase-2 (COX2) gene (Joyce *et al.*, 2001). However in cattle, BMP15, secreted by the oocyte, is needed for cumulus cell survival during oocyte maturation and cumulus expansion (Hussein *et al.*, 2005). In addition, cumulus expansion enabling factor (CEEF), secreted from the oocyte, is another factor required for FSH-induced cumulus expansion (Salustri *et al.*, 1990). Therefore, it seems that there are a number of secretory growth factors that are involved in the process, the requirements of which are markedly different between species.

Cumulus gene expression has been investigated to attempt to find a correlation to oocyte quality and developmental capacity, although the extent of cumulus expansion is a morphological criterion for oocyte selection in IVF that has been used now for a number of years (Russell and Robker, 2007). Gene expression studies of COX2 and HAS2 for example, have been significantly correlated with blastocyst quality (McKenzie *et al.*, 2004), indicating a possible role in the future for gene profiling as an additional oocyte competence indicator.

Research using rodents suggests that cumulus expansion may be in some way functionally related to oocyte maturation (Wert and Larsen, 1989) and fertilisation (Chen *et al.*, 1993). However, in cattle the relationship between expansion and oocyte developmental capacity has not yet been elucidated (Gutnisky *et al.*, 2007).

1.3.4. Follicle selection and dominance

In monovulatory species, just one dominant follicle from the developing cohort is able to continue all the way to ovulation, while the others regress by a process called atresia. While this system has been studied extensively, and the involvement of gonadotrophins as well as locally produced factors is now widely accepted, the underlying mechanisms of follicular dominance are not yet fully understood (Webb *et al.*, 2003; Webb and Campbell, 2007).

It is widely believed that the primary mechanism by which only one follicle survives from the growing cohort is the decline in circulating FSH, as first suggested by Baird (1983). The largest follicle plays a key role in decreasing FSH to the point where the other smaller follicles are unable to continue growth (Webb *at al.*, 1999a), although it has been shown that the larger follicles of this cohort also play a small part in

this in the initial stages (Gibbons *et al.*, 1997). The growing and selected follicles suppress FSH production by secreting oestradiol and inhibin, although it has been shown in cattle that the effects of these hormones can change depending on the follicular wave (Webb *et al.*, 1999a). FSH has been shown to induce oestradiol production by bovine (Gutierrez *et al.*, 1997) and ovine (Campbell *et al.*, 1996) granulosa cells *in vitro*, further demonstrating the key role of gonadotrophins in the regulation of steroid hormone production, and the paracrine mechanisms by which they are subject to. The dominant follicle has a much higher concentration of oestradiol in its follicular fluid than the subordinate follicles, and it seems that a rapid increase in oestradiol is in fact a key characteristic of dominant follicles (Fortune *et al.*, 2004). Follicular fluid levels of oestradiol in cattle preovulatory follicles reach more than 1μg/ml before they ovulate (Bridges and Fortune, 2003).

It is likely that the dominant follicle develops mechanisms which allow it to survive the fall in FSH, before its subordinate follicles are able to acquire the same ability (Baird, 1983), however this selected follicle still requires basal amounts of FSH to continue growth (Webb *et al.*, 2003). It seems that during its development the dominant follicle is uniquely able to switch its FSH dependence over to LH (Campbell *et al.*, 1999; Webb *et al.*, 2003), as LH receptor mRNA begins to be expressed in the granulosa cells (Bao and Garverick, 1998; Zelenik, 2001). This becomes important as the circulatory LH increases while FSH declines (Kulick *et al.*, 1999), with subordinate follicles gradually becoming atretic under the constantly changing peripheral environment.

Instead of LH receptor being the follicular dominance ‘switch’, it is thought that the IGF system may have a much stronger involvement in dominant follicle selection. Fortune *et al.* (2004) hypothesised that follicular dominance arises because one follicle first acquires proteolytic activity against the IGF binding proteins -4 and -5, and an increase in free IGF and oestradiol, before its subordinate follicles. Free IGF is then able to amplifiy the effects of FSH on the follicle, allowing it to increase oestradiol production and hence decrease circulatory FSH. This then prevents the follicles in the cohort from acquiring the PAPP-A protein needed to decrease IGFBPs and hence increase free IGF. However, this is just one theory, and it is thought that the BMP system is also likely to be involved (Campbell *et al.*, 2009), further illustrating that the process of folliculogenesis relies on complex interactions between gonadotrophins and locally produced growth factors.

Whether or not the granulosa cell LH receptor theory of dominance is true, LH has been shown to be an essential requirement for normal follicular development to ovulation, as well as subsequent luteal function, however it appears that it is not of importance whether the release is pulsatile or continuous (Campbell *et al.*, 2007). In addition to the effects of LH on the dominant follicle itself, LH indirectly modulates the release of FSH from the pituitary, by controlling oestradiol and inhibin A secretion from the ovulatory follicle, and hence the fate of the subordinate follicles which are dependent on FSH (Webb and Campbell, 2007).

The process of folliculogenesis is one that is still only partially understood. It is known that the relationship between cellular proliferation, differentiation and apoptosis is the primary underlying mechanism, but this still requires further exploration. Gonadotrophins are well established as the survival signalling hormones for the follicle, however their action is indirect, through an extremely diverse range of growth factors that result in an ever-changing follicular environment optimal for development at that particular timepoint (Webb *et al.*, 2003). Described by Campbell *et al.* (2009) as an "intrafollicular cascade", it appears that the interactions between hormones and locally produced growth factors are highly temporally organised, and in order for follicular development to progress to the successful ovulation of an oocyte with developmental competence to become fertilised, the timing and extent of these interactions is vital.

1.4. Oocyte-follicle interactions

It is well established that oocytes and their surrounding cumulus cells maintain a very close association from the beginning of folliculogenesis, and that this is a relationship that is vital to maintain normal development (Buccione *et al.*, 1990). The oocyte and follicle grow and develop in a mutually dependant manner, in unison (Gilchrist *et al.*, 2004). The first physiological evidence of this was presented by Pincus and Enzmann in 1935, who believed that the follicular somatic cells maintain the oocyte in meiotic arrest. They reached this conclusion after it was found that oocytes removed from antral follicles would spontaneously resume meiosis in culture, independent of any gonadotrophin stimulus. Subsequent research found that the follicular somatic cells promote the resumption of meiosis and the advance to metaphase II, and hence nuclear maturation. They also enable the oocyte to become fertilised, and

undergo cytoplasmic maturation (Eppig, 2001). Nutrients and regulatory chemical signals are sent to the oocyte by these cells, which ultimately enable the oocyte to acquire developmental competence (Ka *et al.*, 1997). If cumulus cells are removed at the beginning of *in vitro* oocyte maturation for example, maturation, fertilisation and subsequent development are compromised significantly. This effect has been investigated in depth, particularly in cattle (Zhang *et al.*, 1995; Fatehi *et al.*, 2002), and it is widely accepted that follicular granulosa cells supply the oocyte with the nutrients and stimuli needed for growth and development (Buccione *et al.*, 1990). Perhaps of greatest importance is that the oocyte is unable to metabolise glucose efficiently (Leese and Barton, 1984), and so are dependent on the surrounding cumulus cells for pyruvate and other products of glycolysis, as well as amino acids that all are critical to the development of the oocyte (Yeo *et al.*, 2009).

The new perspective that has emerged over the last 15 years is that communication between the oocyte and its surrounding somatic cells is a bi-directional process. Granulosa cell-to-oocyte communication is a fairly well documented research area, however oocyte-to-granulosa cell communication is a much more novel topic (Eppig, 2001). This two-way communication is essential for follicular development and production of mature oocytes capable of undergoing fertilisation and producing a pregnancy to term (Su *et al.*, 2008). In the past, the oocyte was considered to be a passive recipient of granulosa cell-produced developmental signals, however research has indicated that this is not the case (Eppig, 2001).

In fact, it is now well-established that the transition from primary to secondary stage of follicle development, in particular, is a process induced by an autocrine/paracrine regulatory mechanism which involves growth factors that are produced both by the oocyte and the granulosa cells (Sadeu *et al.*, 2008). In addition it is now widely accepted that the oocyte itself acts as an active regulator of follicular development (Su *et al.*, 2008). The ability of the oocyte to regulate most somatic cell functions is lowest in preantral follicles as the oocyte is undergoing its growth stage, then highest during the antral phase of folliculogenesis, followed by a decline after the LH surge and the resumption of meiosis (Gilchrist *et al.*, 2001; 2004). For example, it has been shown that in mice, the capacity of the oocyte to regulate granulosa cell steroidogenesis throughout follicular development varies (Vanderhyden and Macdonald, 1998).

In the murine species, the major benefit of this ability to alter the effect of the oocyte on the somatic cells is that the oocyte can direct its surrounding granulosa cells to first promote its own growth until fully grown, and then actively prevent the cells from causing further growth in order to prevent overgrowth (Carabatsos *et al.*, 1998). The growing oocyte stimulates kit-ligand production by the granulosa cells of preantral follicles, which in turn stimulates further oocyte growth (Packer *et al.*, 1994). Once at full size, the signal switches from stimulation to inhibition, ceasing kit-ligand production and hence preventing further growth (Joyce *et al.*, 1999). Furthermore, it seems that in the mouse it is ultimately the oocyte that dictates the rate of follicular growth, not the granulosa cells (Eppig *et al.*, 2002).

There is still relatively limited knowledge and understanding of the mechanisms by which the oocyte and cumulus cells communicate, and the effect that dynamic changes in this communication may ultimately have on oocyte developmental competence (Gilchrist *et al.*, 2008). However, it is clear that in all species the oocyte plays a vital role in folliculogenesis, secreting paracrine factors that ensure an appropriate microenvironment optimised for the acquisition of its developmental competence (Li *et al.*, 2008a).

1.5. Oocyte-secreted factors

The oocyte plays a major role in the regulation of development and function of follicles of all stages, from as early as the primordial stage (Soyal *et al.*, 2000). It does this by secreting soluble growth factors; oocyte-secreted factors (OSFs), which act on the surrounding cells, regulating a number of granulosa and cumulus cell functions. These functions include promoting the transition from primary to secondary stage (Juengel *et al.*, 2002a; Latham *et al.*, 2004), proliferation and differentiation of granulosa cells before the LH surge (Gilchrist *et al.*, 2003; Otsuka *et al.*, 2005), the transition from preantral to antral stage (Diaz *et al.*, 2007; Orisaka *et al.*, 2006), cumulus expansion and finally ovulation following the LH surge (Diaz *et al.*, 2006; Dragovic *et al.*, 2007).

While oocytes actively promote cumulus cell growth, they also prevent cellular apoptosis. If the oocyte is oocytectomised from a COC, there is a significant increase in cumulus cell apoptosis, an effect that can be reversed using OSF exposure (Hussein *et al.*, 2005). Furthermore, cumulus cell phenotype is maintained by oocyte-secreted paracrine growth factors, as well as regulation of inhibin synthesis and luteinizing hormone receptor expression (Gilchrist *et al.*, 2004).

The extent to which the oocyte has the capacity to deliver, and the granulosa cells to receive, paracrine cell signals, changes throughout the course of follicular development, due to extensive progression in oocyte development and granulosa cell differentiation (Gilchrist *et al.*, 2004).

It is thought that an oocyte-granulosa cell regulatory loop exists, in which there are complementary cell signalling and metabolic pathways. These pathways control the development and the function of the oocyte and the follicular components (Su *et al.*, 2008). Two growth factors that are thought to play major roles in this regulatory loop are growth differentiation factor 9 (GDF9) and growth differentiation factor 9b (GDF9b), better known as bone morphogenetic protein 15 (BMP15). These are two members of the transforming growth factor β (TGFβ) superfamily, which are oocyte-derived key regulators of follicle growth and ovulation in a number of species (Yan *et al.*, 2001; Juengel *et al.*, 2002a; Chand *et al.*, 2006).

1.5.1. Growth Differentiation Factor 9 and Bone Morphogenetic Protein 15

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are members of the transforming growth factor β (TGFβ) superfamily, shown to be secreted by oocytes of humans, rodents and sheep, as well as a number of other species (Aaltonen *et al.*, 1999; Fitzpatrick *et al.*, 1998; Bodensteiner *et al.*, 1999). This superfamily of extracellular signalling molecules consists of over 30 different proteins which include activins A, B and AB, anti-Müllerian hormone (AMH), inhibins A and B, 3 TGFβ isoforms 1, 2 and 3, at least 9 GDFs and around 20 BMPs (Massague and Wotton, 2000). The mature forms of these proteins form disulphide links to form dimers. Most form homodimers, but some are also able to form heterodimers (Knight and Glister, 2003).

They are oocyte-specific proteins (though some non-ovarian expression has been observed), expressed and translated in the growing oocyte as inactive pre-proteins, consisting of a signal peptide, a prodomain and a biologically active mature region at the C-terminal part of the protein (Shimasaki *et al.*, 2004; Veitia and Caburet, 2009). GDF9 mRNA is expressed in primordial follicles of a number of species including sheep, cattle and humans (Galloway *et al.*, 2000; Bodensteiner *et al.*, 1999; Aaltonen *et al.*, 1999), whereas BMP15 expression commences in primary follicles (Dube *et al.*, 1998; Otsuka *et al.*, 2000, McNatty *et al.*, 2001). Unlike other members of the TGFβ superfamily, these growth factors can form

homodimers as well as GDF9/BMP15 heterodimers through non-covalent interactions (Liao *et al.*, 2003; 2004), and as a result their biological action has been suggested as being united (Gilchrist *et al.*, 2008).

The growth factors play critical roles in a wide range of developmental and physiological processes, in particular the control of granulosa cell differentiation (Gilchrist *et al.*, 2004; Juengel and McNatty, 2005a; Li *et al.*, 2008b) and in fact, the absence, deletion or mutation of GDF9 and BMP15 has been shown to result in infertility (Dong *et al.*, 1996; Galloway *et al.*, 2000). Interestingly there are considerable species differences involved in the functions of the growth factors, suggested by gene knockout models. For example in the sheep, it has been found that heterozygous mutations in expression of the growth factors increases ovulation rate, but homozygous mutations result in sterility, as both are required for folliculogenesis (Hanrahan *et al.*, 2004). In contrast, it has been shown that the mouse does not have an absolute requirement for BMP15 as BMP15-null mice are fertile, although they do suffer from subfertility due to decreased ovulation and fertilisation rates (Yan *et al.*, 2001). Yan *et al.* (2001) also found that mice lacking two copies of the BMP15 gene and one copy of the GDF9 gene had an increased prevalence of oocyte loss, and a decreased number of later stage follicles. In mice lacking both copies of the GDF9 and BMP15 genes, there was arrest of follicle growth at the primary stage, similar to that seen in GDF9 knockout mice (Sadeu *et al.*, 2008), indicating that GDF9 is vital for murine fertility, but not BMP15.

Sheep in particular have proven to be an extremely valuable model for the use in studying GDF9 and BMP15, as there are a number of breeds that have been discovered to possess naturally occurring genetic mutations of the expression of these factors and their receptors. These alterations in expression primarily affect ovulation rate, as well as elements of folliculogenesis. Several breeds of sheep including Cambridge, Belclare, Inverdale, Thoka, Hanna and Lacaune possess different polymorphic sequence variations in both GDF9 and BMP15. In addition the Booroola breed is of particular interest due to its genetic mutation of the activin-like kinase receptor ALK6, which is a TGFβ-related receptor. These animals with the $FecB^{B}$ allele have decreased ALK6 receptor function, which causes ovarian follicles to ovulate much earlier than the non-carrier animals. The homozygous carriers' follicles ovulate at 3-4mm rather than 7mm, at more than 5 ovulations per cycle as opposed to one or two (Davis *et al.*, 1982), and their granulosa cells differentiate earlier so that follicular maturity is not compromised as a result of shortened folliculogenesis (Baird and Campbell, 1998).

In the human, it seems that GDF9 and BMP15 play a crucial role in the regulation of fertility. Abnormal expression of GDF9 is linked to polycystic ovarian syndrome and polycystic ovaries (Teixeira Filho *et al.*, 2002), BMP15 mutations are associated with premature ovarian failure (Di Pasquale *et al.*, 2004; Dixit *et al.*, 2006), and GDF9 mutations are associated with increased incidence of dizygotic twinning (Palmer *et al.*, 2006) and primary ovarian insufficiency (POI) (Welt, 2008).

The substantial difference between species growth factor expression patterns and responses is likely to reflect the considerable differences between local signalling requirements in monovulatory and polyovulatory species (Rodrigues *et al.*, 2008).

There has been extensive research into the functions of GDF9 and BMP15 *in vitro*, however conclusions of such studies have often proved contradictory. This is likely to be due to discrepancies between culture method, as well as differences between species of origin of growth factor and cells (Juengel and McNatty, 2005). Some studies have concluded that GDF9 and BMP15 have a mitogenic effect on granulosa and thecal cells (McNatty *et al.*, 2005a), and also may inhibit granulosa and theca cell progesterone production under stimulation from gonadotrophins (Yamamoto *et al.*, 2002). More specifically, it is thought that the growth factors regulate cholesterol biosynthesis within cumulus cells (Su *et al.*, 2008).

Through investigation using different doses of recombinant BMP15, GDF9 and fibroblast growth factor 8B (FGF8B) on cumulus cells *in vitro*, it was found that adding BMP15 and FGF8B to the culture promotes glycolysis by increased expression of glycolytic enzymes (Sugiura *et al.*, 2007). This indicates additional importance of the oocyte-cumulus cell symbiotic relationship, as the oocyte is deficient in the glycolysis pathway and hence relies on cumulus cells to provide the glycolytic products it needs to develop normally (Li *et al.*, 2008a).

Using an ovine model, BMPs 2, 4 and 6 have been shown to be ineffective in stimulating the differentiation of granulosa cells in the absence of FSH, however there does appear to be a role for these BMPs in regulating cellular differentiation when FSH is present, as aromatase activity is closely related to BMP and IGF exposure in culture (Campbell *et al.*, 2006).

1.5.2. GDF9 and BMP15 signalling

GDF9 and BMP15 utilise two distinct signalling pathways, both through the SMAD intracellular cascade. The growth factors can be in the form of homo or heterodimers, binding to either type-I receptors; activin receptor-like kinase (ALK), or to BMP type-II receptors (Edwards *et al.*, 2008) on the cell surface. Both of these pathways lead to receptor phosphorylation and then phosphorylation of signal transducers called SMADs (Shimasaki *et al.*, 2004). There have been 7 type I, and 5 type II receptors identified in mammals, and because there is a substantial amount of interaction between TGFβ superfamily ligands and receptors, the system underpinning the process is extremely complex (Knight and Glister, 2003).

BMP15 uses the BMP pathway, which activates the SMAD 1/5/8 messengers, and GDF9 uses a combination of this and the TGFβ/activin pathway, which activates the SMAD 2 and 3 messengers (Hussein *et al.*, 2006). After phosphorylation, these SMADs combine with SMAD 4, and are then translocated to the nucleus, where they interact with transcriptional regulation elements (Juengel and McNatty, 2005a). Figure 1.6 below illustrates this process.

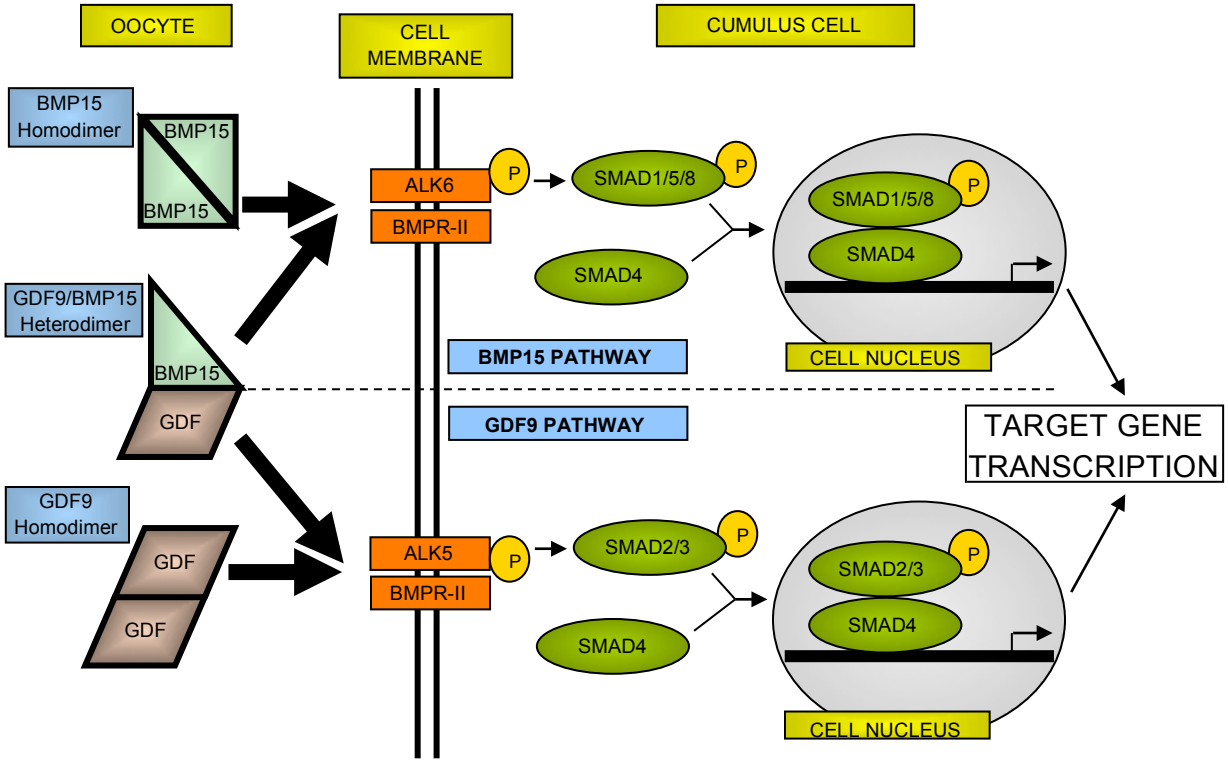

Figure 1.6. Molecular basis of oocyte-cumulus cell signalling pathway.

The TGFβ superfamily members BMP15 and GDF9 are two key oocyte-secreted factors that regulate folliculogenesis. Their paracrine signalling pathway is illustrated in figure 1.7. BMP15 binds to the BMP type-II receptor (BMPR-II) and activin receptor-like kinase 6 (ALK6), which becomes phosphorylated. Receptor-regulated signal transducers called SMADs then become phosphorylated, which activates the receptor-independent SMAD4, leading to target gene expression in the cell nucleus. GDF9 binds BMPR-II and ALK5, which leads to a slightly different SMAD intracellular cascade.

Table 1.1 below summarises the expression patterns of the TGFβ ligands and their receptors mentioned in this chapter. Notably, all of the receptors are expressed from the start of folliculogenesis, and are expressed by granulosa cells at different stages. This allows the oocyte to act in an autocrine and paracrine fashion (McNatty *et al.*, 2006).

Ligand/Receptor	**Stage of follicular development**			
	Primordial	Primary	Preantral	Antral
GDF9	Oocyte	Oocyte	Oocyte	Oocyte
BMP15	-	Oocyte	Oocyte	Oocyte
BMP6	Oocyte	Oocyte	Oocyte	Oocyte, GC?, TC?
ALK5	Oocyte	Oocyte	Oocyte, GC, TC	Oocyte, GC, TC
ALK6	Oocyte	Oocyte, GC	Oocyte, GC, TC	Oocyte, GC, TC
BMPRII	Oocyte, GC	Oocyte, GC	Oocyte, GC, TC	Oocyte, GC, TC

Table 1.1. Expression of GDF9, BMP15, BMP6 and their receptors in ovine oocytes, granulosa and theca cells. Redrawn from McNatty *et al.* (2006), data from Juengel and McNatty (2005).

Despite the knowledge of the BMP15 and GDF9 pathways, much of their cellular biology remains to be understood, in particular the ways in which the growth factors interact with each other, other OSFs, and the established hormones associated with folliculogenesis (Gilchrist *et al.*, 2008). It has been shown, however, that GDF9 and BMP15 interact with a factor called kit ligand (KitL) (Rodrigues *et al.*, 2008). Although KitL, GDF9 and BMP15 each have distinct roles during folliculogenesis, research has revealed that there are considerable interactions between them (Otsuka and Shimasaki, 2002), forming a

regulatory loop (figure 1.7). GDF9 is thought to inhibit KitL expression (Joyce *et al.*, 2000; Wu *et al.*, 2004), in contrast to BMP15 which is thought to act as a promoter of KitL expression (Otsuka and Shimasaki, 2002). KitL then acts to inhibit BMP expression, in a negative feeback loop (Hutt *et al.*, 2006). In addition, Thomas *et al.* (2005) have shown that FSH regulates BMP15 expression indirectly through Kit signalling, indicating a role for FSH in controlling oocyte- and granulosa cell- secreted factor expression, a theory supported by Yang *et al.* (2003).

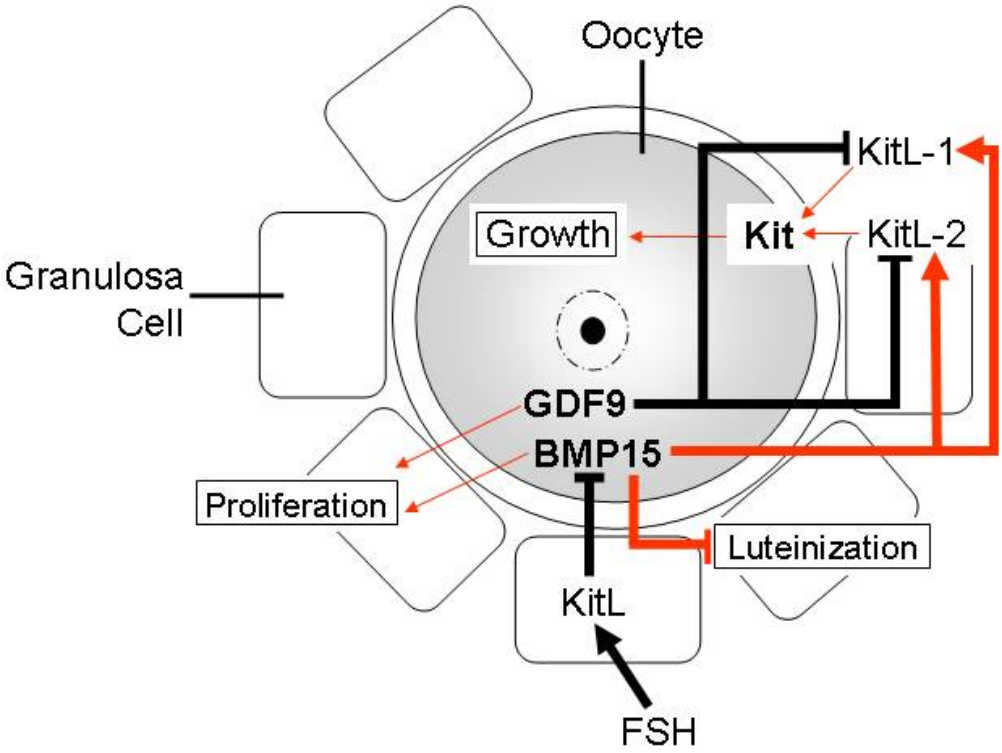

Figure 1.7. KitL, BMP15 and GDF9 interactions in oocyte follicular development

(Redrawn from Thomas and Vanderhyden, 2006)

FSH regulates the oocyte- and granulosa cell-secreted factors needed for oocyte growth and development. GDF9 and BMP15 stimulate granulosa cell proliferation. BMP15 inhibits luteinisation and promotes KitL-1 and KitL-2 expression, regulated by KitL. In contrast, GDF9 inhibits KitL-1 and KitL-2 expression. Red arrows depict actions, black arrows depict effect on mRNA expression.

1.5.3. Bone morphogenetic protein 6

Bone morphogenetic protein 6 (BMP6) is expressed in both oocytes and granulosa cells of follicles at secondary stage onwards (Vanderhyden and Macdonald, 1998; Glister *et al.*, 2004; Knight and Glister, 2006). The complete range of functions is yet to be uncovered (Gilchrist *et al.*, 2006), however recent research has revealed a number of roles in the dynamics within the follicle, in particular concerning steroidogenesis.

In the mouse, BMP6 seems to act in the promotion of follicle survival, by preventing premature luteinisation. It does this by regulating the biological effects of FSH on the granulosa cells, through inhibition of FSH-induced progesterone production by the mural granulosa cells and inhibiting cAMP synthesis, which occurs without affecting oestrogen production (Otsuka *et al.*, 2001; Miyoshi *et al.*, 2007).

In contrast to the mouse, an *in vitro* study by Campbell *et al.* (2006) illustrated that in sheep granulosa cells, BMP6 (as well as BMP2 and 4) is a powerful promoter of FSH-stimulated oestradiol production. In addition, the study demonstrated that the BMP system has a significant interaction with IGF-I, as FSH-induced oestradiol production increased only when both BMP6 and IGF-I are present. The study also revealed that BMPs promote theca cell differentiation at very low doses, but inhibit differentiation at higher doses, suggesting a higher sensitivity to these growth factors.

BMP6 is thought to acts alongside BMP15 to prevent cumulus cell apoptosis, through the intricate network of paracrine-secreted BMP growth factors and a number of binding proteins such as follistatin and gremlin (Hussein *et al.*, 2005). As a result it has a similar physiological role to BMP15, but acts through differing functional mechanisms. However, unlike some of the other oocyte-secreted factors such as BMP15 and GDF9, in most species BMP6 is not a promoter of granulosa cell proliferation, or involved in oestradiol synthesis regulation, although in the mouse BMP6 does weakly induce granulosa cell proliferation (Otsuka *et al.*, 2001). This suggests that there are probably species-specific functional roles.

There are, in fact, some fairly major differences in functional roles between species, for example in chickens it has been shown that BMP6, originating from the theca cells, actually enhances gonadotrophin-dependant progesterone secretion (Al-Musawi *et al.*, 2007).

The BMP system is not the only group of growth factors thought to be heavily involved in the process of folliculogenesis. The insulin-like growth factor (IGF) family is another system of local growth factors that is relatively well characterized (Webb *et al.*, 2007) and is believed to interact with both the BMPs and gonadotrophin hormones in the complex mechanisms that underlie follicular development.

1.5.4. Insulin-like growth factor

The insulin-like growth factor (IGF) family is believed to play a primary role in regulating gonadotrophin action on follicular somatic cells. In particular, IGF-I is thought to be involved with the control of preantral follicle growth, with IGF binding proteins (IGFBPs) regulating extra-ovarian IGF bioavailability (Webb *et al.*, 2004; Webb and Campbell, 2007). Furthermore, IGF-I stimulates growth of bovine preantral follicles *in vitro* (Gutierrez *et al.*, 2000). IGF-II, which stimulates steroidogenesis, is not expressed in theca cells until early antral stage (Armstrong *et al.*, 2000; Spicer *et al.*, 2004), when it seems the whole IGF system becomes functional. There are several species differences regarding the IGF system, however it seems that in all species the interaction between gonadotrophins and IGFs is important in regulating the development of follicles to the point of ovulation (Webb *et al.*, 2007). Furthermore, Campbell *et al.* (2006) demonstrated a clear interaction between BMPs and IGFs in the stimulation of granulosa cell differentiation, indicating that the inter-relationships between locally produced growth factors have a key role in the mechanisms underlying folliculogenesis (figure 1.8).

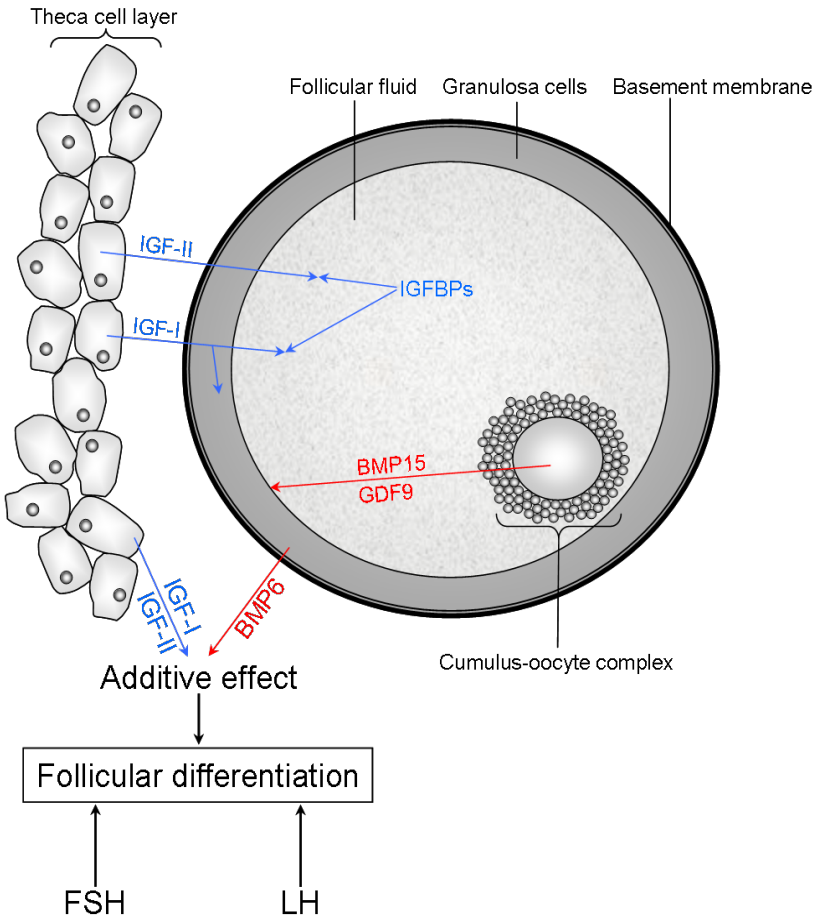

Figure 1.8. Summary of the IGF and BMP systems

(Redrawn and adapted from Webb *et al.*, 2003 and 2007)

1.6. Follicular cell expressed markers

During clinical *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), the cumulus cells, follicular fluid and granulosa cells are discarded, because currently they serve no further purpose in the process. However, the cells are easily accessible and plentiful, making them ideal candidates for analysis for the assessment of oocyte quality and embryo developmental potential (Li *et al.*, 2008a).

In order to establish additional measures of oocyte and embryo quality to be used for selection, a significant amount of research has been directed towards evaluating follicular cell signatures to be used as molecular predictors of oocyte developmental competence (McKenzie *et al.*, 2004; Zhang *et al.*, 2005; Feuerstein *et al.*, 2007; Bettegowda *et al.*, 2008).

Following the development of high throughput gene analysis technologies such as microarray, it has become relatively easy to identify the transcriptome of granulosa cells. As a result, several research groups have found a number of potential markers of oocyte developmental competence in cattle. These consist of GDF9 target genes such as HAS2, tumour necrosis factor alpha-induced protein 6 (TNFAIP6), prostaglandin-endoperoxidase synthase 2 (PTGS2) and gremlin 1 (GREM1) (Elvin *et al.*, 1999a; Pangas *et al.*, 2004a; Varani *et al.*, 2002), as well as inhibin βA (INHBA), epidermal growth factor receptor (EFGR), betacellulin (BTC) and CD44 (Assidi *et al.*, 2008; reviewed in Li *et al.*, 2008a). Expression of HAS2 and GREM1 in particular, is thought to correlate to oocyte developmental competence (Cillo *et al.*, 2007), and this has encouraged the search for other molecular markers with the same relationships to oocyte quality. Other identified cumulus cell markers associated with oocyte competence include steroidogenesis genes such as ferredoxin 1 (FDX1) and cytochrome P450 (CYP19A1), as well as a number of genes involved in apoptosis (Hamel *et al.*, 2008).

Recently, there has been research on bovine cumulus cell markers of poor oocyte competence, in particular the cathepsin family of cysteine proteinases, which have been proposed as apoptosis-inducing genes. Inhibitors of these proteases could be used to reduce cumulus cell apoptosis and therefore improve oocyte developmental potential (Bettegowda *et al.*, 2008).

There have been a number of discrepancies between the conclusions of the studies concerning cumulus cell gene profiling, probably as a result of experimental differences, such as different endpoint measures (e.g. early cleavage, blastocyst rate, etc.), cell sources (pooled cumulus cells or cells from individual COCs) and analysis methodology and equipment (Li *et al.*, 2008a). As a result, more research is needed in order to clarify which markers are capable of being used as predictors of oocyte competence, embryo development and pregnancy outcome.

1.7. Angiogenesis

In order for cells to survive, a continuous supply of oxygen and nutrients must be provided, as well as a removal system for metabolic waste products. In addition, a transport system is needed to transfer endocrine hormones from glands to target cells (Stouffer *et al.*, 2001; Tamanini and De Ambrogi, 2004). Sufficient transfer of small, soluble molecules such as oxygen and glucose can only occur through

diffusion for short distances of a few hundred micrometers, beyond which vascularisation is needed (Neeman *et al.*, 2007). The process by which vascularisation is established is angiogenesis.

In the adult, the vascular system is generally quiescent, apart from in the female ovary, where there is substantial angiogenesis. Angiogenesis in the ovary is critical for the processes of follicular development, ovulation and corpus luteum formation (Reynolds and Redmer, 1999; Tamanini and De Ambrogi, 2004; Fraser, 2006). Vasculature in the ovary is not evenly distributed around the follicle population, as blood flow requirement is dependent on the developmental stage of the follicle. It is thought that the dormant primordial and preantral follicles rely on blood vessels in the stromal tissue and acquire their oxygen and nutrients through simple diffusion, as they do not possess their own vascular network (Robinson *et al.*, 2009). As the follicles develop further, blood vessels spread from the surrounding ovarian stromal tissue to vascularise the follicle, between the theca cell layers. As the antral cavity develops, one layer of the vascular sheath forms below the basement membrane, and the other forms in the theca externa (Martelli *et al.*, 2006). The vessels do not penetrate to the granulosa cell layer as they are separated by the basement membrane (Fraser and Wulff, 2001), and yet despite this, in the corpus luteum each luteinized granulosa cell is, in fact, adjacent to an endothelial cell (Duncan *et al.*, 2008).

It is often observed that the dominant follicle has a more vascularised theca layer than that of the subordinates (McNatty *et al.*, 1981; Zeleznik *et al.*, 1981), which suggests that the development of increased vasculature may be one of the determining factors in acquiring follicular dominance (Redmer and Reynolds, 1996; Aerts and Bols, 2010). Studies by Acosta *et al.* (2004; 2005) demonstrated that the dominant follicle in mares had increased blood flow prior to achieving dominance, and in cattle the subordinate follicles had decreased blood flow than the dominant follicle. There is evidence to suggest that the acquisition of vasculature around the developing antral follicle is a rate-limiting factor in terms of follicle selection and maturation prior to ovulation (Stouffer *et al.*, 2001), as there would be differing accessibility to oxygen, nutrients and hormones for individual follicles at this stage, resulting in the promotion of dominance in one follicle, and limited development and atresia in another.

Research on angiogenesis began in cancer tissue, with the first angiogenic factor to be discovered in the ovary being basic fibroblast growth factor (bFGF) by Gospsodarowicz *et al.* (1985), which is expressed in the mature follicle and corpus luteum. The FGF family is thought to play an important role in

folliculogenesis (Buratini *et al.*, 2005), although the extent to which these growth factors are a requirement has yet to be elucidated. It is thought that there are a wide range of functions, including stimulation of endothelial cell proliferation and inhibition of granulosa cell apoptosis, thereby favouring angiogenic factor production (Bikfalvi *et al.*, 1998). However, unlike other angiogenic factors, FGFs do not appear to have a critical role in angiogenesis (Stouffer *et al.*, 2001).

Feedback on a microenvironmental scale is required for the balance of angiogenesis with the requirements of the tissue and this is primarily under control of vascular endothelial growth factor-A (hereafter VEGF) expression by the hypoxic cells (Shweiki *et al.*, 1995). The key microenvironmental factor affecting angiogenesis is oxygen. Under low oxygen (hypoxic) conditions, the transcription factor hypoxia inducible factor-1 (HIF-1) is stable, inducing VEGF expression. When oxygen supply is adequate, the oxygen causes destabilisation of the α-subunit of HIF-1, which suppresses its activity (Neeman *et al.*, 2007).

1.7.1. Vascular endothelial growth factor

VEGF has an established central role as the primary angiogenic growth factor. Its immediate effects are vasodilation and an increase in vessel permeability, and after a longer duration, endothelial cell proliferation, migration and differentiation, which results in the formation of new capillaries (Neeman *et al.*, 2007). Angiogenesis is then further supported by VEGF by means of signalling to endothelial cells of the newly formed capillaries to enable them to survive (Benjamin *et al.*, 1999). VEGF is produced in a number of different isoforms, $VEGF_{165}$ playing the most important role in the ovary (Tesone *et al.*, 2005). In addition, two other soluble isoforms are produced as a result of gene splicing; $VEGF_{121}$ and $VEGF_{145}$, as well as two larger, membrane-bound forms; $VEGF_{189}$ and $VEGF_{206}$ (Stouffer *et al.*, 2001). In the primate, VEGF mRNA is completely absent in primordial, primary and early secondary follicles, until the vascular network forms in the secondary follicle stage, although in cattle it seems that VEGF may be involved in primary to secondary follicle transition, indicating species-specific roles (Yand and Fortune, 2007). It is expressed more weakly in the theca cells of antral follicles, and the granulosa cells next to the oocytes of preovulatory follicles (Stouffer *et al.*, 2001; Taylor *et al.*, 2004). In cattle and pigs, VEGF expression occurs during early folliculogenesis, becoming stronger in the granulosa and theca cells of the dominant

follicle (Kaczmarek *et al.*, 2005), hence expression increases with follicular size (Barboni *et al.*, 2000; Ferrara *et al.*, 2003).

The importance of VEGF during folliculogenesis has been demonstrated by a number of research groups, who showed that VEGF inhibition during the follicular phase, using VEGF Trap, significantly reduces follicle growth, and results in antral follicles that are small and poorly vascularised. As a result no dominant follicles emerge (Wulff *et al.*, 2002; Fraser and Duncan, 2005).

mRNA/protein expression of VEGF has been found to be stimulated significantly by the LH surge in granulosa cells (Koos, 1995; Christenson and Stouffer, 1997). LH is thought to act indirectly to induce follicular angiogenesis, by modulating VEGF expression (Gutman *et al.*, 2008). In addition, treatment using GnRH antagonists results in the same outcome as that of VEGF inhibition, which indicates gonadotrophins as regulators of VEGF expression in preovulatory follicles (Kaczmarek *et al.*, 2005; Lam and Haines, 2005; Duncan *et al.*, 2008). In addition to the interaction with LH, it seems that IGF-I may also have a role in VEGF production, at least in bovine and primate granulosa cells (Schams *et al.*, 2001; Martinez-Chequer *et al.*, 2003). However, the mechanisms underlying the regulation of VEGF in the mammalian ovary have still not become apparent.

It has been suggested that hypoxia is one of the primary regulators of ovarian VEGF expression, using culture of luteal cells (Tropea *et al.*, 2006). In the situation where VEGF expression is mediated by hypoxia, there is significant up-regulation of a number of specific transcription factors in the ovary. The most notable of these transcription factors is hypoxia-inducible factor-1 (Forsythe *et al.*, 1996).

1.7.2. Hypoxia-inducible factor-1

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that is of particular importance in angiogenesis, as it regulates and maintains expression of a number of factors involved in the process. It is of primary importance in the induction of gene expression for factors that allow cellular adaption and survival in an environment that is continuously shifting from normoxia to hypoxia (Semenza, 1998).

HIF-1 was first identified following the discovery of a hypoxia-responsive element (HRE) in the erythropoietin gene enhancer, which stimulates red blood cell proliferation under hypoxic conditions (Goldberg *et al.*, 1988). Later studies identified the protein that binds to the HRE during hypoxia as HIF-1.

HIF-1 is composed of a HIF-1α and a HIF-1β subunit (Wang and Semenza, 1995), which form a heterodimeric complex to allow DNA binding and the transactivation of HIF-1 (Jiang *et al.*, 1996; Kallio *et al.*, 1997). It is up-regulated under hypoxic conditions, during which its proteosomal degradation is prevented, resulting in an accumulation which allows for binding of HIF-1α to HIF-1β. This process is summarised in figure 1.9.

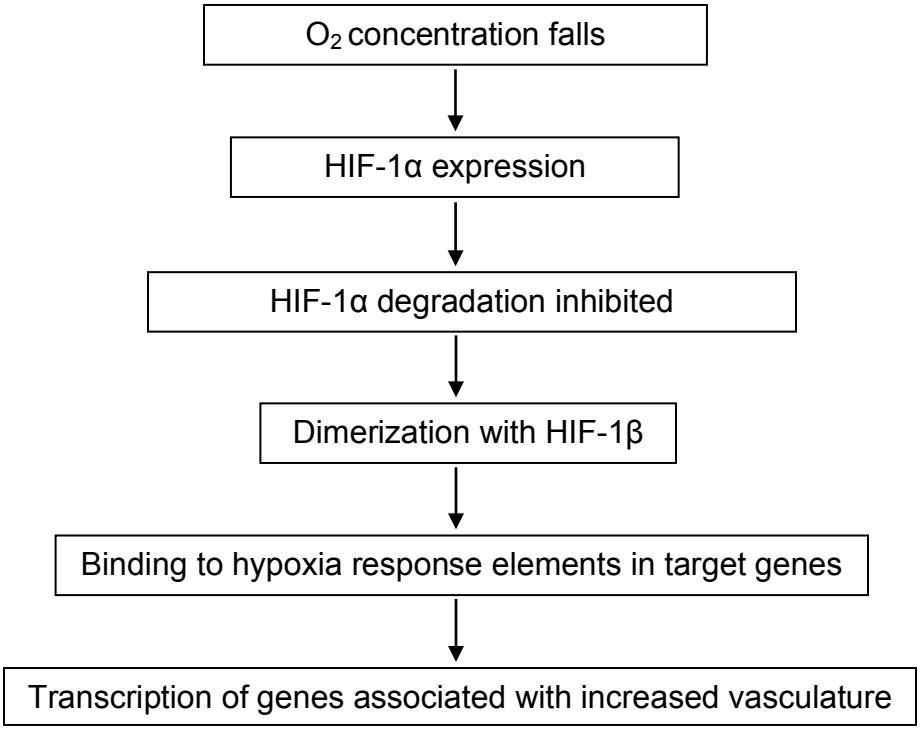

Figure 1.9. The HIF angiogenic pathway

O_2 concentration decreases as the metabolic demands of the developing follicle increases, leading to an increase in HIF-1α expression. Cells that normally produce HIF then quickly degrade the protein in non-hypoxic conditions, cease protein degradation, resulting in accumulation of HIF-1α. This then dimerizes with HIF-1β, and binds to hypoxia response elements within target genes, and activates transcription of angiogenic growth factors (Hirota and Semenza, 2006).

HIF-1 is a critical transcription factor regulating angiogenic gene expression, controlling the expression of over 40 genes (Hirota and Semenza, 2006), which encode a wide range of proteins including glycolytic enzymes, glucose transporters and erythropoietin, as well as target genes which promote vascularity such as VEGF (increasing vessel permeability) and leptin (inducing endothelial cell proliferation), as a more long term solution (Semenza, 1998; Alam *et al.*, 2009; Fong, 2009). Its primary role involves the activation of the multiple physiological responses to hypoxic conditions in order to counteract the deficiency of oxygen.

Besides the role of HIF-1 in hypoxic conditions, under normoxic conditions, it has been shown that HIF-1 upregulates inhibin-α, LH receptor and VEGF in granulosa cells stimulated with FSH (Alam *et al.*, 2004). Expression of HIF-1 is rarely seen in somatic cell nuclei in growing follicles, and even in the dominant pre-ovulatory follicle, but then expression increases dramatically in the short time before, and during the process of ovulation (Fraser and Duncan, 2009; Duncan *et al.*, 2008). However, its patterns of expression and the mechanisms underlying expression during hypoxia are still not fully understood, and will require further exploration. For example, HIF-1 expression in follicular somatic cells has not yet been examined in a study relating to IVF outcome, but would be an ideal candidate to compare expression levels to ultrasound-derived indices of follicular vascularity, as well as oocyte and embryo quality.

HIF-1β is expressed constitutively, with its mRNA and protein levels continuously maintained, independent of oxygen availability within the cell (Kallio *et al.*, 1997), whereas the HIF-1α has a very short half-life, and is completely dependent on oxygen conditions (Salceda and Caro, 1997). Although HIF-1α transcription and synthesis are continuous, and unaffected by oxygen, the proteins rapidly degrade under normoxic conditions (Wang *et al.*, 1995). In hypoxic conditions, HIF-1α stabilises and is able to translocate from the cytoplasm to the nucleus. Here it binds to HIF-1β to form the transcriptionally active HIF complex (Huang *et al.*, 1996). Gene expression is then induced by the HIF complex associating with the HREs in the target gene regulatory regions, binding the coactivators (Lando *et al.*, 2002).

1.7.3. Follicular blood flow

Follicular vasculature, measured by blood flow power Doppler ultrasound, has been correlated to oocyte developmental competence, and even pregnancy potential (Chui *et al.*, 1997; Van Blerkom, 1998). It seems that follicular vasculature has a significant influence on the oocyte that resides within (Mercé *et al.*, 2006), which would be explained by the increased metabolism, respiration and removal of waste products. However, it is not known whether it is primarily the follicle driving the formation of vasculature through production of angiogenic growth factors, or vice versa with increased vasculature formation leading to the development of the dominant follicle.

Oocytes from highly vascularised follicles have fewer chromosomal abnormalities (Bhal *et al.*, 1999), as there are fewer defects in spindle organisation, cytoplasmic structure and chromosomes. Furthermore, development in poorly vascularised follicles is hindered considerably as they have reduced capacity in progressing past the 6-8 cell embryo stage (Van Blerkom *et al.*, 1997). Oocytes are very sensitive to hypoxic stress, and so it is crucial to develop an adequate network of vasculature for normal oocyte development to occur (Monteleone *et al.*, 2008).

As VEGF is the primary angiogenic growth factor in the ovary, measuring its concentration in the follicular fluid would also seem to be a potential measure of the extent of peri-follicular blood supply. Indeed, VEGF concentrations in the follicular fluid seem to have a positive relationship with these oocyte quality, embryo development parameters as well as pregnancy rates (Van Blerkom *et al.*, 1997), indicating a possible role for follicular VEGF measurement and power Doppler ultrasound as tools for oocyte selection in IVF (Moneteleone *et al.*, 2008). There has, however, been some controversy in this area, as some studies found that VEGF in follicular fluid was, in fact, not related (Kim *et al.*, 2004), or even negatively correlated (Barroso *et al.*, 1999; Ocal *et al.*, 2004) to IVF outcome in stimulated cycles. As a result, further research is needed to elucidate these relationships. The problem with a number of the studies is that the authors were unable to track the embryo developmental progress and hence relate the ultrasound and assay data to the individual embryos and consequentially, pregnancy rates (Shrestha *et al.*, 2006). As a result, individual embryo culture and single embryo transfer would be advantageous, to enable embryo development tracking and allow the data to be related to pregnancy outcome.

1.8. Experimental models

There is a wide range of methods commonly used for *in vitro* culture that have been used in follicular somatic cell culture, each with its own advantages and drawbacks. However, a reliable culture system that allows normal development from the very first stages of folliculogenesis up to antral stage has still not yet been developed. Although the culture systems explained below have been improved significantly over recent years, further work to develop more *in vitro* models is needed in order to make progress in elucidating the mechanisms underlying early follicular development (Webb and Campbell, 2007).

Research using animals with naturally occurring or articifially produced mutations, such as those deficient in GDF9 and/or BMP15, has revealed a great deal of information regarding the role of these growth factors *in vivo*. In particular, sheep have become an extremely popular species for use as an experimental model, as in previous years there have been several breeds identified with genetic mutations that occur naturally, with considerable effects on ovulation rate, as well as aspects of folliculogenesis from the very first stages (McNatty *et al.*, 2006). This has allowed research to reveal the considerable differences between the underlying mechanisms of follicular development in different species. For example mice and sheep with GDF9 deficiency (Dong *et al.*, 1996; Hanrahan *et al.*, 1998), and sheep (but not mice) with BMP15 deficiency (Martinez-Royo *et al.*, 2008) are sterile due to a block of folliculogenesis at the primary stage. This is due to a failure of the granulosa cells to proliferate, and a lack of antrum and theca cell layer formation (Dong *et al.*, 1996; Carabatsos *et al.*, 1998), indicating that these are a vital requirement for follicular development in these species. Furthermore in the mouse, GDF9 deficiency results in excess oocyte growth, with consequent loss of the oocyte, and formation of steroidogenic granulosa cell clusters (Elvin *et al.*, 1999b). However, these knock-out models can be limited in their usefulness, as they do not allow the study of the roles of the growth factors in later folliculogenesis involving oocyte capacitation, due to the cessation of development (Gilchrist *et al.*, 2008).

Research using human tissue has been held back by the fact that it is difficult to obtain normal, healthy tissue that has not been treated with high doses of gonadotrophins *in vivo*. As a result, animal models form the basis for research in this field, in order to overcome this problem. In particular, sheep and cows are widely used because they are predominantly monovular, in abundance, relatively inexpensive, and are large enough to obtain sufficient amounts of tissue for study (Campbell *et al.*, 2003). Mice are also

frequently used, however the relevance of the rodent model to human physiology can be somewhat limited.

In vitro experiments have been an extremely valuable source of information concerning the functions of OSFs and their effects on the follicular cells. Recombinant OSFs have been used to treat ovarian cells, yielding a considerable amount of new information, however in the past researchers have been met with extensive problems regarding being able to source high quality recombinants and reliable antibodies and immunoassays (Gilchrist *et al.*, 2008). As a result, there is a great deal of inconsistency between laboratories, and hence their findings have often proved contradictory. Because of these difficulties, the current preferred method of study relies upon bioassay.

1.8.1. Native OSF bioassays

The underlying principal of OSF bioassays is to culture ovarian cells with denuded oocytes (DOs), and compare the response to cells cultured without DOs. The effects on co-culture of follicular cells with DOs are marked, as granulosa and cumulus cells completely alter in function, indicating OSFs as the source for regulation of cell function. In addition, cells can be cultured in medium which has previously contained oocytes; oocyte-conditioned medium (OCM), which elicits a cellular biological response (Buccione *et al.*, 1990). Furthermore, if more DOs are added to the co-culture, the response increases (Hussein *et al.*, 2005).

Figure 1.10 shows the types of bioassay commonly used, where the target cells are in 3 different conditions; as a monolayer on the plate or dish surface [1 and 2], as oocytectomized complexes (OOX) [3], or as complete COCs [4]. Oocytectomy involves removing the oocyte contents from the COC, which creates a hollow ball of cumulus cells with the zona pellucida inside (Buccione *et al.*, 1990). Without the presence of an oocyte to secrete paracrine phenotype-stabilising factors, these cumulus cells become more characteristic of mural granulosa cells by decreasing DNA synthesis and secreting progesterone, suggesting that this may be their default developmental pathway (Li *et al.*, 2000).

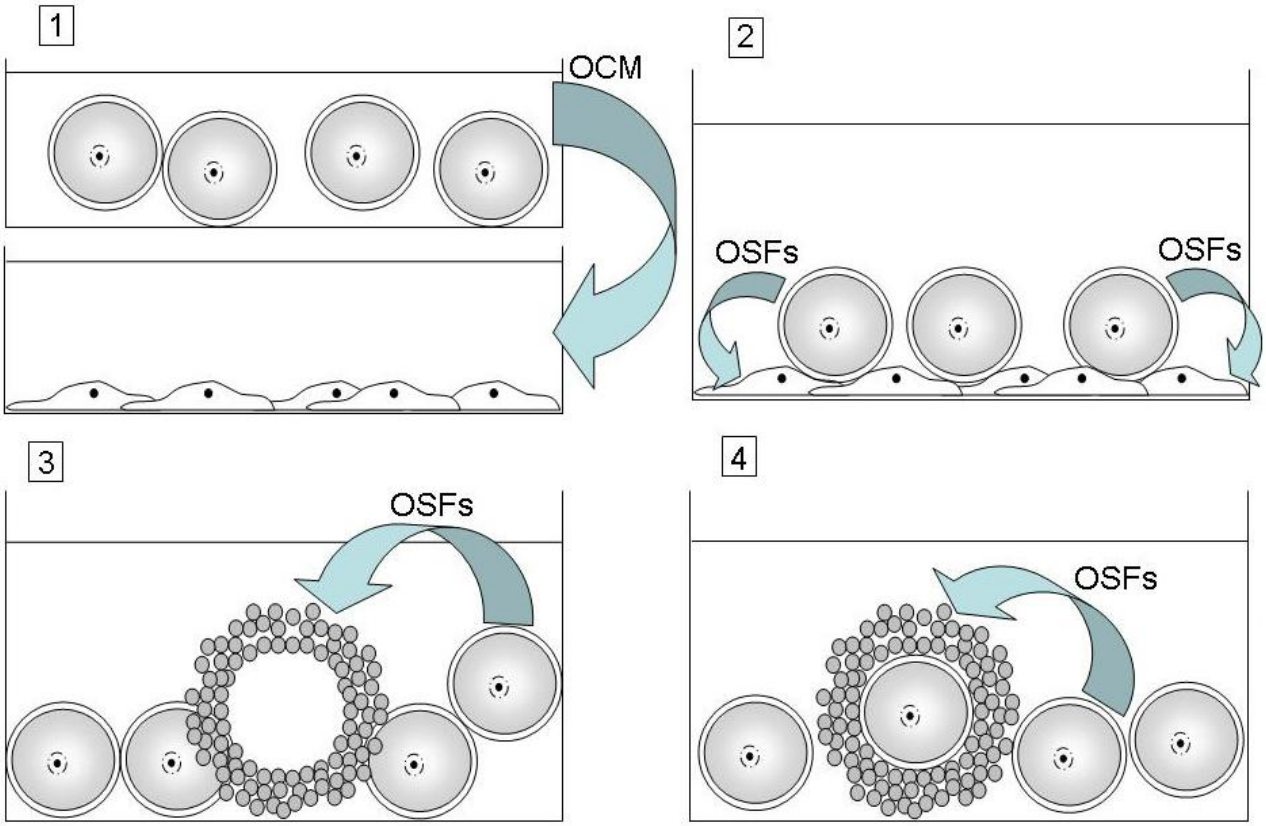

Figure 1.10. Bioassays of native oocyte-secreted factors (Redrawn from Gilchrist *et al.*, 2008)

In all of the models illustrated above the source of oocyte-secreted factors is DOs, made by removing the cumulus cells of COCs which are then cultured at high density. [1] shows target cells being treated with medium previously containing DOs. [2] and [3] show co-culture of DOs with target somatic cells, which can be either as a monolayer or as an oocytectomized complex respectively. [4] shows culture of DOs with a complete COC. (DO, denuded oocyte; COC, cumulus-oocyte complex; OCM, oocyte-conditioned media).

The problem with models 1-3 illustrated in figure 1.10 above is that the trans-zonal projections and gap junctions between the oocyte and cumulus cells are destroyed; hence their physical association is lost. However the major advantage of all of the systems is that because oocytes rather than recombinants are

used as the source of OSFs, the cell responses observed are much more physiological (Gilchrist *et al.*, 2008).

Using the cow model for oocyte and ovarian cell studies is a viable option to using human material, due to the extensive similarities between the reproductive mechanisms and embryonic development (Adams and Pierson, 1995; Bettegowda *et al.*, 2008). Using bovine material is particularly advantageous as it avoids the considerable ethical implications that are associated with research on humans, without compromising experimental relevance.

1.8.2. Cell culture systems

The development of a reliable and repeatable cell culture system for use in follicular somatic cell studies has facilitated research in the field of follicular dynamics. The system in place in our laboratory was conceived by Campbell *et al.* (1996), and was designed in such a way as to mimic physiological conditions as closely as possible, so that the results obtained from experiments based on challenges to the cells are physiologically relevant. As a general rule, cells obtained from physiological tissues are able to adapt to some extent to the environment in which they are cultured (Mandel, 1986), however it is vital to use a system in which cells secrete the same hormonal profile and respond as they would *in vivo*.

The ovine culture system used in our laboratory utilises serum-free culture medium, and allows for granulosa cell FSH-responsive production of oestradiol by undifferentiated cells from small antral follicles, as well as maintained production by differentiated cells from large antral follicles (Campbell *et al.*, 1996). In the past, medium has been supplemented with serum, from animal or human origin, as a means to provide the cultured cells with the different factors required for cell metabolism including growth factors, proteins, vitamins and minerals. However, the use of serum is frequently frowned upon, firstly because of the ethical issues associated with collecting foetal calf serum, secondly because of contamination by viruses, and thirdly due to the unreliable batch-to-batch variation of commercially available serum (van der Valk *et al.*, 2010). These variations often cause experimental discrepancies and alter results; for example, the inclusion of serum in culture of follicular cells has been shown to affect oestradiol production significantly, as well as affecting FSH responsiveness (Campbell, 1989). As with many cell culture mediums in research, the medium that is in use in our laboratory is supplemented with insulin, transferrin

and selenium. It has been known since the 1920s that insulin is a vital ingredient for cell culture (Gey and Thalhimer, 1924) for stimulation of glycolysis. Transferrin is required for iron transfer across cell membranes (Bjare, 1992), and selenium is used to protect the cells from oxidative stress which is associated with extended cell culture (Helmy *et al.*, 2000). In addition, bovine serum albumin is included in the medium as this is a lipid carrier, which also facilitates cell adhesion (Taub, 1990), as well as L-glutamine which is an essential protein synthesis precursor and respiratory fuel (Zielke *et al.*, 1984).

1.9. Ultrasonography

Following the original pioneering work performed by Edwards and Steptoe over 30 years ago, transvaginal ultrasound is now used routinely in assisted reproductive treatments in order to estimate the ovarian reserve and assess ovarian status, so that the ovarian response to stimulation can be both predicted and monitored respectively (Edwards and Craft, 1990).

Conventionally, ultrasound is performed in 2 dimensions, and follicle size estimation performed by taking an average of two diameter measurements. However, as ovarian follicles are three-dimensional structures, it is more accurate to measure the mean diameter in three dimensions, or even measuring volume as a proxy for follicular size. As a result, 3D ultrasound techniques have been developed in order to more accurately deduce the size of ovarian follicles (Ata *et al.*, 2011), as well as an extremely diverse range of other applications.

The development of power Doppler technology in conjunction with 3D ultrasound has been developed to enable the non-invasive measurement of blood flow within a target area, which is more sensitive than the more established colour Doppler technique (Schulten-Wijman *et al.*, 2008).

1.9.1. Power Doppler

Transvaginal power Doppler ultrasonography is a technique that has become well established as a valuable research tool in the field of reproductive medicine. The blood flow in the female ovary can be monitored accurately and non-invasively using Doppler ultrasound imaging, and the measurements that the technique gives could be used as predictors of fertility treatment outcome. Power Doppler technology

is relatively new in the field, and offers the advantages over conventional colour Doppler that the colour image produced is mapped on the amplitude of the frequency shift in terms of the integrated power of the Doppler spectrum, therefore facilitating measurement of low flow volumes and velocities that colour Doppler is unable to detect (Murphy and Rubin, 1997; Bhal *et al.*, 1999). As a result, it is possible to visualize very small blood vessels (MacSweeney *et al.*, 1996), for example those that surround the developing follicles.

Relatively recent studies measuring peri-follicular blood flow have revealed that each fully developed follicle has individual blood flow characteristics and vessel configuration, indicating that power Doppler is able to detect differences between follicles that conventional ultrasound would not (Borini *et al.*, 2001).

Studies examining follicular vascularity have found that preovulatory follicles can be classed according to the extent of vascularity that surrounds them. In fact, Bhal *et al.* (1999) created an index of perifollicular vascularity, which was based on the percentage of the follicle circumference that depicted an echo signal. This group found a link between vascularity index and oocyte retrieval rate, fertilisation rate and pregnancy rate, as well as a negative correlation to chromosomal abnormalities, a result also supported by Borini *et al.* (2001).

Huey *et al.* (1999) found that colour Doppler indices relate to oocyte developmental competence, by measuring pulsatility index (PI), resistance index (RI) and S-D ratio (peak systolic frequency shift [S] - end diastolic frequency shift [D]). Although the study was based on just 16 patients (79 oocytes), peri-follicular vascularity was significantly correlated to embryo cleavage at day 3, though there was no significant correlation to embryo morphology. There were a number of trends observed, however due to a relatively small sample size these were not significant.

The combination of power Doppler with three-dimensional (3D) ultrasonography enables quantification of blood flow, within a specified area, which can be an extremely sensitive technique (Pairleitner *et al.*, 1999). There are three indices calculated by the ultrasound computer; vascularisation index (VI), flow index (FI) and vascularisation flow index (VFI) (Schulten-Wijman *et al.*, 2008), which can be used to give an arbitrary indicator of blood flow around the ovary. These indices signify the extent of vascularity, and therefore could be used to predict the developmental competence of the oocyte within the follicle of interest (Monteleone *et al.*, 2008).

1.9.2. Antral Follicle Count

True ovarian reserve reflects the number and quality of the primordial follicle pool within the ovary, and as a result is not possible to measure whilst maintaining fertility (Visser and Themmen, 2005). Chronological age is correlated to ovarian reserve (Broekmans *et al.*, 2004), and a model based on this has been produced (Wallace and Kelsey, 2010), but this alone is an inadequate basis for accurate ovarian reserve prediction. A number of tests have therefore been developed and researched extensively in order assess the ovarian function of the individual patient (Broekmans *et al.*, 2009; Hansen *et al.*, 2011). These include serum FSH level (Toner *et al.*, 1989), clomiphene citrate challenge test (Loumaye *et al.*, 1990), gonadotropin-releasing hormone agonist stimulation test (GAST) (Winslow *et al.*, 1991), serum oestradiol level (Lucciardi *et al.*, 1995), serum inhibin-B level (Seifer *et al.*, 1997), and more recently ultrasound antral follicle count at the start of the menstrual cycle (Chang *et al.*, 1998; de Carvalho *et al.*, 2008) and serum anti-Müllerian hormone (Seifer *et al.*, 2002; Jayaprakasan *et al.*, 2010b).

The number of growing follicles is thought to be directly related to the number of primordial follicles in the pool itself (Gougeon, 1996); consequently, counting the number of visible small antral follicles on ultrasound scan has become a popular method of estimating ovarian reserve. Transvaginal ultrasonography is now used routinely in assisted reproduction treatments to assess ovarian status and estimate ovarian reserve in order to predict and monitor ovarian response to stimulation (Moawad *et al.*, 2009). Conventionally, 2D assessment of the ovarian follicles, which has been studied in great depth, is performed manually by a skilled operator, with findings now suggesting that it may be both inaccurate (Amer *et al.*, 2003) and time-consuming (Jayaprakasan *et al.*, 2008; Lamazou *et al.*, 2010). The antral follicle count involves estimating the total number of visible follicles from 2mm in diameter, to 10mm by convention (Deb *et al.*, 2010), although some groups have used a lower cut-off of 5 or 6mm diameter (Chang *et al.*, 1998; Pellicer *et al.*, 1998; Haasdma *et al.*, 2007) due to the hypothesis that smaller follicles are considered to form the so-called ‘functional’ ovarian reserve. These are the follicles that have the potential to develop to maturity and ovulate, and as a result these may be the most reflective of the true ovarian reserve (Jayaprakasan *et al.*, 2009; 2010b).

Thesis rationale, objectives and hypotheses

The rationale for this thesis is to investigate the relationship between the oocyte and its microenvironment, focussing on the bi-directional communication between the oocyte and somatic cells, and also the effect of vasculature on the individual follicle and oocyte within. The thesis will be based on a multi-faceted approach to further investigate the interactions that occur within the ovarian follicle, with a focus on oocyte-secreted factors and blood supply.

Our overall hypothesis is that the measurement of growth factors secreted by the oocyte (for example BMP15 and GDF9), angiogenic growth factors from somatic cells (for example VEGF and HIF), and also constituents of the follicular fluid, such as steroid hormones and oocyte-secreted factors, could reflect the quality and developmental potential of the oocyte contained within. Assessment of follicular blood flow by 3-dimensional power Doppler ultrasonography is a tool that could also be used for this purpose as follicular blood flow is thought to have a considerable influence on the development of the oocyte within. As the method is objective and non-invasive, and closely related to follicular development, we propose that it could serve as a good predictor of oocyte competence and embryo development so that the embryo with the best developmental competence can be selected for transfer.

The overall objective of the thesis is to elucidate some of the mechanisms underlying follicular development relating specifically to oocyte development and quality. This will be achieved by completing the following specific objectives:

1. Development of *in vitro* culture models to examine the molecular mechanisms regulating the expression of oocyte-secreted and angiogenic factors and their effect on follicular somatic cells cultured under controlled conditions. This will form the basis of the investigation of interactions between the oocyte and somatic cells, as well as the effects of hypoxia on a cellular scale. These will be examined on a molecular level using gene expression analysis, as well as measuring levels of steroid hormones and growth factors associated with angiogenesis. The hypothesis is that the oocyte influences the overall development of the follicle by producing factors that act locally to control the microenvironment in which it is subjected.
2. Investigation into the effects of passively immunizing animals against oocyte-secreted factors on follicular development by directly infusing antiserum into the ovarian vasculature. This will further

examine the roles of BMP15 and GDF9 *in vivo*, which we hypothesise will have a significant synergistic effect on folliculogenesis.

3. Development of phantom-based models in order to determine the accuracy and relevance of ultrasound assessment of ovarian blood flow, and also the 3D ultrasound measurement of follicular size and antral follicle count. Power Doppler ultrasound techniques will form the basis of the investigation of ovarian and peri-follicular blood flow on a histological scale, using a bovine phantom tissue model. Currently, the blood flow parameters given by the ultrasound machine are arbitrary in terms of units and so through relating true blood flow and vascularity information determined in perfused bovine ovaries, we hope to clarify the meaning of these ultrasound parameters. In addition, the accuracy of automated follicular measurement and antral follicle count will be examined using another phantom model.
4. Amalgamation of the findings of the previously mentioned experiments in the form of a small scale clinical trial in human subjects undergoing fertility treatment. Patients will undergo ultrasound assessment of peri-follicular blood flow, and then the oocytes cultured individually in order to maintain individuality, so that the embryological data can be related back to that of the blood flow scans. Follicular fluid and somatic cells will be collected at oocyte retrieval and examined against the ultrasound and embryological data. This trial has been designed to examine the relationships between oocyte-secreted and angiogenic growth factors and hormones, as well as ultrasound blood flow assessment parameters, as well as IVF treatment outcome. We hypothesise that the levels of growth factors and steroid hormones in the follicular fluid will correlate to ultrasound blood flow parameters, as well as consequent oocyte and embryo quality.

Chapter 2 – Materials and Methods

2.1. Cell Culture

2.1.1. Follicle dissection and granulosa cell isolation

Approximately 20-30 bovine ovaries were obtained from the abattoir and transported to the laboratory in a flask containing warmed dissection medium. They were washed in 70% ethanol, then warmed Dulbecco's PBS (DPBS), and then transferred to fresh warmed dissection medium (see appendix). Depending on the type of culture being used, follicles of various sizes were roughly dissected from the ovaries and transferred to petri dishes of warmed dissection medium using fine curved forceps.

Once approximately 200 follicles had been obtained, they were cleaned of excess extraneous tissue if necessary, using fine forceps. The follicles were then hemisected in approximately 10ml of DPBS using fine bow scissors and forceps under a stereo microscope, to release the fluid and cells from within.

The follicle halves were transferred into 25ml universal tubes and then flushed repeatedly using a 1ml syringe. After allowing the follicle shells to settle, the supernatant cell suspension was transferred to 15ml centrifuge tubes containing 5ml warmed granulosa culture medium (see appendix), along with the DPBS used for hemisecting. The tubes were centrifuged at 800rpm for 10 minutes, the supernatant discarded, and the cell pellets resuspended in 10ml warmed granulosa culture medium. This process of flushing the follicle shells was repeated to obtain more granulosa cells.

Once the granulosa cells had been collected, a viable cell count was performed using trypan blue (Sigma T8154, 0.4%). 50µl of cell suspension and 50µl trypan blue were mixed, and used to fill a haemocytometer. Only transparent cells (not blue) were considered to be viable and hence included in the cell count. These were counted in all 25 small squares of the haemocytometer, multiplied by 2 (dilution factor), and then by 10^4 to calculate the cells per ml.

2.1.2. Granulosa cell culture

Granulosa cells were cultured according to the serum-free method developed by Campbell *et al*. (1996). This method mimics physiological conditions in the follicular phase due to the inclusion of physiological amounts of FSH, IGF, insulin and LH (see appendix for full list of additives). Cells were seeded at 75,000

per well of a 96 well plate, in 250µl of granulosa cell culture medium, and incubated at 37°C with 5% CO_2 for 8 days. Culture medium was changed every 48 hours, and used medium was kept and stored at -20°C for later analysis. Where the culture required a cell number estimation was to be performed, a flask of cells containing approximately 2 million cells in 5ml of granulosa medium was also cultured to be used at the end of the culture to construct a standard curve for neutral red staining. If cells were to be used for PCR, they were lysed and prepared at the end of the culture, as per the method outlined in 2.2.4.1., hence in this case cell counts were not performed.

2.1.3. Estimation of viable cell count

At the end of the culture, the total viable cell count was performed using neutral red staining (Sigma N6264), which is based on the uptake of dye that accumulates in viable cell lysosomes (Chiba *et al.*, 1998). The culture medium was removed and replaced with fresh culture medium containing neutral red, and incubated for 2 hours to allow uptake of the dye. Following this the neutral red culture medium was removed and replaced with 200µl per well of formol calcium (4% w/v formaldehyde and 1% w/v calcium chloride) to fix the cells to the bottom of the wells. This was then replaced with 200µl of acetic acid-ethanol (50%v/v ethanol, 1% v/v glacial acetic acid), which lyses the cells. Absorbance at 540nm was then measured on a plate reader against a standard curve as prepared below.

Cells were cultured in a flask under similar conditions to the main culture. At the end of the cell culture the medium was removed and replaced with 5ml of medium containing neutral red, and incubated for 2 hours to allow dye uptake. Following this the medium was discarded and replaced with 5ml trypsin-EDTA (0.2% w/v trypsin and 0.08% w/v EDTA in DPBS) for approximately 20 minutes, until the cells had become detached from the flask surface. The cell suspension was then transferred to a 15ml centrifuge tube with 1ml of foetal calf serum to inactivate the enzyme. This was then centrifuged at 800rpm for 10 minutes, the supernatant discarded, and 2ml culture medium used to resuspend the pellet. A cell count was performed using trypan blue as previously described to estimate the total number of cells.

The cells were centrifuged at 800rpm for 10 minutes, and the supernatant replaced with 2ml acetic acid-ethanol solution. A serial dilution in acetic-acid ethanol was used to create a standard curve in 6ml bijou tubes, and 200µl of each concentration was transferred in quadruplicate into a 96 well plate. Absorbance

was read at 540nm by spectrophotometer, and the cell numbers from granulosa cell cultures estimated in comparison to the relative standard curve.

2.2. Sample analysis

2.2.1. Steroid Hormone ELISA

Oestradiol Plasma Extraction

Extraction on plasma samples was performed to improve the detection limit of the assay, by preventing interference from sex-hormone-binding globulins (Dighe and Sluss, 2004). A similar method to that of androstenedione was used for extraction of oestradiol. 400µl of plasma sample of QC was pipette into 100x13mm glass tubes, and 4ml diethyl ether was added to each tube. The tubes were then vortexed for 1 minute to allow mixing. They were then placed into a bath of IMS and dry ice in order to freeze the plasma. The supernatant was then poured off into 75x12mm glass tubes, and then dried down using a heated sample concentrator. 250µl of assay buffer was added to each tube to resuspend the oestradiol.

ELISA

Similar methods were used for oestradiol and progesterone ELISAs. Oestradiol intra-assay CV was 5.3% and 6.6% respectively, and inter-assay CV 8.0% and 9.3% respectively. Both had been optimised and validated in-house.

Plate coating

100µl of diluted oestradiol or progesterone antibody (ABCAM ab1024 and SAPU R7044X) at 1:64,000 for oestradiol or 1:32,000 for progesterone, in coating buffer (appendix 2) was pipette into each well of a 96 well plate (Nunc F96 Maxisorp, Denmark), and incubated overnight at 4°C.

Plates were washed 4 times using wash solution, on a plate washer, and then 100µl of blocking solution (PBS/3%BSA) was pipette into each well. They were then incubated for 2 hours at room temperature. Following this, the plates were washed a further 4 times using a plate washer. 100µl of standard, sample or QC were pipette into each well (samples and QCs diluted 1:4). 100µl E2-HRP conjugate (ABIOX ED-

006) at 1:64,000 in assay buffer was added to each well and the plates incubated for 2 hours at room temperature.

Plates were then washed 4 times on a plate washer, and then 50µl of TMB (Sigma T0440) was pipette into each well. They were incubated at room temperature for approximately 10 minutes and then 50µl of stopping solution (1M H_2SO_4) was added into each well. Plates were read at 450nm absorbance (referencing at 620nm) and sample concentrations estimated compared to the standard curve.

Reagents

Standards were made from stock solutions as follows:

- 10µl 'Stock B' (10µg/ml oestradiol [Sigma E2758] or progesterone [Sigma P0130] in ethanol) into 10mls culture medium or assay buffer to obtain 1000pg/100µl.
- Double diluted in culture medium or assay buffer to obtain 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9pg/100µl. Assay buffer is 'zero' standard. Stored frozen in LP4 tubes.

2.2.2. AMH, BMP15 and GDF9 ELISA

Follicular concentrations of AMH, BMP15 and GDF9 were measured in samples collected from patients in the clinical trial using ELISA kits.

The human AMH kit from Cusabio Biotech Ltd. (CSB-E12756) was a simple competitive ELISA with a detection range of 8ng/ml to 0.32ng/ml. All reagents were supplied with the kit, and the plate was to be read at 450nm with a correction wavelength of 570nm. 50µl of undiluted samples were added in duplicate to the wells, and all fell within the detectable range with a CV of less than 10% between replicates.

The human BMP15 and GDF9 ELISA kits from USCN Life Science Inc. (E92107Hu and E90427Hu) were sandwich enzyme immunoassays which used a biotinylated antibody and avitin conjugated to horseradish peroxidase. Both kits had a detection range of 10ng/ml to 0.156ng/ml. All reagents were supplied with the kit, and the plate was read at an absorbance of 450nm. 100µl of follicular fluid sample was used both neat and diluted 1:10 in PBS for BMP15 ELISA, and used undiluted, 1:10 and 1:100 in PBS for GDF9 ELISA in duplicate. All samples diluted 1:10 for BMP15 fell within the detectable range with a CV of less than 10% between replicates. GDF9, as later discussed, was not detectable in the follicular fluids, however a

standard curve was constructed with an r^2=0.9992, indicating that concentrations in the follicular fluid were too low to be measured using this method.

2.2.3. Radioimmunoassay

Radioimmunoassays for FSH and LH were performed as previously described by Campbell *et al.* (1994) and Kendall *et al.* (2004). Intra-assay variation for the radioimmunoassays was 8.4% for FSH and 4.4% for LH, and inter-assay variation 12.6% and 11.6% respecitvely, as assessed by internal QCs.

FSH radioimmunoassay

Standards were set up using follicle stimulating hormone (NIDDK, USA oFSH-19-SIAFP) in assay buffer (0.01M PBS, 0.1% BSA and 0.1% sodium azide) at 0, 0.26, 0.52, 1.04, 2.08, 4.17, 8.34 and 16.67ng/ml. 200µl of each standard or sample/QC was pipetted into LP4 plastic tubes in triplicate or duplicate respectively. A further 200µl of assay buffer was added to the tubes. 50ul of FSH antibody (Tucker Institute, ter.oFSH.AB.1) at 1:175,000 in assay buffer was added to each sample, QC and standard tube. 50µl of FSH antigen-labelled I^{125} (iodination performed in house) was added to each sample, QC, standard, total binding and non-specific binding tube. The label was used at between 10,000 and 12,000cpm. Tubes for non-specific binding (NSB) contained 450µl assay buffer with label, and total count (TC) tubes contained label only. Both were prepared in triplicate. The tubes were incubated at room temperature overnight.

100µl of second antibody mixture was added to each tube (apart from TC), and the tubes were then left overnight at 4°C once again. The second antibody solution consisted of donkey anti-rabbit serum (DARS) (SAPU anti-rabbit IgG 5022-220) at 1:80 and normal rabbit serum (NRS) (Sigma R4505) at 1:1600 in assay buffer. 500µl of assay buffer was added to each tube (apart from TC), and these tubes were centrifuged at 4°C for 30 minutes at 3000RCF. The supernatant was then aspirated from the tubes (apart from TC), and the counts per minute were measured relative to the standard curve on a gamma counter.

LH radioimmunoassay

Standards were set up using luteinizing hormone (NIDDK, USA oLH-I-4) in assay buffer (0.01M PBS, 0.1% BSA and 0.1% sodium azide) at 0, 0.156, 0.31, 0.625, 1.25, 2.5, 5, 10, 20 and 40ng/ml.

100µl of each standard or sample/QC was pipetted into LP4 plastic tubes in triplicate or duplicate respectively. A further 400µl of assay buffer was added to the tubes. 100ul of LH antibody (NIDDK, USA anti-oLH-1) at 1:420,000 in assay buffer was added to each sample, QC and standard tube. 100µl of LH antigen-labelled I^{125} (iodination performed in house) was added to each sample, QC, standard, total binding and non-specific binding tube. The label was used at approximately 10,000cpm. Tubes for non-specific binding (NSB) contained 600µl assay buffer with label, and total count (TC) tubes contained label only. Both were prepared in triplicate. The tubes were incubated at room temperature overnight.

200µl of second antibody mixture was added to each tube (apart from TC), and the tubes were then incubated overnight at 4°C. The second antibody solution consisted of DARS at 1:80 and NRS at 1:400 in assay buffer. 500µl of assay buffer was added to each tube (apart from TC), and these tubes were centrifuged at 4°C for 30 minutes at 3000RCF. The supernatant was then aspirated from the tubes (apart from TC), and the counts per minute were measured relative to the standard curve on a gamma counter.

Androstenedione Plasma extraction

Androstenedione was first extracted from the samples and QCs as outlined in 2.2.1. Radioimmunoassay was performed as previously described by Campbell *et al.* (1994).

200µl extracted plasma sample or QC (low, medium and high) was pipetted into 75x12mm glass test tubes in duplicate, and 2ml hexane/diethyl (4:1) added. The tubes were then vortexed for 1 minute to allow mixing. The tubes were then placed into a bath of IMS and dry ice in order to freeze the plasma at the bottom of the tube. The solvent supernatant was then poured off into 75x10mm glass tubes, and then dried down using a heated sample concentrator. 100µl of PGel buffer was then added to each tube to resuspend the androstenedione.

Androstenedione Radioimmunoassay

Standards were made up using androstenedione (Sigma A9630) in PGel buffer at 0, 0.039, 0.078, 0.156, 0.31, 0.625, 1.25, 2.5, 5 and 10ng/ml. 200µl of each standard or sample/QC was pipetted into 75x10mm glass tubes in triplicate or duplicate respectively. 100ul of androstenedione antibody ('Rabbit C' prepared in house) (1:15,000) was added to each sample, QC and standard tube. 100µl of androstenedione antigen-labelled I^{125} (Glasgow Royal Infirmary, Scotland) was added to each sample, QC, standard, total binding and non-specific binding tube. The label was used at between 10,000 and 12,000cpm. Tubes for non-specific binding (NSB) contained 200µl Pgel buffer with label, and total count (TC) tubes contained label only. Both were prepared in triplicate. The tubes were incubated at 4°C overnight.

250µl of second antibody mixture was added to each tube (apart from TC), and the tubes were then left overnight at 4°C once again. The second antibody solution consisted of DARS at 1:80 in PGel, with NRS at 1:1600 in PGel. 250µl of Pgel buffer was added to each tube (apart from TC), and these tubes were centrifuged at 4°C for 30 minutes at 3000RCF. The supernatant was then aspirated from the tubes (apart from TC), and the counts per minute were measured relative to the standard curve on a gamma counter.

The intra-assay coefficient of variation was 9.1%, and inter-assay variation 10.6%.

2.2.4. Reverse Transcriptase PCR

2.2.4.1. mRNA extraction from somatic cells

The cell lysates in RLT buffer with β-ME were first homogenized using QIAshredder columns (Qiagen 79654). Briefly, the cell lysate was loaded onto the columns and centrifuged at 13,000rpm for 2 minutes, into a 2ml collection tube. This homogenized lysate was then used for mRNA extraction.

The commercially available Qiagen RNeasy mini kit (Qiagen 74104) was used for mRNA preparation from the cell lysate, which uses a spin column containing RNA binding membrane. An equal volume of 70% ethanol was added to the cell lysate before use, and then the mixture was loaded onto the RNeasy spin columns and centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded and 350µl of buffer RW1 added to the columns. They were then centrifuged again at 10,000rpm for 15 seconds, and the flow-through discarded. At this point a DNase (Qiagen 79254) digestion step was used in order to remove unwanted DNA from the sample. The DNase for each column was prepared by adding 10µl of

DNase stock solution to 70µl RDD buffer supplied in the kit. 80µl of the DNase solution was added to each column and left at room temperature for 15 minutes. 350µl of RW1 buffer was added to the columns to remove the DNase, and then centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded and 500µl RPE buffer added to each column, then centrifuged at 10,000rpm for 15 seconds. The flow-through was again discarded, and 500µl of RPE buffer was added to each column and centrifuged at 10,000rpm for 2 minutes. The columns were transferred to fresh 2ml collection tubes, and then centrifuged once more at 13,00rpm for 1 minute to remove all unwanted cell material. The columns were then transferred to 1.5ml Eppendorfs and 40µl RNase-free water (Qiagen 129112) added to elute the mRNA. The columns were centrifuged at 10,000rpm for 1 minute, and then the columns were discarded, leaving the mRNA in the RNase-free water. These tubes could then be stored at -80° for later analysis, or used straight away in the next step of the process; cDNA synthesis.

2.2.4.2. cDNA synthesis

cDNA was synthesised from mRNA by adding 11 µl of sample to 2µl random hexamer primers (Promega C1181). This was incubated at 70°C for 5 minutes and then immediately held on ice for a minimum of 2 minutes to stop the reaction. 7µl of mastermix was then added to make a 20µl reaction, and incubated first at 47°C for 35 minutes, then 75°C for 10 minutes.

Mastermix was prepared using the following (per sample):

5x first strand buffer (Invitrogen Y00146)	4µl
0.1M DTT (Invitrogen Y00147)	1µl
4dNTP mix (Invitrogen 10297-018)	1µl
RiboLock™ RNase inhibitor	0.5µl
M-MLV reverse transcriptase (Invitrogen 28025-013)	0.5 µl

cDNA samples were then stored at -20°C for later PCR analysis.

2.2.4.3. Reverse-transcriptase PCR

Mastermix was created using the following (per sample):

SYBR Green (Applied Biosystems 4367659)	12.5µl
Sense primer	0.5µl
Antisense primer	0.5µl
cDNA sample	2µl
RNase-free water (Qiagen 129112)	9.5µl

18S was used as a housekeeping gene, to be compared with the gene of interest (e.g. HIF-1α, VEGF, aromatase). Primers were diluted at 1:4 for 18S, 1:10 for HIF-1α and VEGF or 1:4 for aromatase. Samples were also diluted 1:10.

The reaction conditions were as follows:

Holding stage	95°C for 10 minutes
Cycling stage (x35)	95°C for 20 seconds
	60°C for 45 seconds
	72°C for 1 minute

2.3. Tissue processing and staining

The ovine ovarian tissue collected from the immune-neutralisation study animals post-mortem was fixed in Bouins and prepared for histological staining by processing through a series of increasing concentrations of alcohol, followed by xylene and wax in an automated tissue processor (appendix 3). Serial 5µm sections were cut from the tissue blocks, and 2 sections mounted per slide (Superfrost® Plus; BDH Laboratory Supplies, Dorset, UK) and incubated overnight at 37°C to allow adequate adhesion. 4 slides were created from each tissue block, and each tissue block comprised of either a half or a third of an ovary, depending on its size.

2.3.1. Hemotoxylin & eosin staining

Follicle counts were performed on sections following haemotoxylin and eosin (H&E) staining (see appendix 3) according to a similar criteria to that defined by Lundy *et al.* (1999) (figure 2.1);

- Primordial follicle – Single layer of flattened pre-granulosa cells
- Transitional follicle – Single layer of granulosa cells with one or more with cuboidal structure
- Primary follicle – Single layer of all cuboidal granulosa cells
- Secondary follicle – Two layers of cuboidal granulosa cell layers with no antrum present
- Preantral follicle – Three or more layers of cuboidal granulosa cell layers with no antrum present
- Antral follicle – Multiple cuboidal granulosa cell layers with a visible antral cavity present

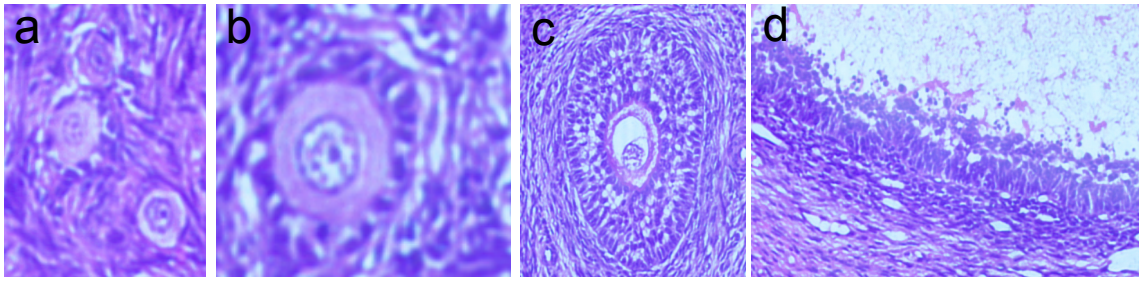

Figure 2.1. H&E stained ovine (a) primordial and transitional follicles, (b) primary follicles, (c) small antral follicle and (d) large antral follicle wall.

(Original magnification (a,b) x400; (c,d) x200)

The tissue was initially mounted on slides using glycerine albumin to aid adhesion to the slide surface, followed by DPX (Sigma 44581). One slide was stained per ovarian section (2 or 3 per ovary), with 2 tissue sections per slide.

2.3.2. Aromatase immunohistochemistry

Immunohistochemistry was used to detect aromatase expression on intermittent slices through the ovarian cortex using an automated staining machine (Leica BOND-MAX™, Milton Keynes, UK). Epitope retrieval was performed on the machine, before the Bond staining protocol was initiated (appendix 3). Homologous antibody for ovine aromatase is not commercially available, and so a monoclonal mouse

anti-human cytochrome P450 aromatase was used (MCA2077S, AbD Serotec, Oxford, UK). The Bond Polymer Refine Detection Kit (DS9800, Leica Microsystems, Milton Keynes, UK) was used in the machine, which included a 1 hour incubation with the antibody. This comprises of a peroxide block, post primary rabbit anti-mouse IgG linker, anti-rabbit poly-HRP-IgG, DAB (3,3'-Diaminobenzidine) chromogen and haematoxylin counterstain. The slides then undergo a dehydration process using alcohol and xylene, and are mounted with a coverslip using DPX mountant (Sigma 44581).

2.4. Ultrasound

For all of the phantom experiments used to study the efficacy of automated follicle measurement and counting, the bovine ovaries were obtained from the abattoir and assessed within two hours of collection in order to try and maintain blood vessel patency and restrict shrinkage. Bovine ovaries were selected for use in the phantom studies because of their morphological similarity to stimulated human ovaries; in particular, the dominant follicle in cattle grows to approximately 18-20mm, which is comparable in size to human stimulated ovarian follicles.

For ultrasound scanning of bovine ovaries, a 5-9MHz transvaginal probe was used in combination with the Voluson E8 Expert ultrasound machine (GE Medical systems, Austria). This particular set-up is routinely used in the fertility clinic for ovarian assessment.

Ultrasound gel was used on the surface of the tissue in order to ensure appropriate contact between the probe and the scanning surface, to avoid attenuation caused by air gaps. The ultrasound settings were kept constant for all scanning procedures, which was the default setting used in the department for patient examination.

2.4.1. SonoAVC™

The same analytical method for ovarian assessment was used for the bovine phantom studies as for human clinical use. The technique utilises SonoAVC™ software application to a 3 dimensional dataset, which can be acquired at a time previous to analysis.

The first stage of the process of ovarian assessment using this technique is to load the dataset into the software. Then the ovary is then centered in all three planes of view so that the whole ovary is included in the analysis, but any outlying areas are excluded (see figure 2.2 below).

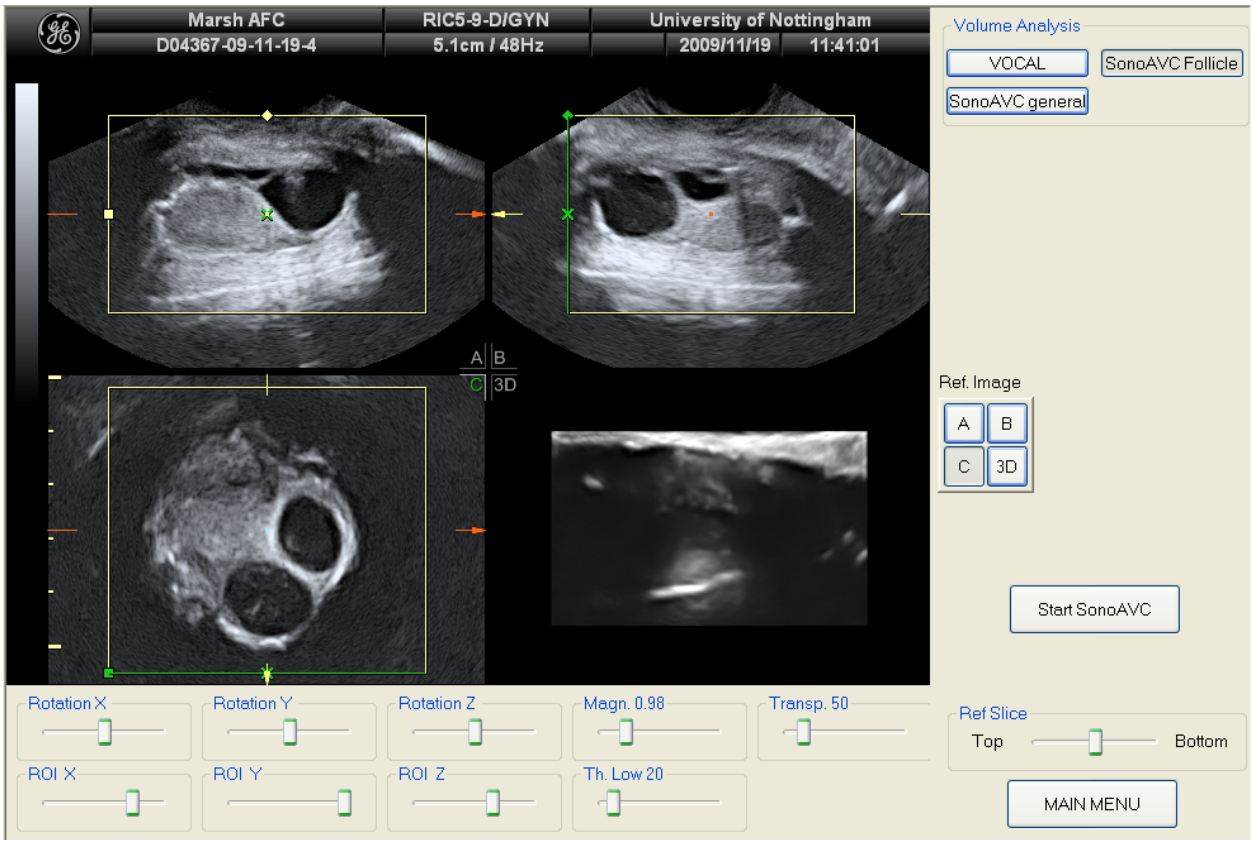

Figure 2.2. 3D ultrasound scan image of bovine ovary with follicles visible as dark circular appearance

The first step in the process is to prepare the image for SonoAVC™ by centering the yellow box over the ovary, and sizing it to eliminate as much of the extraneous tissue as possible.

The SonoAVC™ software is then applied to the image and the follicle interior wall is drawn around in all three planes automatically, to form a 3D structure (figure 2.3). The software then measures each follicle in three dimensions and gives a mean value for the diameter (m-d), a volume (V), and a relaxed sphere diameter (d(V)) which is the diameter of the follicle calculated from the volume (measured in 3D pixels called voxels). The relaxed sphere diameter is generally used in clinical practice rather than the mean diameter.

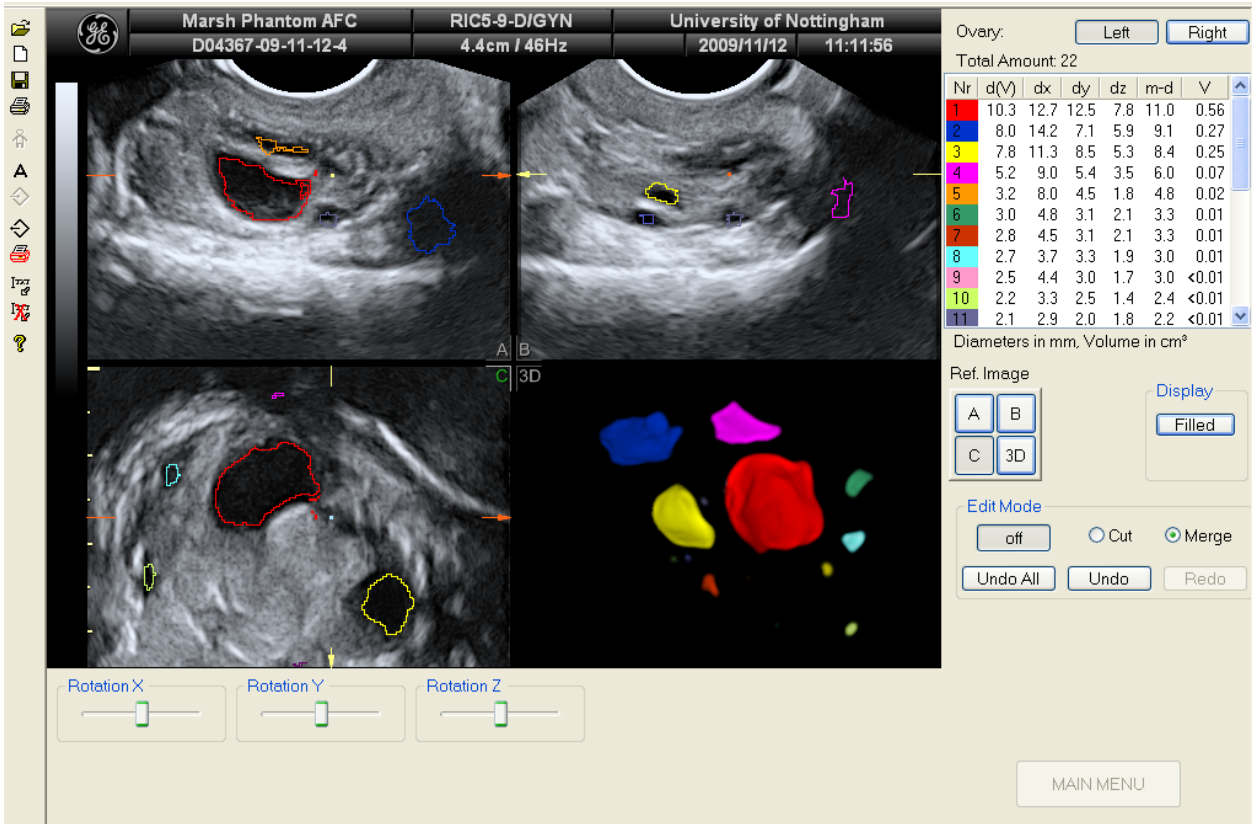

Figure 2.3. Semi-automated SonoAVC™ measurement of bovine ovarian follicles

The software identifies hypoechoic regions and draws around the inner surface. Each follicle is assigned a different colour and its dimensions are displayed in the upper right hand corner. The diameter by volume [d(V)], mean diameter [m-d] from the dx, dy and dz planes, and volume [V] are given for each follicle detected by the software.

2.4.2. VOCAL power Doppler analysis

VOCAL analysis is a technique commonly used for the assessment of blood flow in a target organ. In these experiments this target organ was the ovary. A bovine ovarian perfusion phantom was used to study the relevance of power Doppler analysis in VOCAL, and the same analytical technique was then used to assess blood flow around a target ovarian follicle in IVF patients for the purposes of the clinical trial. The only methodical differences were that the volume of interests analysed included the whole ovary or the selected follicle respectively.

The computerised histogram facility in 4D View software was used for all power Doppler analyses (GE Medical Systems, Austria), whereby the scan is performed in 3D and the image stored on the computer to be analysed 'offline'. Figure 2.4 below shows the first stage of the process in whole ovary analysis.

The user selects a volume of interest (VOI) using the Virtual Organ Computer-aided Analysis (VOCAL) tool (figure 2.4 below).

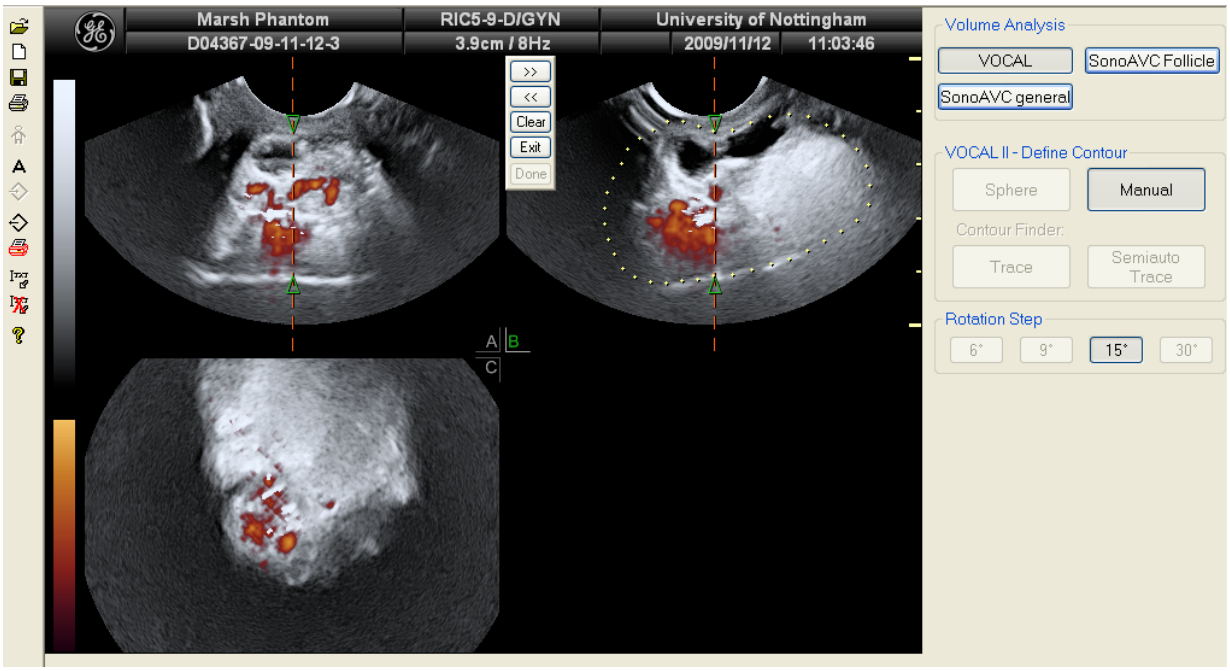

Figure 2.4. A 3D ultrasound scan of a perfused bovine ovary in the VOCAL image analysis tool

The 3D scan is able to be analysed in 3 planes; A, B and C in order to attain the most accurate outline of the target organ. The target of interest is drawn around in a series of 15 degree rotations (yellow dots on top left scan image), around a central point (red dashed line on top two images) until the whole organ is included in the selection.

Following the selection of the target organ by a series of manual drawing steps in 15° rotations, VOCAL measures the amount and intensity of the Power Doppler signal on the target volume of interest. The measured vascular indices are displayed as a histogram (Raine-Fenning *et al.*, 2008b). The three indices calculated are vascularisation index (VI), flow index (FI), and the vascularisation flow index (VFI) (figure 2.5).

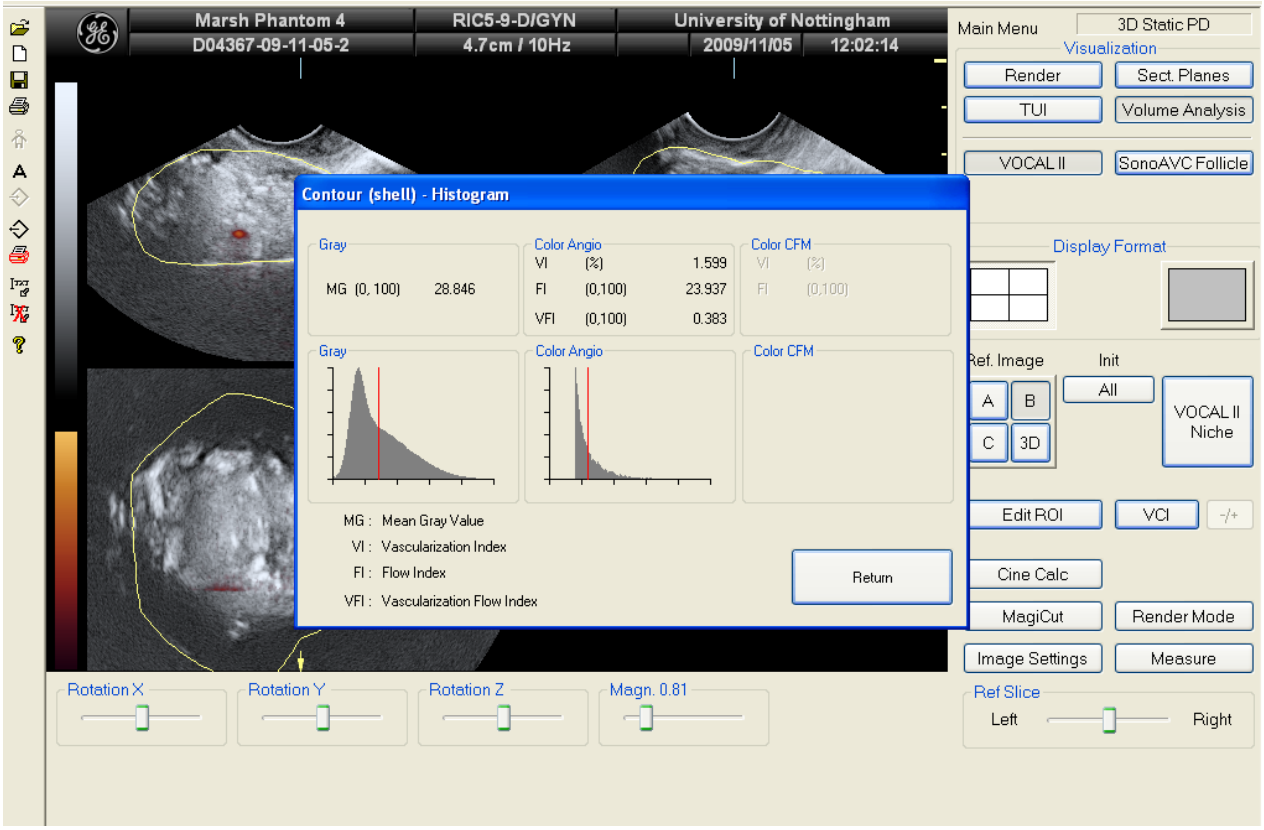

Figure 2.5. VOCAL analysis of Power Doppler blood flow in perfused bovine ovary

The vascularisation index, flow index and vascularisation index are displayed in the Color Angio box in the top centre. The colour histograms are also displayed as graphs.

Vascularisation index (VI) is based on the ratio of colour voxels to total voxels in the volume of interest, flow index (FI) is the mean power Doppler signal intensity for the defined volume, and the vascularisation flow index (VFI) is the multiplication of the VI and FI divided by 100. In order to measure peri-follicular blood flow around a selected follicle, a similar procedure was used for that of whole ovarian analysis. The

inner wall of the follicle was drawn around in the A plane, in order to select this as the volume of interest, in a series of 15° rotations (figure 2.6).

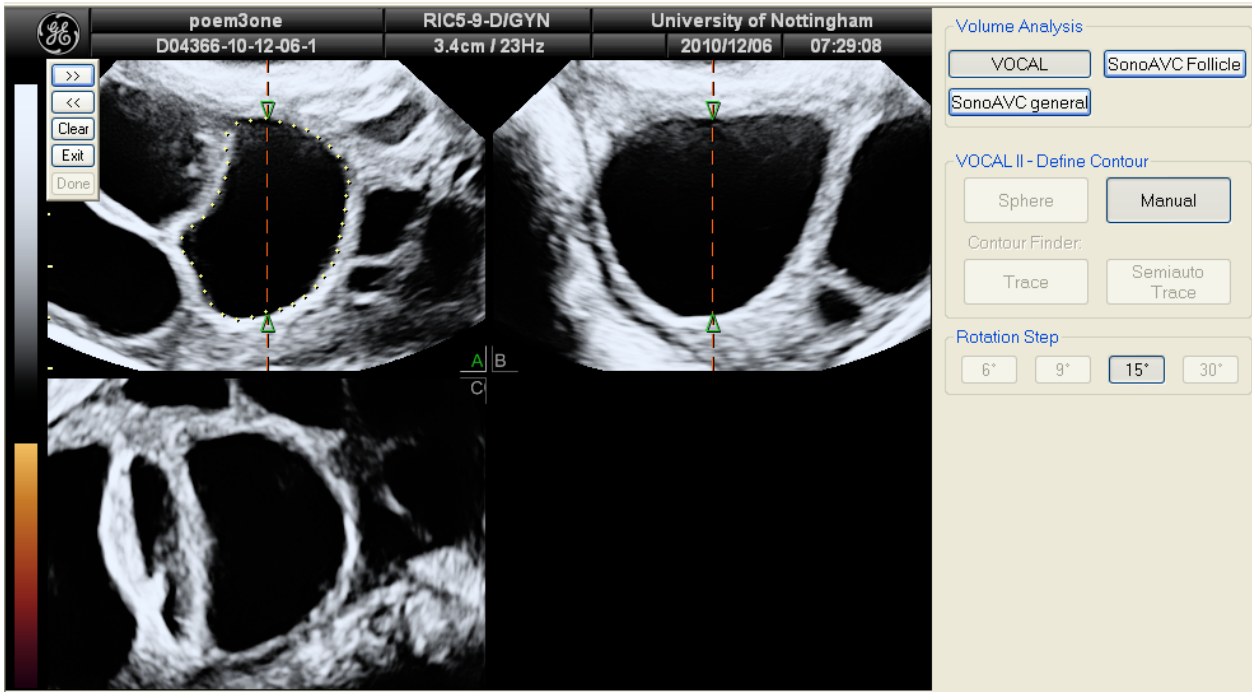

Figure 2.6. The first stage of highlighting a follicular volume of interest in the human ovary, involving a series of 15° rotations around a central line of reference

The next stage of the process is to correctly align the selected area with the follicular wall, as this is required to be relatively accurate for a reliable analysis (figure 2.7).

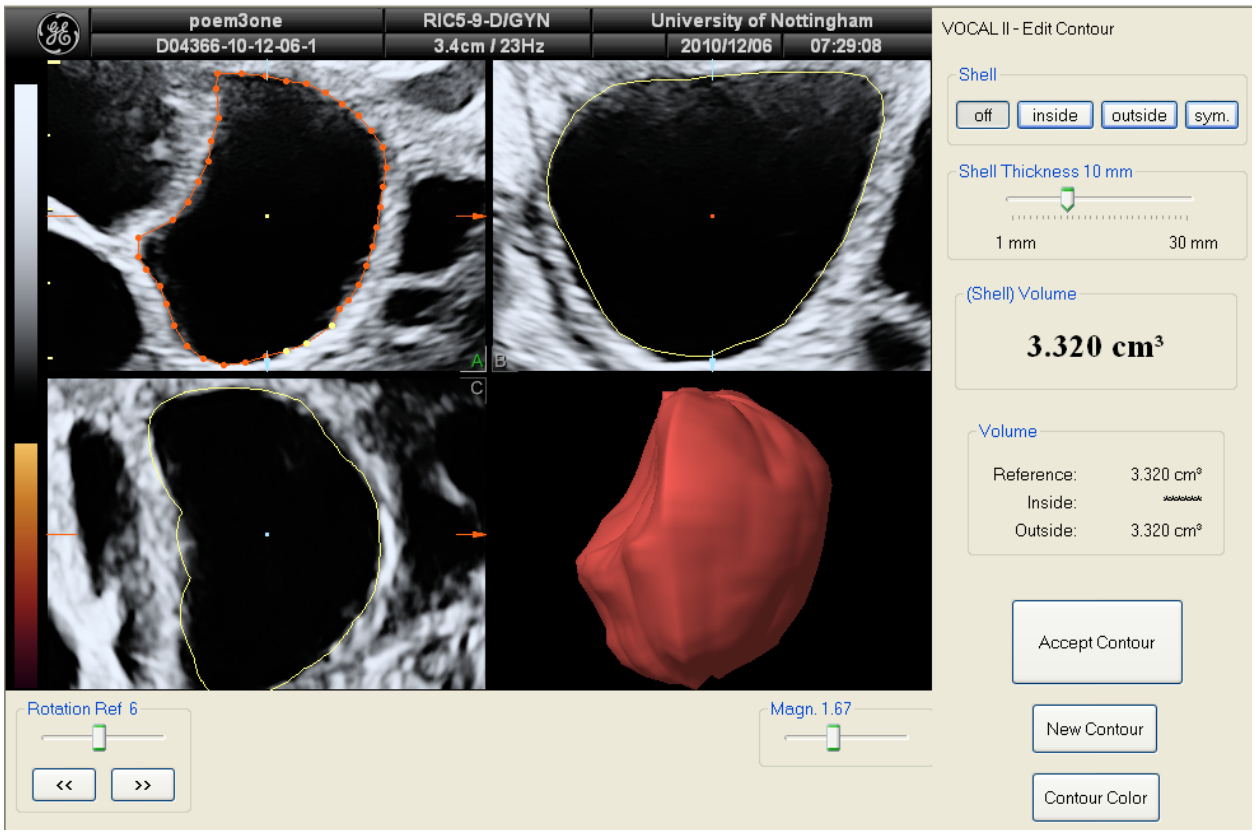

Figure 2.7. VOCAL contour selection adjustment in human ovarian assessment to achieve an accurate line trace around the interior follicle wall

The contour is adjusted to perfectly align with the inner follicular wall. The volume of interest is displayed on the lower right as a 3D image.

Once the line drawn correctly matches with the inner follicular wall, the contour is accepted, and the volume of the follicle displayed on the right hand side of the screen. A shell is then applied to the contour in order to analyse the area around the follicle for power Doppler signal. It was decided to use a 1mm shell around the follicle in order to include the entire relevant signal, but exclude signal from vessels of other follicles. In addition, an inner shell of 1mm was included because the nature of the power Doppler

signal means that the areas of flow present as spreading areas of colour that can infiltrate the inner follicle wall (figure 2.8).

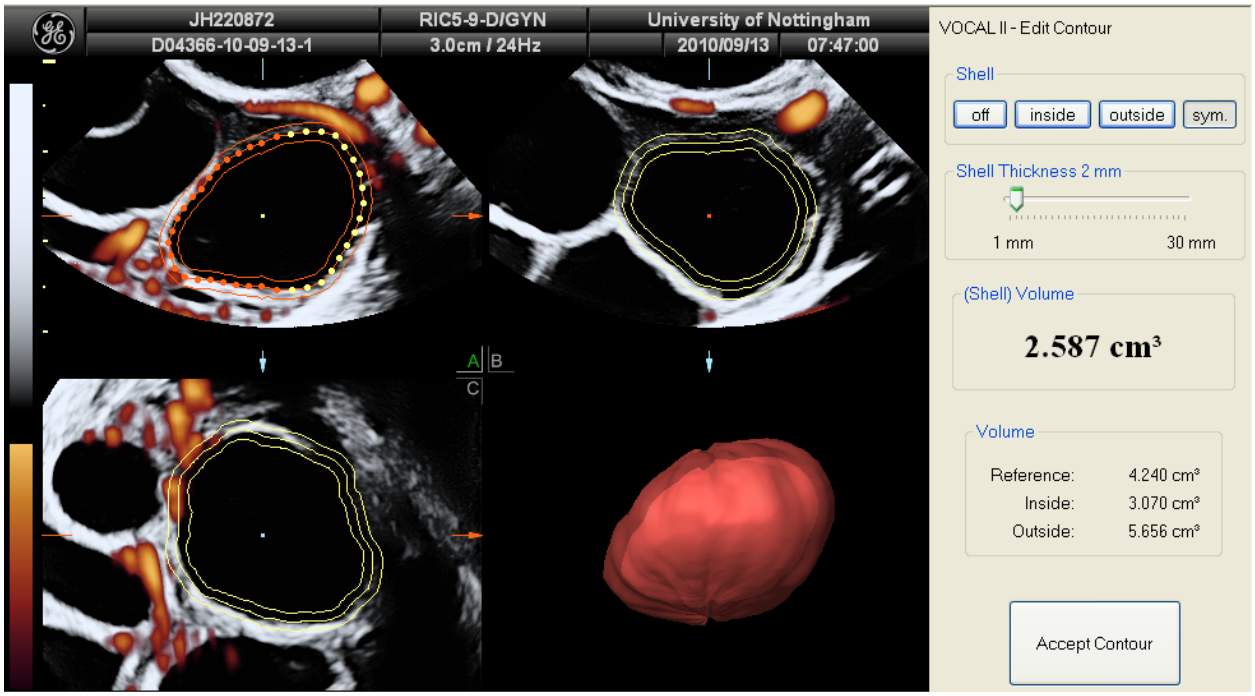

Figure 2.8. VOCAL shell application to human ovary for power Doppler analysis of peri-follicular blood flow

A 2mm shell was applied across the contour so that an area of 1mm either side was included in the analysis.

Once the shell had been applied, this was selected as the volume of interest for the power Doppler analysis (figure 2.9), and the program could then measure the signal in this target area.

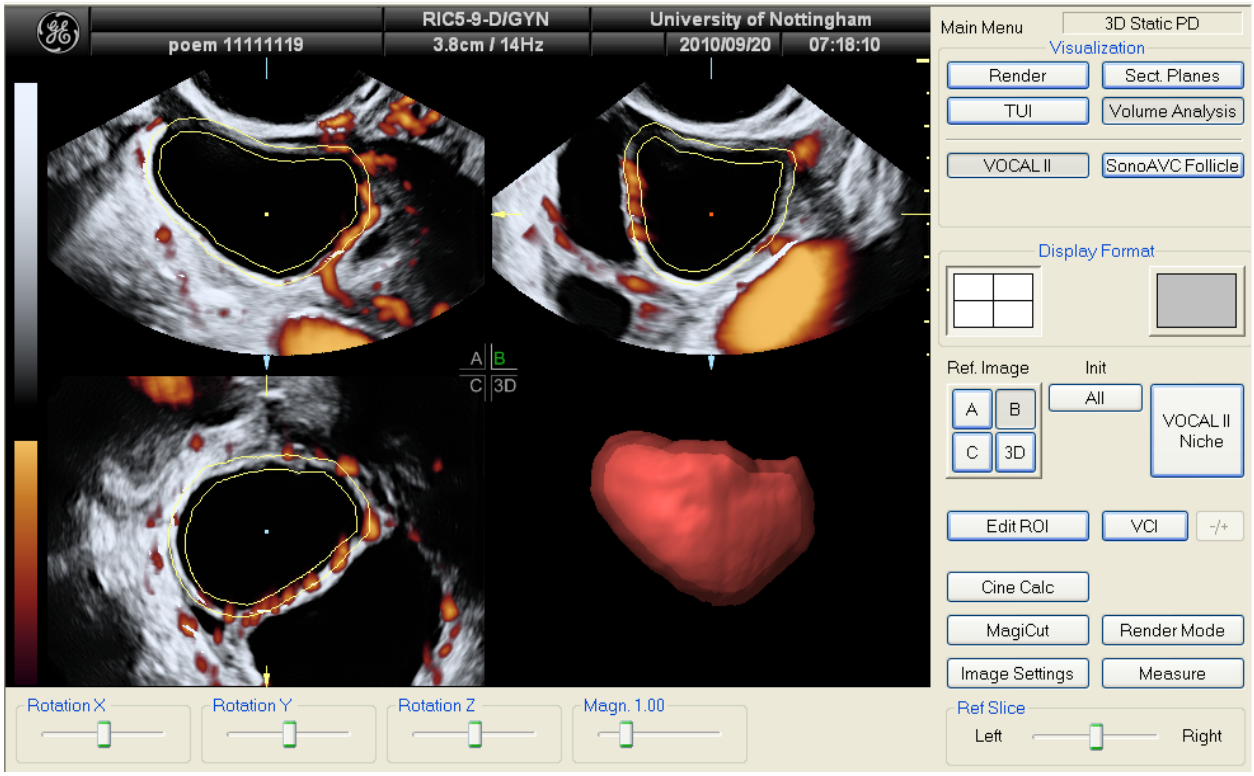

Figure 2.9. VOCAL selection of power Doppler peri-follicular blood flow in a human ovary

Measurement of the selected follicle mean diameter was performed in 4D View by taking an average of the x and y measurements in the A plane, and the z measurement in the B plane (figure 2.10). These were taken at the largest points in each case.

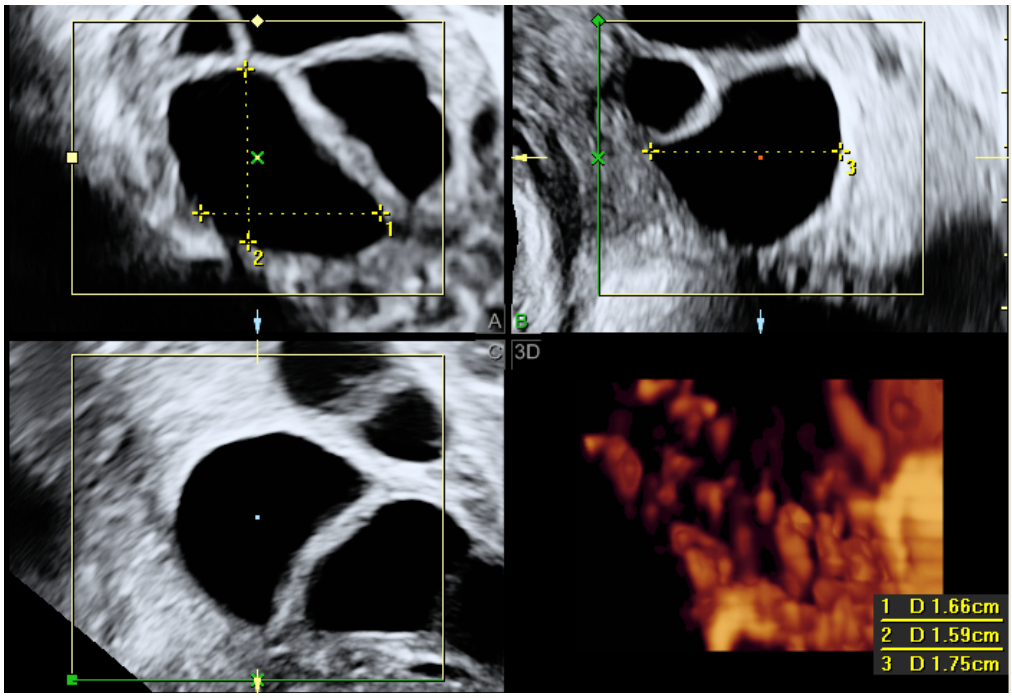

Figure 2.10. Human ovary mean follicular diameter measurement in 4D View

The longest horizontal and vertical measurements are taken in the A and B plane, and displayed in the bottom right hand corner.

Chapter 3 – The Oocyte Effect on Granulosa Cells

3.1. Introduction

It is well established that the oocyte and somatic cells within the follicle share a close association from the beginning of folliculogenesis, and that this is a relationship that is critical in order to maintain normal development (Buccione *et al.*, 1990). Oocyte-somatic cell communication is a bi-directional process, as the somatic cells produce vital nutrients and regulatory signals required by the oocyte to achieve developmental competence (Ka *et al.*, 1997). In addition, the oocyte itself is known to play a key role in the developmental and functional regulation of the follicle, at every stage of folliculogenesis (Soyal *et al.*, 2000). It does this by secreting paracrine factors such as BMP15 and GDF9, which are thought to act synergistically to maintain the oocyte in a consistently optimal microenvironment for development, by regulating granulosa cell function (Moore and Shimasaki, 2005; Su *et al.*, 2008). These oocyte-secreted factors act on the surrounding somatic cells, regulating a number of granulosa and cumulus cell functions. Most notably, the oocyte is thought to regulate proliferation and differentiation of granulosa cells before the LH surge (Gilchrist *et al.*, 2003; Otsuka *et al.*, 2005).

In addition to factors secreted by the oocyte, granulosa cells are also subject to endocrine signalling by gonadotrophins such as follicle-stimulating hormone from the pituitary, which, as the name suggests, promotes follicular development in the follicular phase. In the early stages of folliculogenesis, follicles of 250μm in diameter are responsive, but not dependant on gonadotrophin stimulation by FSH. As follicular development progresses the follicles enter a critical phase in which they become completely dependant on FSH for survival, from around 2-4mm in diameter in cattle, and 1-2mm in sheep (Campbell *et al.*, 1995; Webb *et al.*, 1999a; Markstrom *et al.*, 2002).

The aim of this series of cell culture experiments was to investigate the effect of the oocyte and the factors it produces on granulosa cell proliferation and differentiation. An ovine model was chosen to base these experiments because of the similarities to the human in terms of certain aspects of reproductive physiology. In the first experiment aimed to investigate the effect of the cumulus-oocyte-complex on granulosa cells *in vitro*, and then to examine the additional effects of follicle-stimulating hormone on the

growth of granulosa cells from follicles at different stages of development in experiment 2. Finally, in experiment 3, the effect of oocyte-secreted factor supplementation on granulosa cells was explored. The effect on granulosa cell proliferation and differentiation was assessed.

3.2. Materials and Methods

3.2.1. Experiment 1

Granulosa cells were harvested and cultured as previously described, according to the method developed by Campbell *et al.* (1996). Follicles of approximately 2-3mm in diameter were dissected from the ovaries for granulosa cell culture, and then COCs were collected by aspiration of the remaining ovaries using a 19G needle and syringe. Only the highest grade oocytes collected were selected for culture. Granulosa cells were seeded at 75,000 per well.

Follicles of other sizes were also dissected from the ovaries, and were individually hemisected in separate dishes of dPBS and the follicle shell snap frozen for storage at -80°C. The follicular fluid in dPBS was snap frozen in Eppendorf tubes and stored at -20°C. These were later used for angiogenic growth factor expression described in chapter 4.

3.2.2. Experiment 2

Ovine ovaries were collected from the abattoir follicles of all sizes were dissected. The follicles were then separated into 4 different size groups; <1mm, 1<2mm, 2<3.5mm and >3.5mm. Granulosa cells were harvested from the follicles as previously described, with the groups kept separate. COCs were also collected.

Granulosa cells from the 4 follicle size groups were cultured for 192 hours as previously described, seeded at approximately 75,000 cells per well. Granulosa cells pooled from 1-3mm follicles were also cultured in wells with a COC in each well. In addition, COCs were cultured in individual wells at one per well with no additional cells. In addition, the culture medium (see appendix) was either supplemented with or without FSH (at 1ng/ml).

After 8 days, cell counts were performed, and oestradiol in the spent culture medium at 144 hours was measured by enzyme-linked immunosorbent assay (ELISA). This experiment was repeated a further two times.

3.2.3. Experiment 3

Ovine ovaries were collected from the abattoir and follicles of less than 3mm in diameter were dissected to harvest the granulosa cells using the method previously described. The cells were cultured for 192 hours under the conditions also previously described, at 75,000 cells per well in a 96 well plate. 3 different doses of growth factor were added to the culture media, with 4 replications per treatment dose. BMP15 and GDF9 were added to the media each at 1, 10 and 100ng/ml. After the first experiment it was decided to add additional intermediate doses of the combination of BMP15 and GDF9.

The experiment was then repeated a further 3 times with these intermediate combination doses in order to investigate whether or not there was an additive effect of having 2 growth factors present.

The BMP15, AMH and BMP6 used were all recombinant human (5096-BM, 1737-MS and 507-BP, R&D Systems Inc, USA), whereas GDF9 was recombinant mouse (739-G9, R&D Systems Inc, USA).

The cells were plated according to table 3.1 below (COC+GC and GC control had no treatment added);

BMP15	1ng/ml	1ng/ml	1ng/ml	10ng/ml	10ng/ml	10ng/ml	100ng/ml	100ng/ml	100ng/ml
GDF9	1ng/ml	1ng/ml	1ng/ml	10ng/ml	10ng/ml	10ng/ml	100ng/ml	100ng/ml	100ng/ml
AMH	1ng/ml	1ng/ml	1ng/ml	10ng/ml	10ng/ml	10ng/ml	100ng/ml	100ng/ml	100ng/ml
BMP6	1ng/ml	1ng/ml	1ng/ml	10ng/ml	10ng/ml	10ng/ml	100ng/ml	100ng/ml	100ng/ml
BMP15+GDF9	1ng/ml	1ng/ml	1ng/ml	10ng/ml	10ng/ml	10ng/ml	100ng/ml	100ng/ml	100ng/ml
BMP15+GDF9	1ng/ml	1ng/ml	1ng/ml	5ng/ml	5ng/ml	5ng/ml	50ng/ml	50ng/ml	50ng/ml
COC+GC	NT	NT	NT	NT	NT	NT	NT	NT	NT
GC control	NT	NT	NT	NT	NT	NT	NT	NT	NT

Table 3.1. 96 well plate format for TGFβ dose response culture. NT=No treatment. n=4 for each dose, n=12 for granulosa cells with a COC in each well, for and granulosa cell control.

The doses were selected in 10 fold increments from 1 to 100ng/ml in the culture medium. 100ng/ml was used as the highest dose because although higher doses had been used in other studies published in the literature (Spicer *et al.*, 2006), this culture was of a significantly longer duration, and so treatment

differences were likely to be observed at these lower concentrations. In addition, it was thought unlikely that the growth factors would be present in the follicular fluid at concentrations as high as those used in other published studies, and so our cell culture model should, in theory, be more physiologically relevant.

Oestradiol and progesterone were measured in the culture media take-off every 48 hours by ELISA. Cell numbers were estimated using neutral red staining as previously described.

All results were analysed using ANOVA in SPSS (Version 15, SPSS Inc). Significance was set at $P<0.05$ for all experimental analyses. Total cell numbers in experiment 1 were compared between the three groups; Granulosa cell control, Granulosa cells with a COC in each well, and individually cultured COCs. Within-group data from experiment 2 was analysed for effect of inclusion of FSH using t-testing in Microsoft Excel 2007, and comparison between the five treatment groups performed by ANOVA in SPSS. All data in experiment 3 was analysed using ANOVA in SPSS to investigate effect of dose response compared to the granulosa cell control.

3.3. Results

3.3.1. Experiment 1

The total cell numbers after 192 hours in culture can be seen in figure 3.2 below. The co-culture of a COC with the granulosa cells was observed to significantly increase cellular proliferation ($P<0.05$).

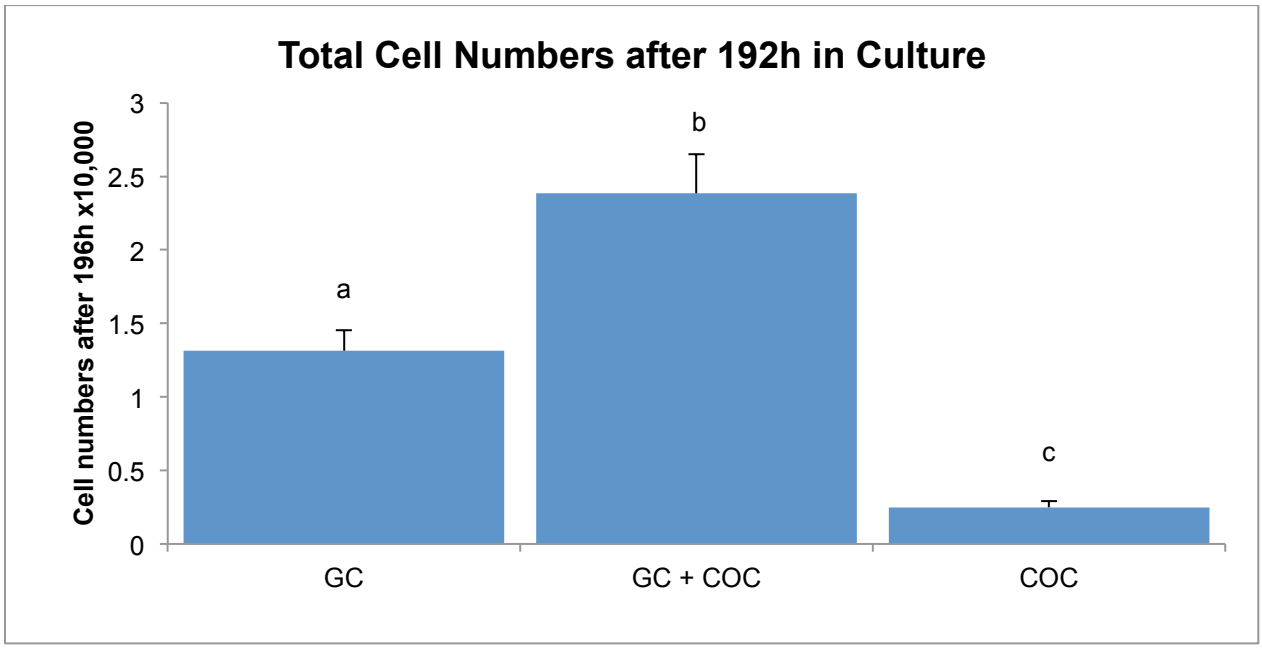

Figure 3.1. Total cell number estimation of ovine granulosa, granulosa with COC, and COC alone, after 192 hours in culture

Error bars indicate SEM. (a = $P<0.05$ compared to COC, b = $P<0.05$ compared to GC control). n=14 for each group.

3.3.2. Experiment 2

The data shown in figures 3.2 to 3.4 is from the average across the 3 experimental repeats.

The cell numbers after 8 days of culture were estimated by neutral red staining based on a standard curve. Overall, FSH had no significant effect on cellular proliferation, as cell numbers after 8 days were not different in the cell groups that had been cultured with FSH and the control groups.

Total granulosa cell number was retained in follicles 1-3.5mm to the end of culture, however cell numbers from follicles less than 1mm in diameter and greater than 3.5mm in diameter were significantly lower than those 1-3.5mm, and in fact, the total cell number at the end of the culture was lower than at the start.

Cell numbers in the COC wells were significantly lower than the other treatment groups as would be expected, and the number of cells in this group was estimated to be around 10,000 per well.

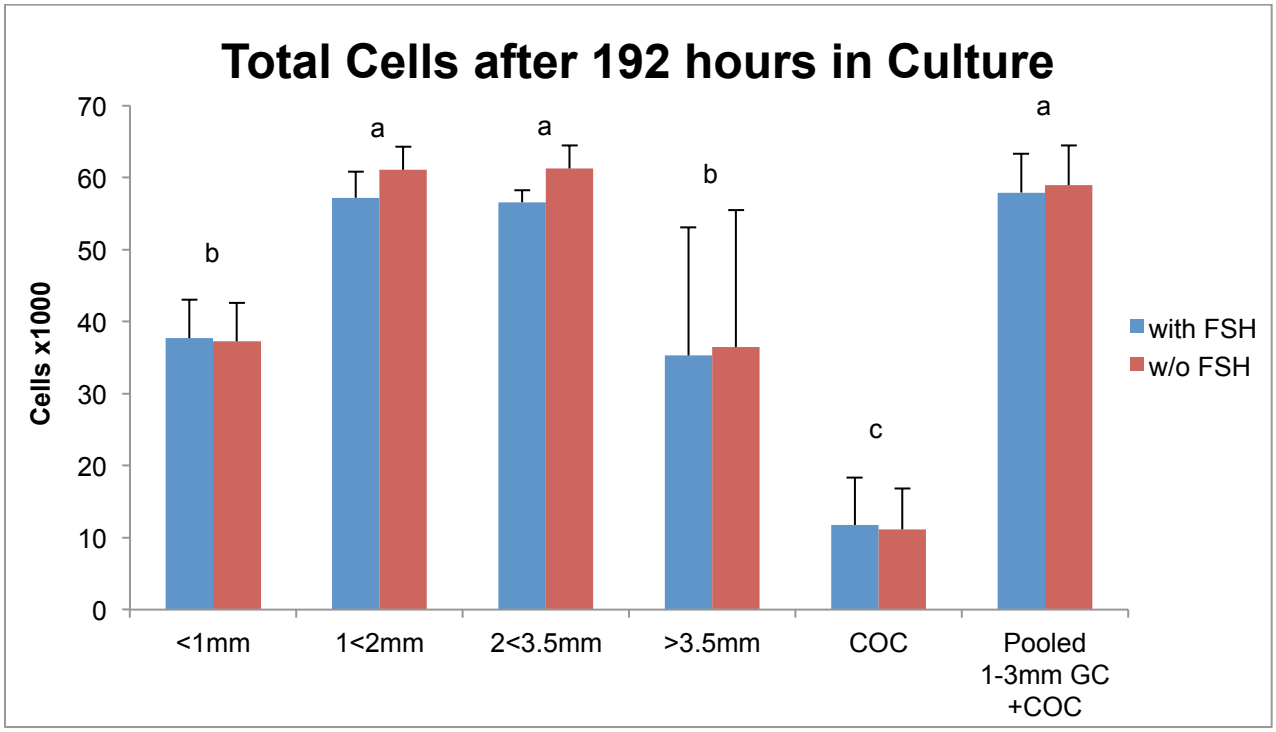

Figure 3.2. Mean total cell numbers after 192 hours in culture for granulosa cells from different sized follicles, with and without FSH

Error bars indicate SEM. Different letters indicate significant difference in cell number across follicle sizes regardless of the presence of FSH ($P<0.05$).

Although FSH did not significantly affect cellular proliferation in this series of experiments, the results across the size classes were analysed. Total cell number was significantly lower for granulosa cells originating from follicles <1mm diameter than all other size classes other than >3.5mm, as well as the GC + COC group. The largest follicle group cell totals were also significantly lower than than those from follicles 1-2mm and 2-3.5mm diameter.

Oestradiol in the spent culture medium at 192 hours (after a previous 48 hour media change) was measured by ELISA. The presence of FSH in the culture medium increased oestradiol production, with the greatest effect seen in cells from the largest follicle group, and was significant in the presence of a COC (P<0.05) (see figure 3.3).

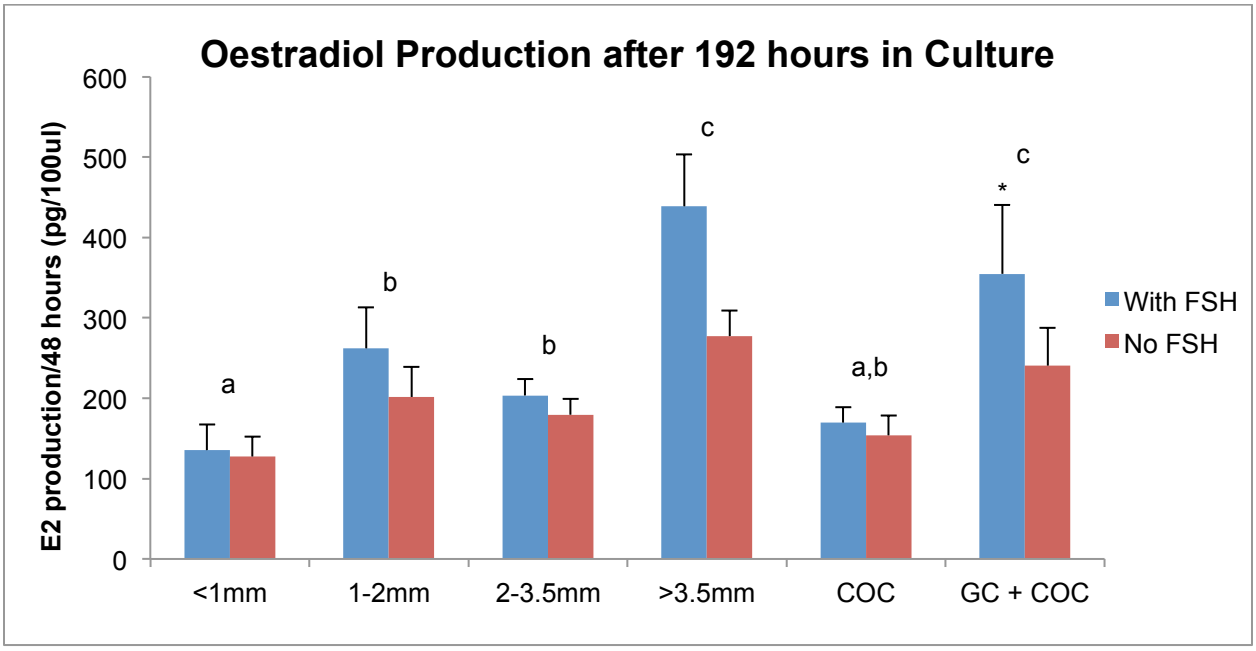

Figure 3.3. Oestradiol production by granulosa cells originating from different follicle sizes after 192 hours in culture with or without FSH

Error bars indicate SEM. * denotes P<0.05 FSH compared to absence of FSH. Different letters denote significant difference across follicle sizes regardless of the presence of FSH (P<0.05).

Oestradiol production per 10,000 cells was calculated in order to determine whether the effects of FSH observed were due to increased oestradiol production or as a result of increased cell numbers.

As seen in figure 3.4, oestradiol production per cell was highest in the large follicle group (P<0.05). There was no significant difference in oestradiol production per cell observed between the <1mm, 1-2mm and 2-3.5mm follicle size groups. Oestradiol production per cell was highest in the wells in which a COC was cultured alone (P<0.01).

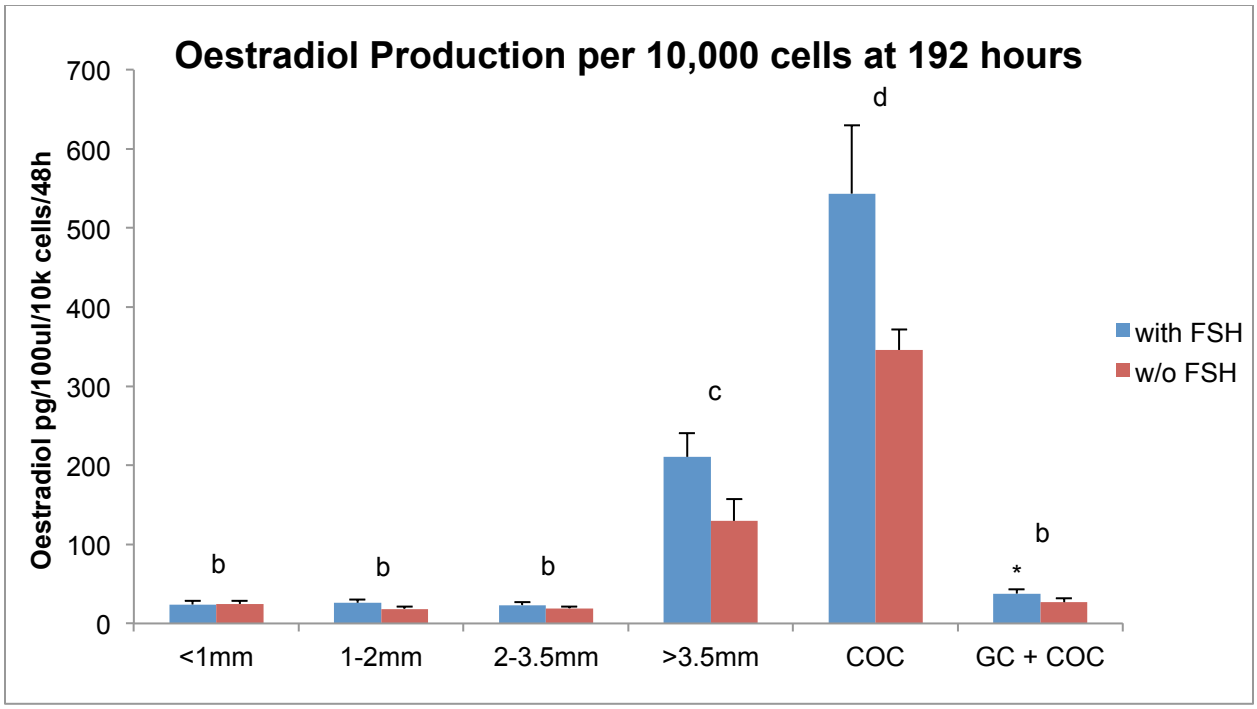

Figure 3.4. Oestradiol production per 10,000 granulosa cells, originating from different sized follicles, after 192 hours in culture with or without FSH

Error bars indicate SEM. *denotes P<0.05 compared to absence of FSH. Different letters denote significant difference across follicle sizes regardless of the presence of FSH (P<0.05).

3.3.3. Experiment 3

The oestradiol (E2) production results across the 4 media change timepoints are shown in figures 3.5 to 3.8 below.

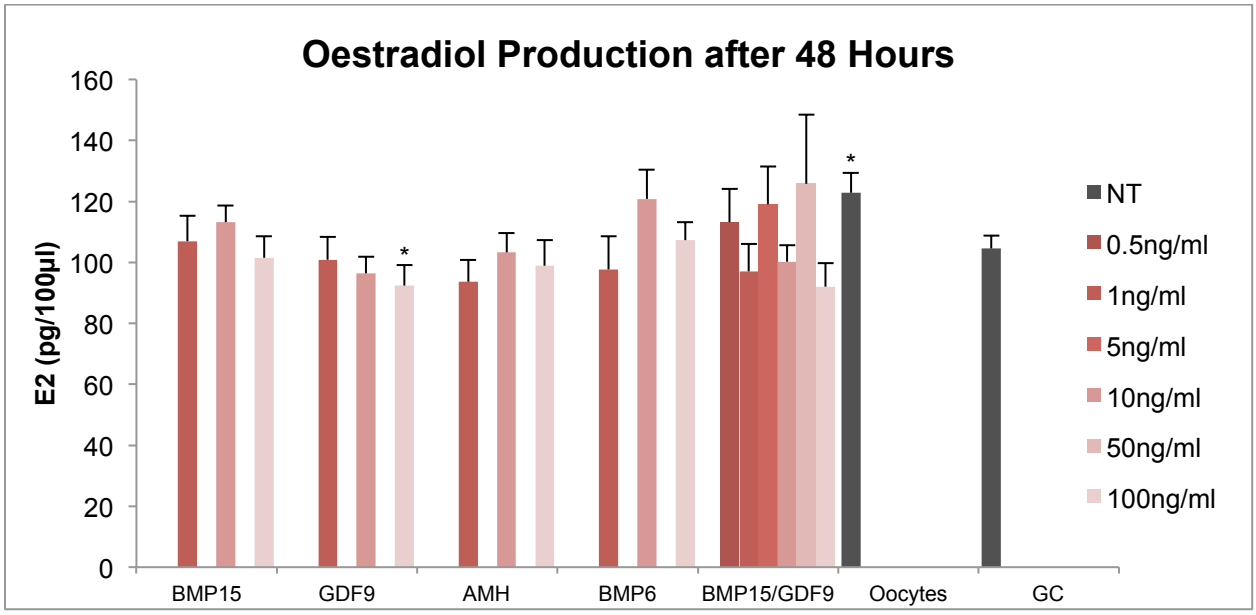

Figure 3.5. Ovine granulosa cell oestradiol production in media after 48 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control in both graphs.

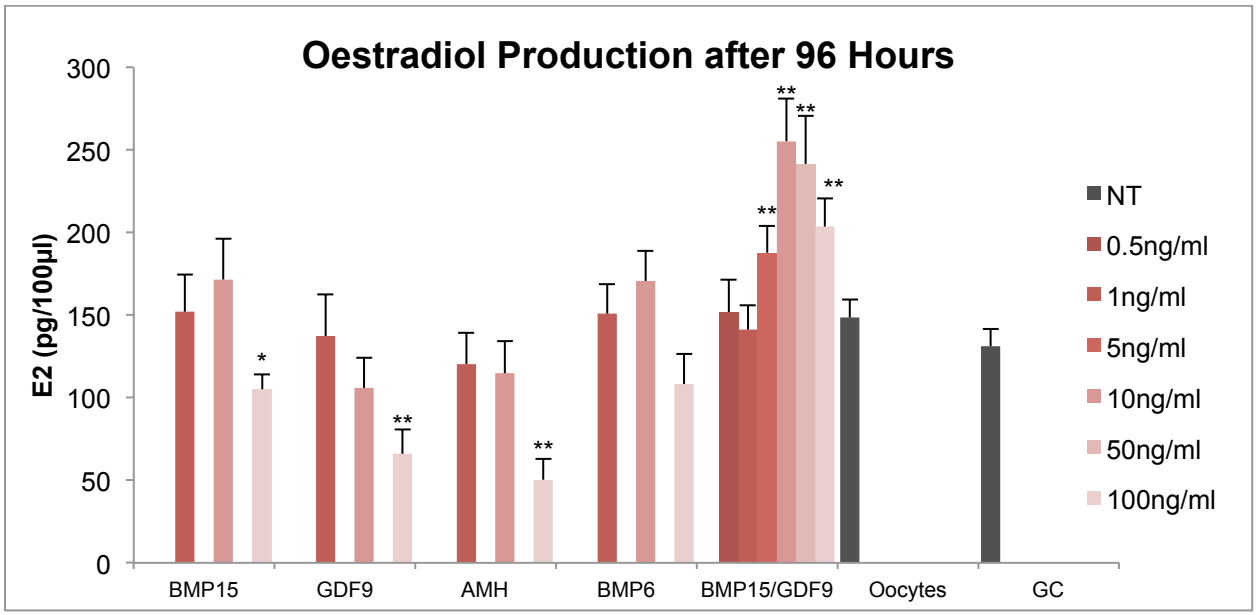

Figure 3.6. Ovine granulosa cell oestradiol production in media after 96 hours in culture

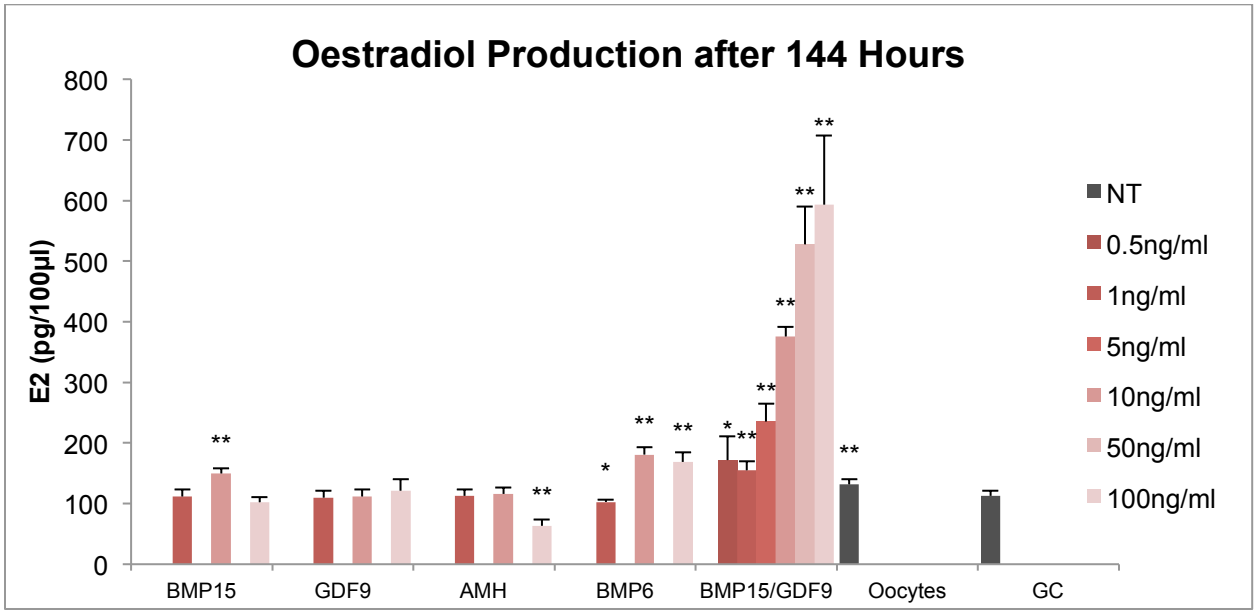

Figure 3.7. Ovine granulosa cell oestradiol production in media after 144 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control in both graphs.

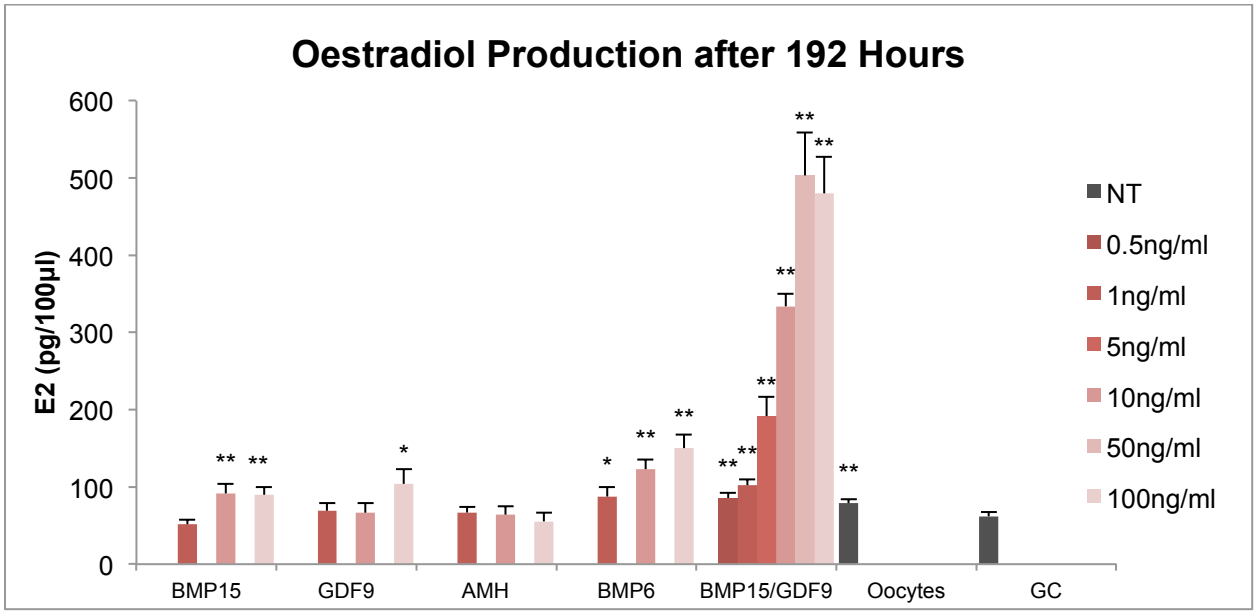

Figure 3.8. Ovine granulosa cell oestradiol production in media after 192 hours in culture

Oestradiol production was significantly increased following treatment using a combination of BMP15 and GDF9 in a dose-dependent manner from 96 hours in culture onwards. The most marked effects were observed at 144 and 192 hours of the culture.

BMP6 also increased granulosa cell oestradiol production in a dose-dependant manner, as did the presence of a COC in culture. Conversely, high dose BMP15, GDF9 and AMH appeared to decrease oestradiol production at 96 hours of culture.

Progesterone in the culture media was also measured every 48 hours following media change. The results of the progesterone ELISA can be seen in figures 3.9 to 3.12 below.

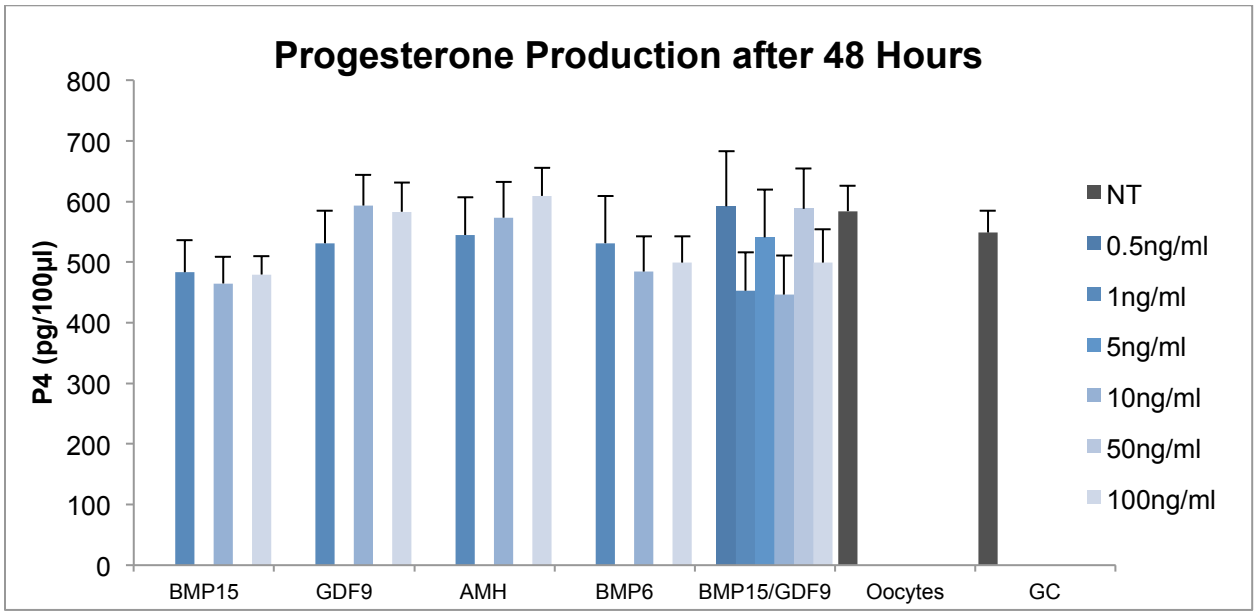

Figure 3.9. Ovine granulosa cell progesterone production in media after 48 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

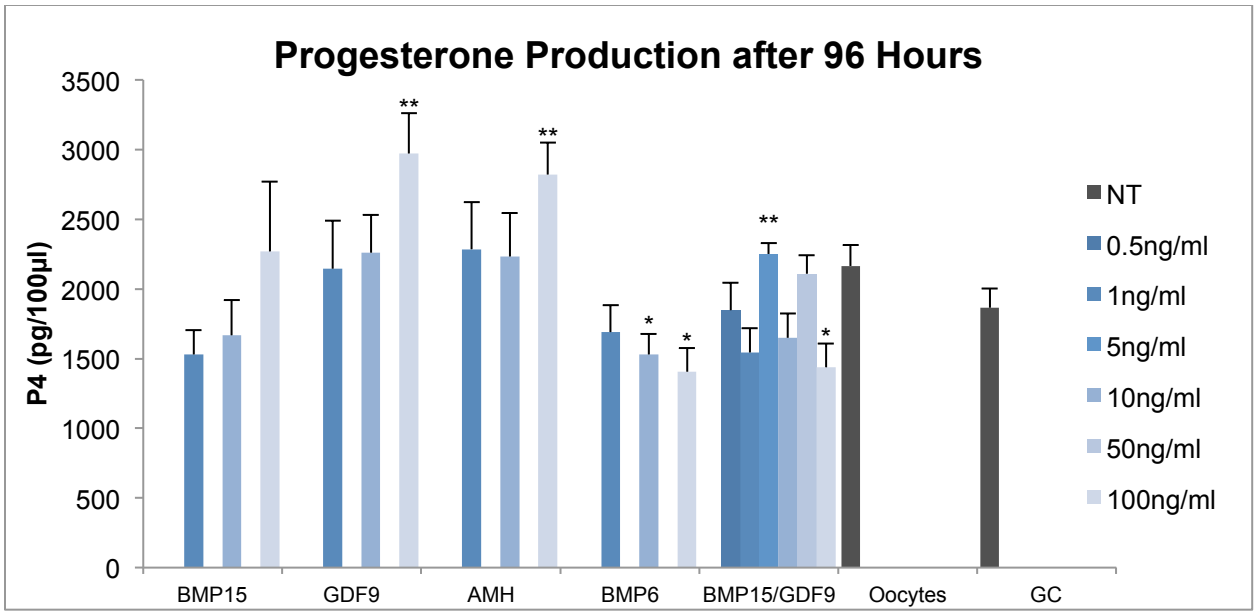

Figure 3.10. Ovine granulosa cell progesterone production in media after 96 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

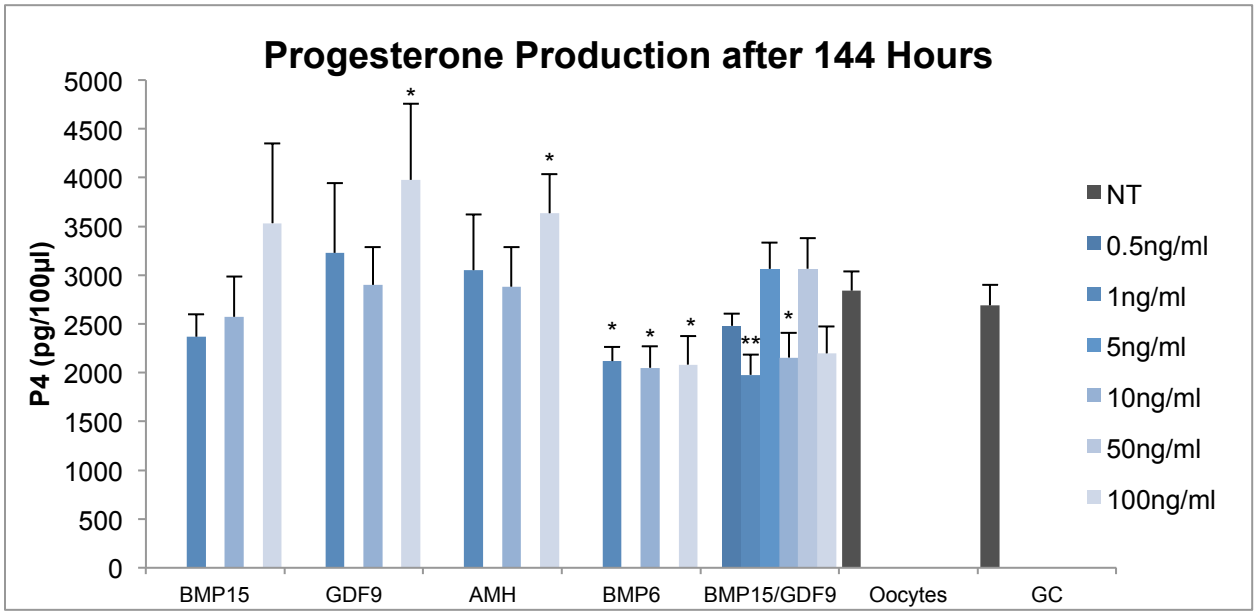

Figure 3.11. Ovine granulosa cell progesterone production in media after 144 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

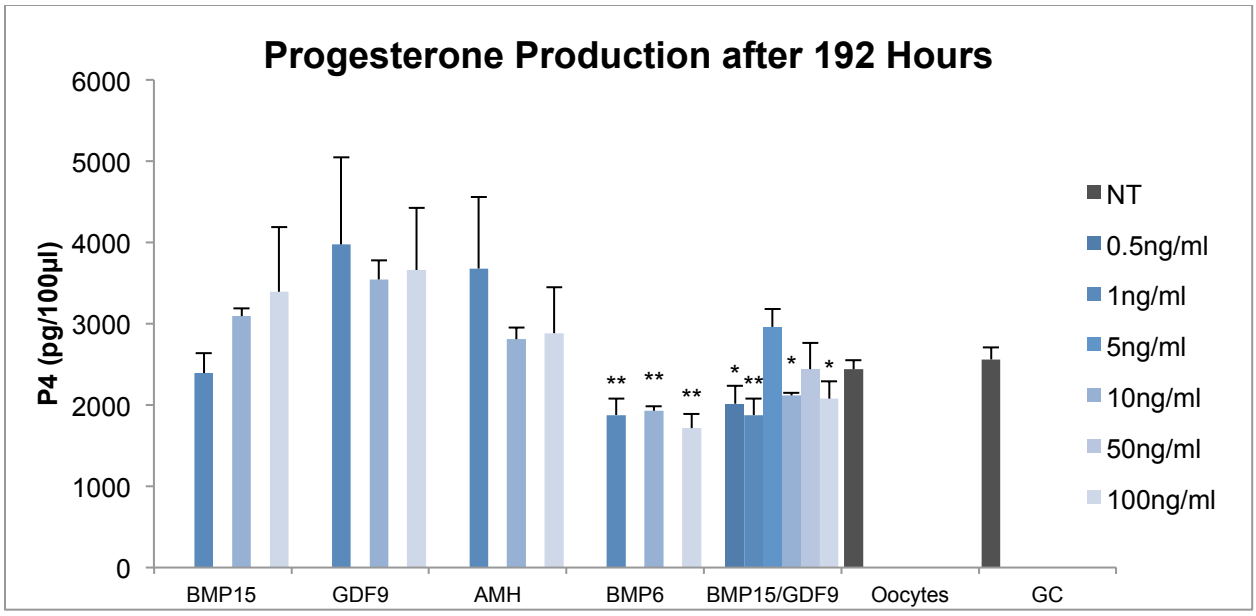

Figure 3.12. Ovine granulosa cell progesterone production in media after 192 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

Progesterone production was significantly decreased by both BMP6 and the BMP15/GDF9 combination treatments (P<0.01) after 96 and 144 hours respectively. Conversely, GDF9 and AMH appeared to increase progesterone production at the highest doses at 96 and 144 hours of culture.

The oestradiol and progesterone production per cell was calculated based on the neutral red estimation of cell numbers at the end of the culture (see figures 3.13, 3.14 and 3.15).

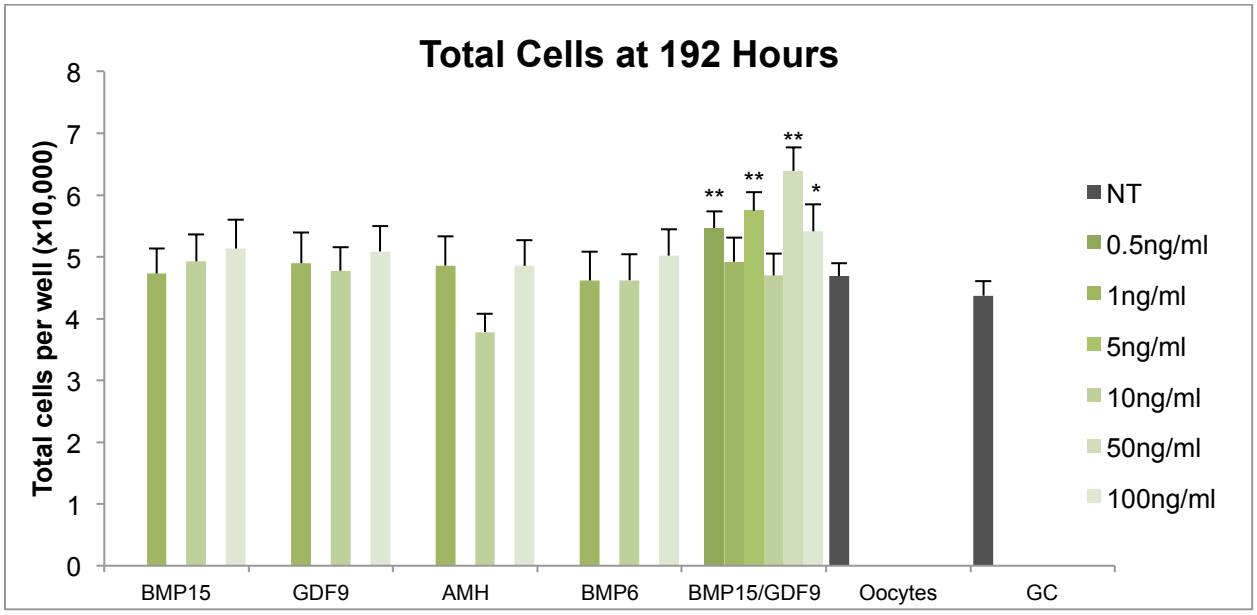

Figure 3.13. Ovine granulosa cell neutral red cell number estimation after 192 hours in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

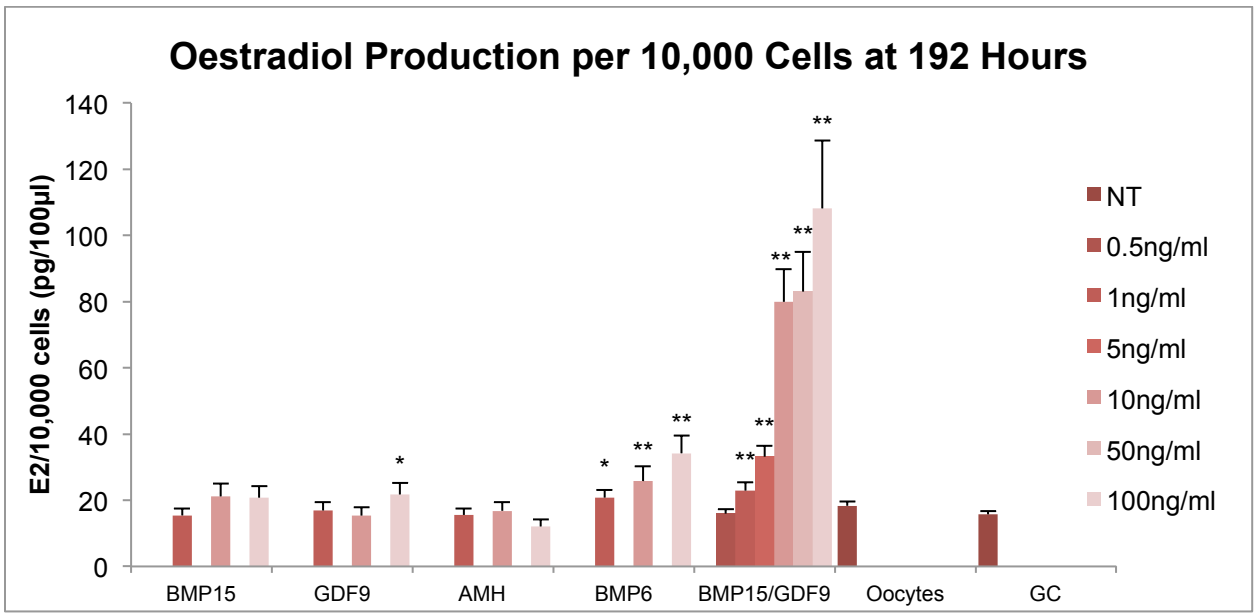

Figure 3.14. Ovine granulosa cell oestradiol production per 10,000 cells after 192h in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

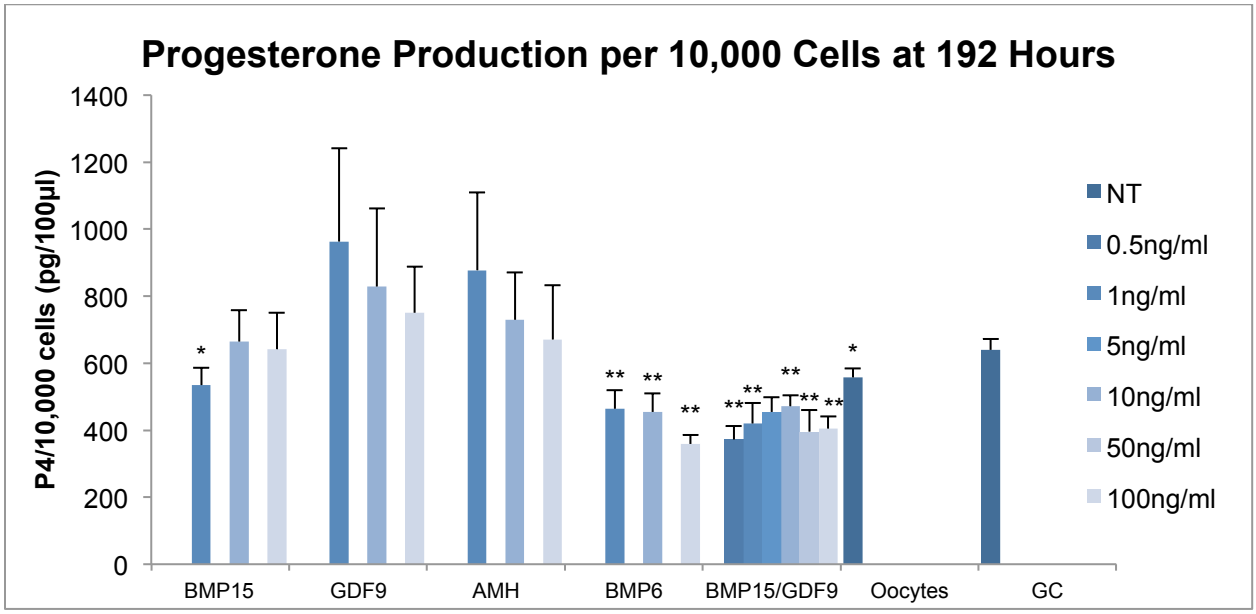

Figure 3.15. Ovine granulosa cell progesterone production per 10,000 cells after 192h in culture

Error bars indicate SEM. *P<0.05; **P<0.01, compared to the granulosa cell control.

Overall it seemed that addition of the various growth factors or a COC did not have a marked effect on cellular proliferation, although cells cultured with the combination dose of BMP15 and GDF9 were significantly greater in number than the control cells cultured alone (P<0.01).

Oestradiol production per cell was significantly increased by both BMP6 and the combination of BMP15/GDF9, in a dose-dependant manner (P<0.01). These two factors also significantly decreased progesterone production (P<0.01). The addition of a COC to the granulosa cells had the same effect on progesterone, although the effect was more subtle than addition of BMP6 and BMP15/GDF9 factors (P<0.05).

In a separate culture, mRNA aromatase expression was measured by PCR after 144 hours in culture, as this was the peak of oestradiol production measured in the culture medium. The treatment doses were 50ng/ml for each factor, and as before, a COC was included as an additional treatment. Figure 3.16 below shows aromatase expression by the granulosa cells at 144 hours, which indicates expression

significantly higher with a 50ng/ml combination dose of BMP15 and GDF9 ($P<0.05$). Aromatase expression was also higher in the GDF9 and COC treatment groups, however this was not statistically significant.

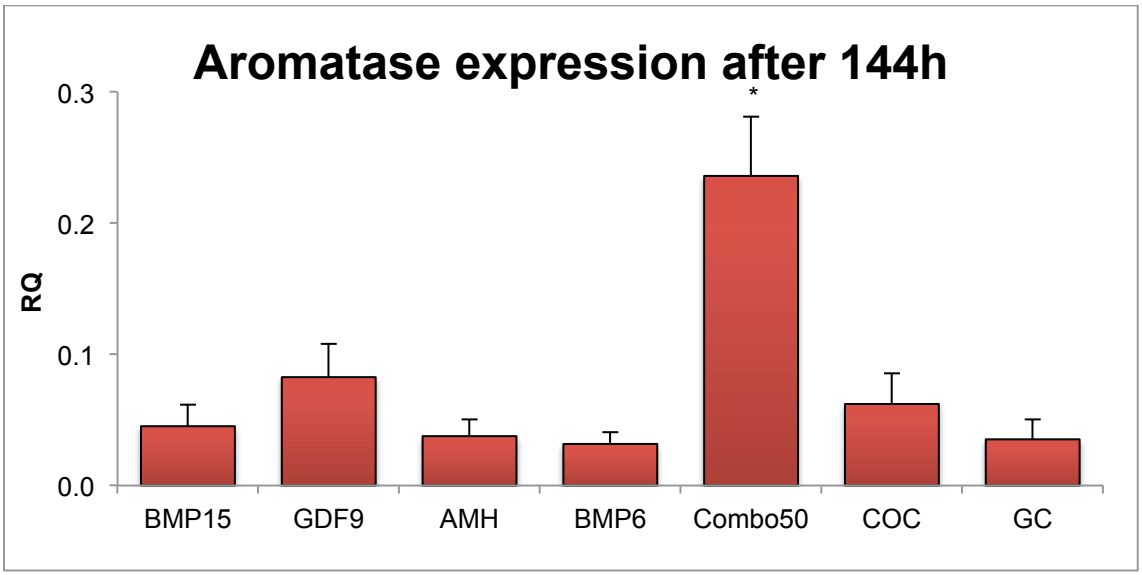

Figure 3.16. Ovine granulosa cell aromatase expression at 144 hours in culture

(Combo50 = Combination dose of BMP15 and GDF9 at 50ng/ml). *$P<0.05$ relative to control.

3.4. Discussion

This study showed that co-culture of COC or exposure to oocyte-secreted factors had a marked effect on granulosa cell proliferation and differentiation, supporting the hypothesis that oocyte is likely to be a key player in regulating follicular development by directly influencing the cells that surround it. Overall, the results of this study have indicated a role for the COC in promoting granulosa cell proliferation, and that factors it secretes act together as powerful promotors of granulosa cell oestradiol production, and suppressors of progesterone production. As such they may have a role in the prevention of follicular luteinisation.

The results from experiment 1 show that granulosa cell proliferation is significantly increased in the presence of a COC ($P<0.01$). This is likely to be as a result of oocyte-secreted factors, such as BMP15 and GDF9, acting on the granulosa cells to increase proliferation, a result also observed by McNatty *et al.* (2005a). Numerous previous studies have found oocytes to increase granulosa cell proliferation in a number of species (reviewed in Gilchrist *et al.*, 2008); using murine, porcine and bovine oocytes (Gilchrist *et al.*, 2006; Hickey *et al.*, 2005; Gilchrist *et al.*, 2003). Notably, in the bovine model it seems that IGF-I is a requirement for this to occur (Gilchrist *et al.*, 2003), which was a constituent of the culture medium used in this experiment.

Although neutral red staining indicated relatively large numbers of cells present in the COC-only wells, this was likely to be partly as a result of the size of the cell, as the oocyte would take more of the staining media than a single granulosa cell, and therefore the actual number of cells present would be a great deal lower than estimated in these wells. However, when in culture the cumulus cells can frequently be seen to gradually detach from the oocyte itself, and differentiate to form monolayers that are similar in appearance to the mural granulosa cells in culture. Consequently, these cells may also behave like mural granulosa cells in culture, and so can proliferate and synthesise steroid hormones. This contrasts the frequently observed lack of proliferation in *in vitro* maturation, where the cumulus cells mucify in response to the gonadotrophin ovulation stimulus (Vanderhyden *et al.*, 1990; Zhuo and Kimata, 2001). When inside the follicle, there must be factors, which may or may not include BMP15, GDF9 and BMP6 that maintain these cumulus cells attached to the oocyte, and once in culture, these factors are not present any longer and so the cells desert the oocyte and begin to behave like mural granulosa cells.

FSH supplementation attenuated granulosa cell proliferation, but increased oestradiol production, as previously seen in ovine granulosa cell cultures by Campbell *et al.* (1996) and bovine granulosa cultures by Gutierrez *et al.* (1997). Follicles of 1-3.5mm exhibited the greatest cellular proliferation, which is consistent with granulosa cell gonadotrophin dependence commencing at around 2-3mm diameter (Scaramuzzi *et al.*, 1993). A fall in proliferation was observed in the largest follicle size group, a result which not seen in the 3rd experimental repeat. This is one of the reasons for the relatively large error bars.

The larger follicles were found to produce more oestradiol, an expected result as these follicles become increasingly gonadotrophin-dependant from around 1-2mm onwards (Webb *et al.*, 1999a) and become highly oestrogenic once they reach 3.5mm in diameter (McNeilly *et al.*, 1991). The presence of a COC appeared to increase cellular proliferation, a result supported by McNatty *et al.* (2005a), but did not have a major effect on steroidogenesis, although levels were slightly elevated. It is thought that certain oocyte-secreted factors, such as BMP6, may increase oestradiol production (Campbell *et al.*, 2009), and others, such as BMP15 and GDF9, could have divergent effects on oestradiol production (Knight and Glister, 2006), therefore these factors may balance in such a way that overall oestradiol production by the granulosa cells was unaffected. Alternatively, the addition of one COC in culture may not have induced a large enough response in the granulosa cells to be measured, and it it thought that the ability of the COC to secrete oocyte-secreted factors *in vitro* may actually decline over time (Sfontouris and Campbell, unpublished observations).

The media taken from the cultured COCs had relatively high concentrations of oestradiol, which would not be expected as there were relatively few cells attached to the oocytes. However, it was observed that in culture the oocyte would separate from the surrounding cumulus cells, and these cells would then visibly change in appearance from a spherical form to a more flattened one, spreading across the plate surface. It is therefore likely that these cumulus cells were differentiating, and hence increasing in steroidogenic capacity, explaining the marked increase in oestradiol production.

BMP6 and the combination of BMP15 and GDF9 had a significant effect on both oestradiol and progesterone production, without affecting cellular proliferation to any great degree, as measured in both the media, and on a per cell basis. These factors significantly increased oestradiol production in a dose-dependant manner, and also decreased progesterone production, although this was not seen to be dose-dependant. Aromatase expression was also significantly increased with treatment of BMP15 and GDF9 in combination (figure 3.16) further confirming the upregulation of oestradiol production. This promotion of oestradiol production was found to mimic the addition of a COC to the culture, which also significantly increased oestradiol production, at the later stages of culture. AMH appeared to have a converse effect on steroidogenesis, suppressing oestradiol production and promoting progesterone production.

Steroidogenesis

Overall, BMP15, GDF9 and AMH alone did not appear to have a major effect on steroidogenesis, although at 96 hours these factors did significantly suppress oestradiol production at the highest dose. GDF9 and AMH significantly increased progesterone production, also at 96 hours as well as at 144 hours for AMH. This result was then not observed for the remainder of the cultures, and did not occur when calculating steroid production on a per cell basis, which suggests that at 96 hours these factors may have become temporarily toxic to the cells at these increased levels.

The presence of a COC significantly increased oestradiol production at 48, 144 and 192 hours, which is consistent with the oocyte-secreted factors BMP6, BMP15 and GDF9 also increasing granulosa cell oestradiol production. However, no effect of addition of a COC on progesterone was observed.

The current literature regarding the effects of BMP15 and GDF9 *in vitro* is conflicting due to discrepancies between species of culture model, origin and dose of oocyte-secreted factor, and culture methodology, and as a result the true role of these factors is still unclear. In comparison to GDF9, there have been relatively few studies published on the effects of BMP15 *in vitro* with reference to steroidogenesis. McNatty *et al.* found ovine BMP15 to decrease progesterone production in bovine granulosa cells (2005a) and rat granulosa cells (McNatty *et al.*, 2005b), but not ovine cells. However, doses of just 4ng/ml and 8ng/ml were used, and so it is unclear as to what the effect at higher doses such as those used in our experiments would be in comparison.

Otsuka *et al.* (2000) observed that hBMP15 depressed progesterone production by rat granulosa cells, and therefore suggested a role for BMP15 in the prevention of premature luteinisation. The fact that in our experiments oestradiol production was increased and progesterone decreased by treatment with BMP15 and GDF9 suggests a likely synergistic role to this effect, thereby inhibiting premature luteinisation and promoting further follicular development. The combination of these two factors had a much larger effect than either one alone, which indicates increased bioactivity, possibly resulting from their heterodimerization, which has been previously demonstrated *in vitro* using an ovine cell culture model (Liao *et al.*, 2003), and also *in vivo* using a mouse model (McIntosh *et al.*, 2008).

Spicer *et al.* (2006) found that in bovine granulosa cells from follicles of all sizes, treatment with high doses (150-600ng/ml) of rat GDF9 significantly decrease both progesterone and oestradiol production. However, this study had several key differences to our own, making comparison relatively difficult. The culture system used had several major differences to ours, in that the granulosa cells were collected for culture by aspiration, were then cultured for 24 hours with serum, and then transferred to serum-free media for 48 hours of treatment. By the authors' own admission, the exposure of the cells to serum may caused partial luteinization, and therefore the luteal activity of these cells may have affected the results. Discrepancies between methodologies such as these could explain the differences in conclusions drawn from *in vitro* cell cultures such in this case.

Interestingly, BMP6 treatment significantly increased oestradiol production in a dose-dependent manner, and decreased progesterone production across all doses, without affecting cellular proliferation (figures 3.13, 3,14 and 3.15). This result suggests that BMP6 mimics the effects seen with BMP15 and GDF9 effect on oestradiol production, albeit in a more subtle manner. Also BMP6 significantly suppressed progesterone production similar to the effects of the combination treatment, and had no significant proliferative effect. This result supports findings of the study by Campbell *et al.* (2006), which showed BMP6 to be a potent promoter of FSH-induced ovine granulosa cell oestradiol production, without affecting proliferation. In this study it was also noted that progesterone production was suppressed (Campbell, unpublished observations). The results are also similar to those found by Glister *et al.* (2004), who used a bovine *in vitro* model to demonstrate that BMP6 enhanced oestradiol production while modulating secretion of progesterone, although in this study a small but significant increase in cell proliferation was observed. It is important to note, however, that FSH was not included in these cell cultures, and as a result our experiments are unfortunately not completely comparable.

There seems to be marked differences in the actions of BMP6 in different species. For example, Otsuka *et al.* (2001) found that recombinant human BMP6 had no effect on oestradiol production, although a decrease in FSH-induced progesterone production in rat granulosa cells was observed, without any effect on cellular proliferation. This response was also observed in a study conducted by Miyoshi *et al.* (2007),

again using rat granulosa cells. In a study conducted by Juengel *et al.* (2006), recombinant human BMP6 had a suppressive effect on progesterone production by ovine granulosa cells, again without affecting cellular proliferation. However, in this study rat granulosa cell progesterone production was again decreased, but proliferation was increased, again highlighting species differences.

It therefore seems that in a number of species, although BMP6 may have different effects on proliferation and oestradiol production, in most cases granulosa cell progesterone production is decreased. This may suggest a universal key role as a premature luteinization-inhibitor for dominant follicles (Otsuka *et al.*, 2001; Juengel *et al.*, 2006), which is likely to be based on the involvement of somatic cell sensitivity to gonadotrophins (Campbell *et al.*, 2009).

Notably, the addition of a cumulus-oocyte complex (COC) to granulosa cells increased oestradiol production, indicating that the oocyte-secreted factors used in this experiment are likely to have the same effects as those induced by a COC.

Treatment of the granulosa cells with AMH had no significant effect on steroidogenesis or proliferation in this study, although a slight dose-dependant depression was observed. Little research has been carried out in this particular area, however Seifer *et al.* (1993) implicated AMH as a potential inhibitor of granulosa cell mitosis after observing a difference in intrafollicular AMH concentrations in relation to granulosa cell proliferation, with 2 different ovarian stimulation regimes.

AMH is produced by the granulosa cells themselves right through to the preovulatory stage in sheep (Durlinger *et al.*, 2002), and so it is likely that higher doses would be needed to induce a response by the cells, which would be expected to result in a decreased responsiveness to FSH, and hence a depression in proliferation and oestradiol production. In fact, the most noticeable effect would have been observed in theca cells, which would serve as a further avenue of research following these experiments.

Cellular proliferation

Cellular proliferation was unaffected by any of the treatments used in this study, apart from an increase at some of the higher combination doses of BMP15/GDF9 (figure 3.13). This result was also observed by McNatty *et al.* (2005a), as this study found the combination of mGDF9 with oBMP15 to increase ovine

granulosa cell proliferation as measured by ^{3}H-thymidine incorporation, although a much higher dose of mGDF9 was used. However, in contrast to our study, this study also found that mGDF9 alone had a small but significant effect of decreasing cellular proliferation, although notably the doses were more than 10-fold higher than those used in our study. This is in contrast to the rat model, in which mGDF9 increases cellular proliferation (Vitt *et al.*, 2000), further indicating species-specific roles for these factors. Interestingly, McNatty *et al.* observed a promotional effect of oBMP15 on proliferation when used alone, which was not seen in our study. However again this may simply further highlight species-specific interactions of BMP15. Spicer *et al.* (2006) also found that GDF9 increased cellular proliferation *in vitro*, however the study used rat GDF9 and bovine granulosa cells, which makes the drawing of comparative conclusions somewhat difficult.

It would be interesting to expand and develop this study to utilise ovine sources of BMP15 and GDF9 with ovine granulosa cells, as this would elucidate the differences between the effects of the origin of the factors and clarify their roles in this particular species, which could be attributed to the mono *versus* polyovulatory nature of the species in question (Galloway *et al.*, 2000). This was not performed in the first instance because of the difficulties in obtaining ovine BMP15 and GDF9.

If indeed the oocyte-secreted factors BMP15 and GDF9 form highly bioactive heterodimers *in* vivo, and play such a key role in the prevention of premature luteinisation through regulation of steroidogenesis, this brings major implications for the role of the oocyte in the hypothalamic-gonadal axis that have not been previously considered (Paulini and Melo, 2011). In fact, it may be possible that the single female gametocyte contained within a follicle of an ovary can at the very least dictate the outcome of its own development, and perhaps even as far as being a key player in regulating the entire follicular cycle.

Chapter 4 – Hypoxia and Angiogenesis in the Ovarian Follicle

4.1. Introduction

In hypoxic conditions, cell metabolism is considerably reduced, as oxygen availability is decreased. Under these conditions the transcription factor HIF-1α is stable, thereby inducing expression of angiogenic growth factors such as VEGF. When oxygen supply is adequate once again, following the formation of new blood vessels, the then adequate supply of oxygen causes destabilisation of the α-subunit of HIF-1, which suppresses its activity (Neeman *et al.*, 2007).

An alternative method for inducing hypoxia in cell cultures is to use chemical agents, such as cobalt chloride and sodium azide. Cobalt chloride is used widely in cell cultures for this purpose, as HIF-1α levels increase as degradation is prevented. The only drawbacks to these chemicals is that they are toxic and so have to be used in strictly controlled quantities in order for them to be used effectively (Semenza *et al.*, 1994).

HIF-1 is a heterodimeric transcription factor of particular importance in angiogenesis, as it regulates and maintains expression of a number of factors involved in the process. It is of primary importance in the induction of gene expression of angiogenic and metabolic growth factors that allow cellular adaption and survival in a continuously changing environment (Semenza, 1998). The patterns of HIF-1 expression during the development of the follicle have not yet been explained. It seems likely that as the follicle develops, HIF-1 expression should increase as the metabolic needs of the follicle increase, however this is difficult to demonstrate as it is thought that follicles approaching or undergoing atresia exhibit different genetic expression patterns. As a result, in this experiment the follicular fluids were collected in order to perform hormone analyses to help indicate the health status of the follicle from which the cells were obtained. It is well established that dominant follicles have increased oestradiol concentrations in the follicular fluid (Fortune *et al.*, 2001; Ryan *et al.*, 2007), due to an elevated steroid hormone synthetic ability, and steroid hormone profiles have been used as indicators of follicular health. However, this must

be done with the consideration in mind that these hormone concentrations change with growth and development, in addition to atresia (Rodgers and Irving-Rodgers, 2010).

Aims:

- To investigate the effect of hypoxic conditions on ovine granulosa cell steroidogenesis in culture, using different volumes of culture medium to mimic hypoxic conditions.
- To investigate the effects of induced hypoxia on ovine granulosa cell steroid hormone production and the expression of factors relating to angiogenic mechanisms, using both physiological and chemical methods.
- To measure HIF-1α expression in ovine follicle shells by real-time PCR to compare expression levels with follicle size, follicular fluid hormone concentrations and angiogenic growth factor expression.

4.2. Materials and Methods

4.2.1. Experiment 1

Follicles of all sizes were dissected from ovine ovaries collected from the abattoir and their granulosa cells harvested as previously described. A 48 well plate was seeded with 220,000 cells/well, with half the wells containing 0.5ml of media (with or without 1ng/ml FSH), and the other half containing 1.25ml of media (with or without 1ng/ml FSH) to simulate hypoxic conditions. The cells were cultured using the same method as in chapter 3. Culture medium was changed every 48 hours, and oestradiol in the culture medium was measured by ELISA.

Statistical comparison of oestradiol production in the two media volume groups, and the inclusion or exclusion of FSH at each timepoint, was performed by ANOVA in Genstat (Version 12.0, VSNi).

4.2.2. Experiment 2

Declaration: This experiment was performed alongside Dr Peter Marsters and Maria Fakiridou for Maria's Masters thesis. As a result, I did not perform the transfections and gene analyses myself.

Three types of culture were set up as follows;

1. High and low density cultures

Granulosa cells were seeded in 24-well plates at 2 different densities; $1x10^6$ cells/well (HD) and $1x10^5$ cells/well as a control (LD). In addition, two further groups of cells were formed; one was cultured with the addition of 250μM cobalt chloride (LDCC) to the media, and the other cultured with the addition of 10μM sodium azide (LDSA), to mimic hypoxic conditions. The cells were harvested after 66 hours and used for gene expression analysis by real-time PCR for HIF-1α and VEGF.

2. Density gradient cultures

Granulosa cells were seeded in 24-well plates at 4 different densities; $1x10^5$ cells/well, $2.5x10^5$ cells/well, $5x10^5$ cells/well and $1x10^6$ cells/well. The cells were harvested after 66 hours. Culture medium was collected and the oestradiol and progesterone content measured by ELISA.

3. Time course cultures with HRE transfections

Granulosa cells were seeded in 24-well plates at $2.5x10^5$ cells/well. Half of the cells were transfected with hypoxia responsive element-luciferase with Tfx-50™ (Cignal HIF Reporter Assay Kit, Tebu-Bio, UK) after 24 hours. Following this, untransfected and transfected cells were split into three groups; one group cultured in the presence of 250μM cobalt chloride, one cultured in the presence of 10μM sodium azide, and the other left untreated. Cells were left for 24 hours to settle, and then harvested at 24 hour intervals for gene expression analysis; 24, 48 and 72 hours after cobalt chloride addition. Cells were then lysed and analysed by luminometer to measure HRE activation.

Statistical analysis was performed by ANOVA using Genstat (Version 12, VSNi), to compare differences between each of the treatment groups for each timepoint.

4.2.3. Experiment 3

Ovine follicles of various sizes from abattoir ovaries were dissected and hemisected individually in dPBS. The individual follicle shells were crushed in liquid nitrogen using a pestle and mortar. They were then put into lysis buffer and allowed to dissolve. mRNA was extracted from the lysed cell suspension using QIA Shredder (Qiagen 79654) columns followed by RNA mini spin columns (Qiagen RNeasy Mini kit 74104). Any excess DNA was digested on the columns using RNase-free DNase (Qiagen 79254). mRNA was eluted from the columns using RNase-free water.

Following cDNA synthesis, reverse transcriptase PCR was performed in triplicate (see methods section for details), in order to quantify the relative expression of HIF in each follicle. A medium sized follicle (follicle no.12) was chosen as a calibrator, in order to compare expression levels in the other follicles.
The follicular fluid from the follicles was individually collected in order to compare steroid hormone concentrations to the results from PCR.

Data was analysed by linear regression using GraphPad Prism (Version 5.0) with significance set at $P<0.05$.

4.3. Results

4.3.1. Experiment 1

Oestradiol production was significantly lower in the high level medium wells across all timepoints ($P<0.01$), and in the normal medium level wells, FSH significantly increased oestradiol production at the later 2 timepoints ($P<0.05$) (figure 4.1). However, when the oestradiol concentrations were corrected for

total well media volume, there was found to be no significant difference in oestradiol production between the high and normal medium, and presence or absence of FSH.

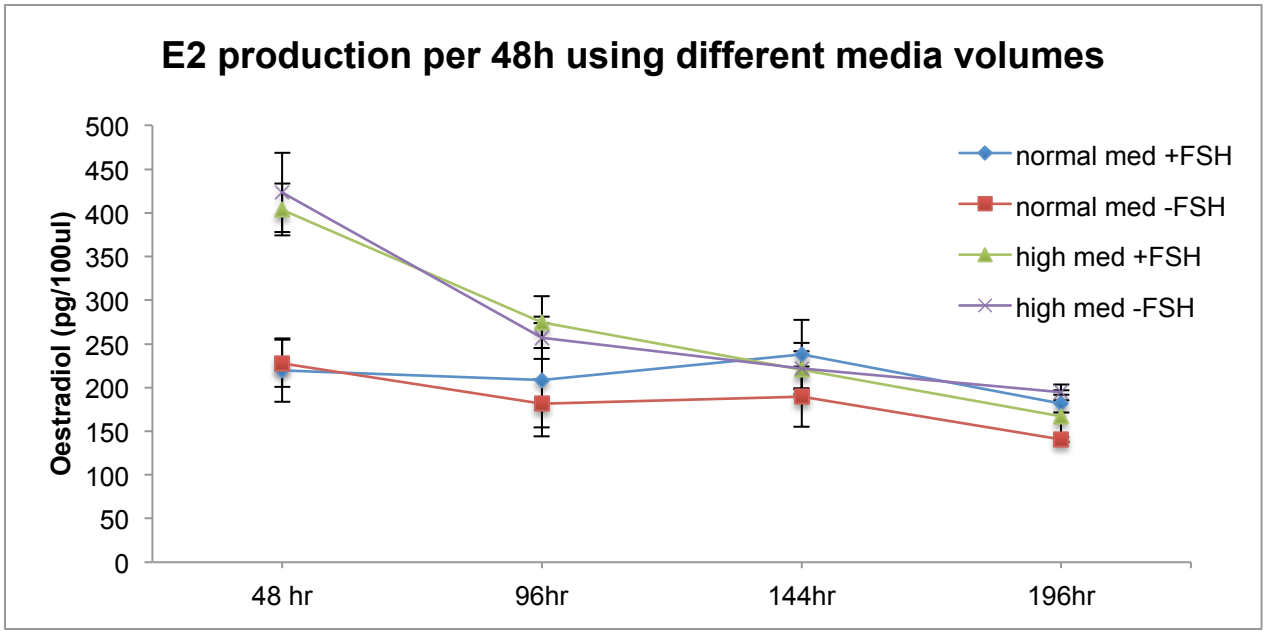

Figure 4.1. Oestradiol production per 48 hours by ovine granulosa cells over a course of 4 timepoints, in the presence of normal or high levels of media, with or without FSH

(Error bars indicate SEM)

4.3.2. Experiment 2

The results from culture 1 can be seen in figure 4.2 below. Gene expression is shown relative to cells cultured at low density. HIF-1α expression was significantly increased in the high cell density group ($P<0.001$) by 2-3 fold, however cobalt chloride and sodium azide had no significant effect on HIF-1α.

VEGF expression was 3-4 fold higher in the high cell density group than in the low density and sodium azide-treated groups ($P<0.001$), and VEGF expression was significantly higher in the cobalt chloride group than in all 3 of the other groups ($P<0.001$).

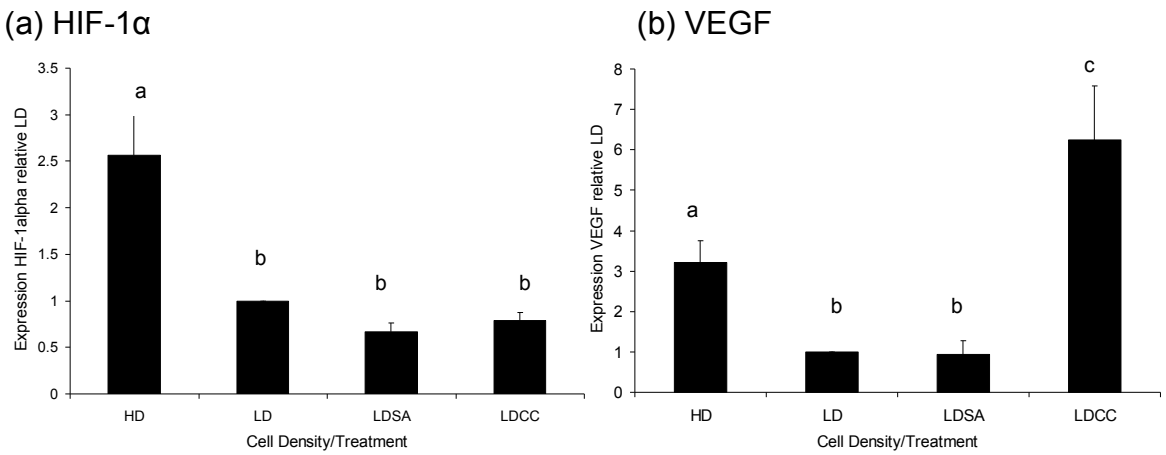

Figure 4.2. Culture 1 ovine granulosa cell (a) HIF-1α expression and (b) VEGF expression relative to low density control

(Groups with the same superscript are not significantly different; groups with different superscripts are significantly different)

The second culture results are shown in figure 4.3. The rate of oestradiol production/million cells/hour decreased markedly as cell density increased ($P<0.001$), although a significant difference was not observed between the two higher cell density groups. There was no significant difference in oestradiol production with addition of cobalt chloride.

The rate of progesterone production also decreased significantly as cell density increased ($P<0.001$), although the rate observed in the $2.5x10^5$ group was slightly lower than expected and as a result did not follow this trend. Cobalt chloride addition significantly reduced progesterone production by the cells across all of the groups ($P<0.001$).

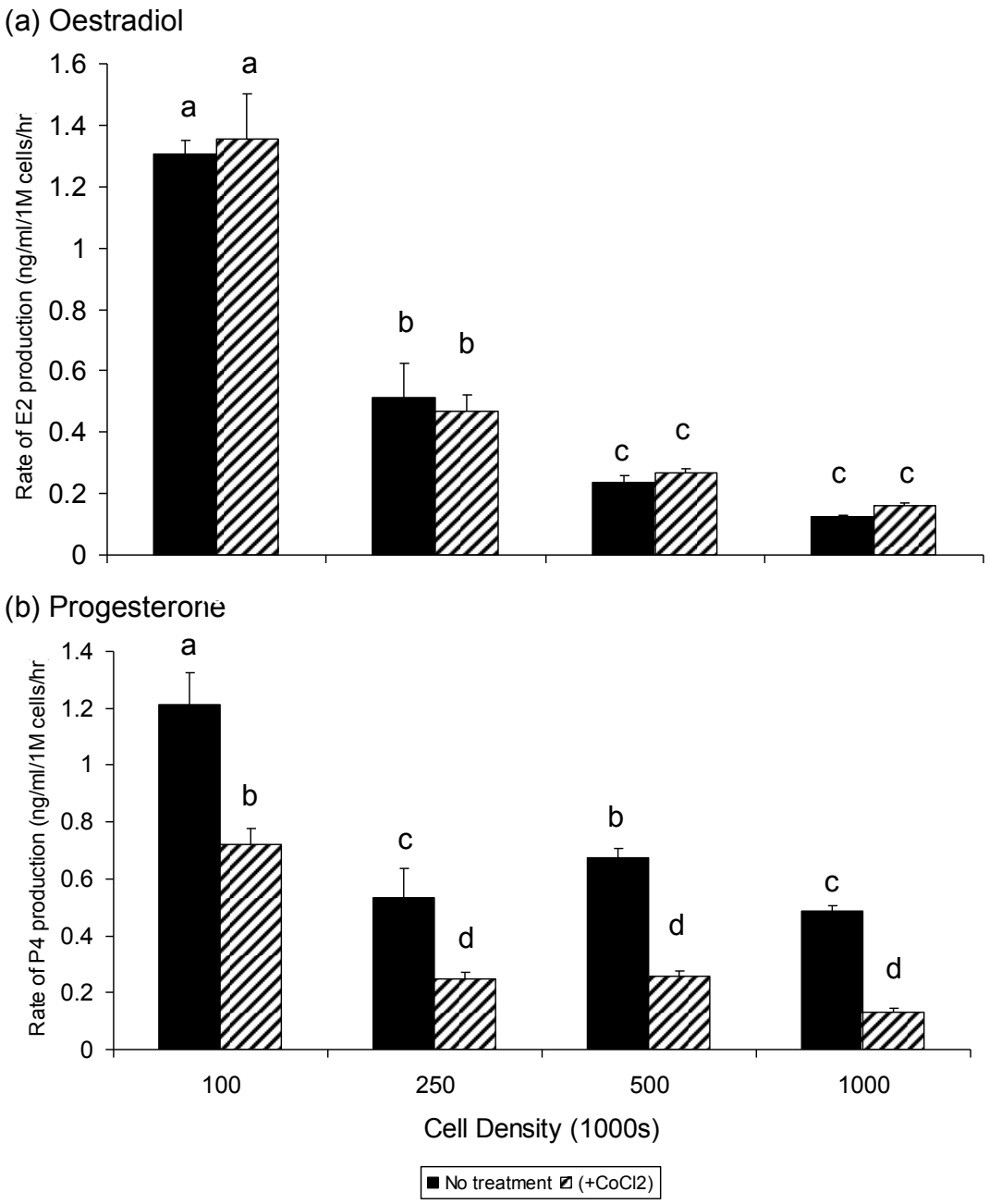

Figure 4.3. Culture 2 ovine granulosa cell (a) oestradiol and (b) progesterone production by cells seeded at different densities and cultures with or without cobalt chloride

(Groups with the same superscript are not significantly different, groups with different superscripts are significantly different)

The results of culture 3 are shown in figure 4.4. The oestradiol production graph (a) shows that in the control group (no treatment, untransfected), oestradiol production rate increases significantly over time ($P<0.001$). Treatment with cobalt chloride on untransfected cells resulted in an initial decrease, then a significant increase ($P<0.001$) in oestradiol production rate after 48 hours of treatment. Following this, a significant depression in production rate was observed ($P<0.001$). Unexpectedly, transfection significantly depressed oestradiol production rate during the first 24 hours in both the treated and untreated groups ($P<0.001$), which remained unchanged for the later timepoints also.

As would be expected, progesterone production rate increased significantly in the control group ($P<0.001$) (no treatment, untransfected), however the rate of production fell significantly after 96 hours in culture ($P<0.001$). The untreated transfected group exhibited a steady increase in progesterone production rate across the timepoints ($P<0.001$), but this was lower than the control group. Cobalt chloride significantly decreased progesterone production rate in both transfected and untransfected cells, although there was a small increase in production rate in the untransfected cell group at 72 hours of culture, which was significant ($P<0.001$).

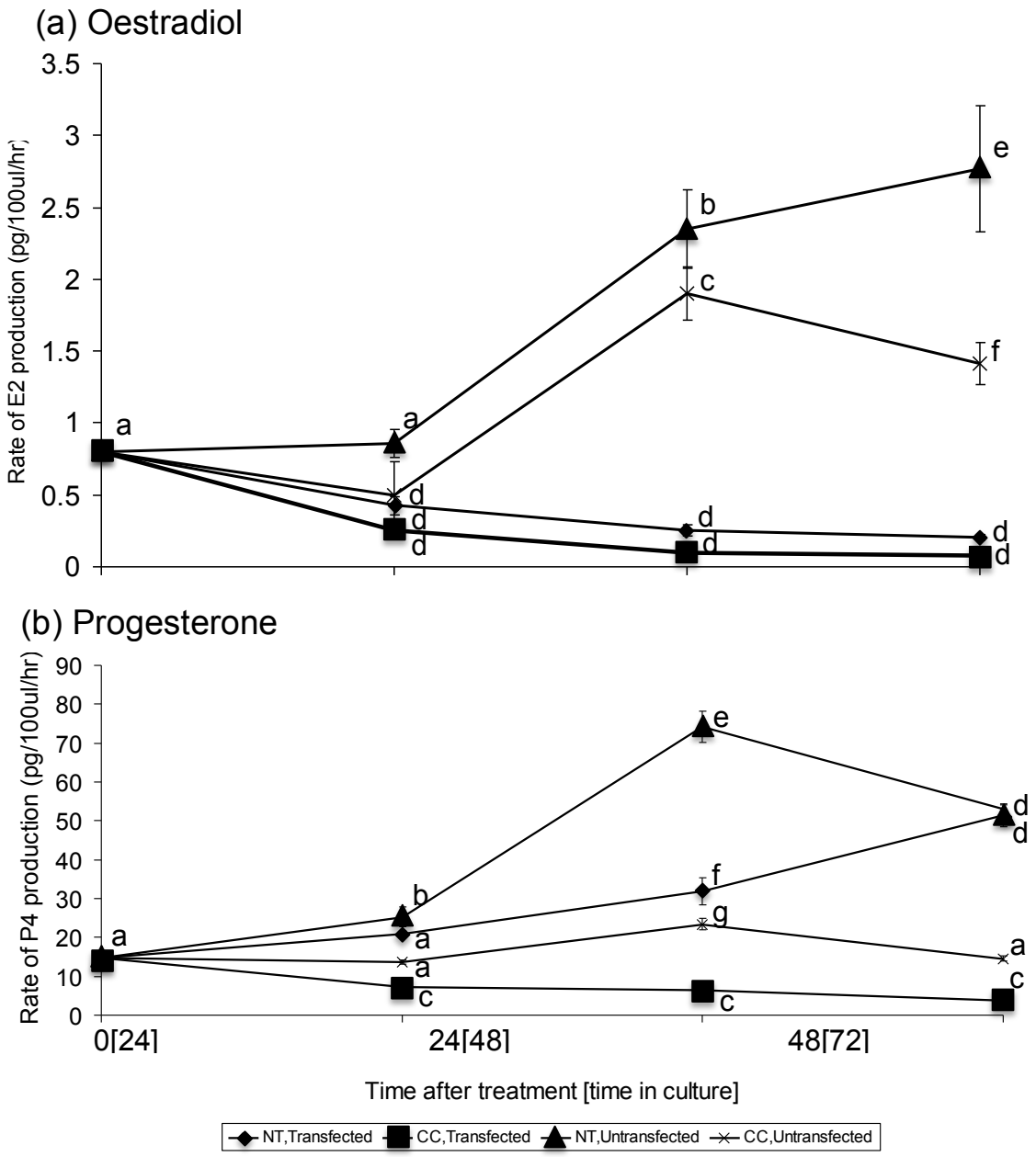

Figure 4.4. Culture 3 rate of ovine granulosa cell (a) oestradiol and (b) progesterone production in transfected and untransfected cells with or without cobalt chloride.

The x axes are numbered as the hours after the treatment (with/without cobalt chloride), numbers in brackets indicate total time in culture.

4.3.3. Experiment 3

Table 4.1 below shows the quantities of mRNA and cDNA, as well as HIF-1α expression and follicular fluid oestradiol concentration in relation to follicular size.

Follicle no.	Size (Grat. Units)	Size (mm)	mRNA (ng/µl)	cDNA (ng/µl)	HIF-1α RQ	E2 concentration (ng/ml)
23	21	1.7	60.5	388.9	1.2075	20.31
28	21	1.7	26.5	371.3	0.3984	20.95
18	22	1.8	49.6	384.5	1.5317	20.52
24	23	1.9	56.4	387.9	2.8469	14.89
27	24	1.9	49.1	382.2	0.2797	10.43
17	25	2.0	22.9	373.4	0.7162	12.77
15	28	2.3	29.9	379.0	0.5659	11.64
26	28	2.3	42.2	368.2	0.5466	7.20
13	30	2.4	10.5	300.3	0.3291	6.24
14	30	2.4	32.7	312.5	0.8677	8.79
16	31	2.5	40.4	380.6	0.3714	12.07
21	31	2.5	169.9	474.1	0.8816	9.30
25	31	2.5	142.8	442.9	1.1272	5.47
12*	35	2.8	104.5	376.0	1	6.52
19	35	2.8	40.4	376.0	0.3635	8.04
20	35	2.8	74.9	402.2	0.938	6.24
22	39	3.2	69.0	394.7	0.3585	4.78
10	50	4.0	90.0	344.0	1.1985	7.49
11	55	4.4	79.0	356.0	0.6721	3.11
7	62	5.0	185.9	420.6	2.443	57.64
8	63	5.1	117.0	363.9	2.1628	40.31
1	66	5.3	33.7	352.9	1.9044	32.77
5	66	5.3	79.5	354.2	0.4698	1.50
9	69	5.6	219.0	437.8	0.5978	2.55
2	70	5.7	274.0	487.6	1.3921	72.93
4	75	6.1	50.3	336.0	0.9214	43.02
3	85	6.9	163.5	415.5	0.6967	44.67
6	85	6.9	95.3	360.3	0.5434	8.87

Table 4.1. HIF-1α expression and follicular oestradiol concentration for a range of follicle sizes. (*reference sample 12)

Granulosa cell mRNA expression of HIF-1α had a significantly positive relationship to follicle size ($P<0.01$), with Pearsons correlation coefficient of 0.11 and coefficient of determination of 0.0122 (figure 4.5).

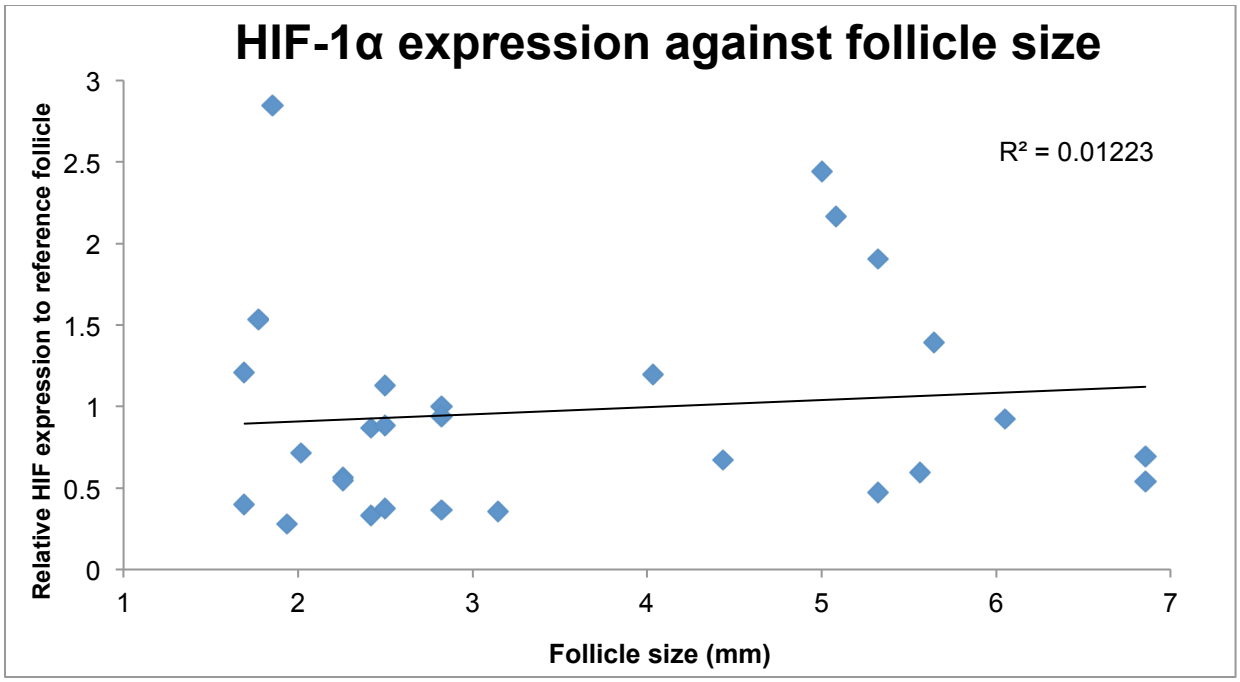

Figure 4.5. Ovine granulosa cell HIF-1α expression against follicular size

Follicular fluid oestradiol was significantly positively correlated to follicular size (P<0.01), with a Pearsons correlation coefficient of 0.47 and coefficient of determination of 0.22 (figure 4.6).

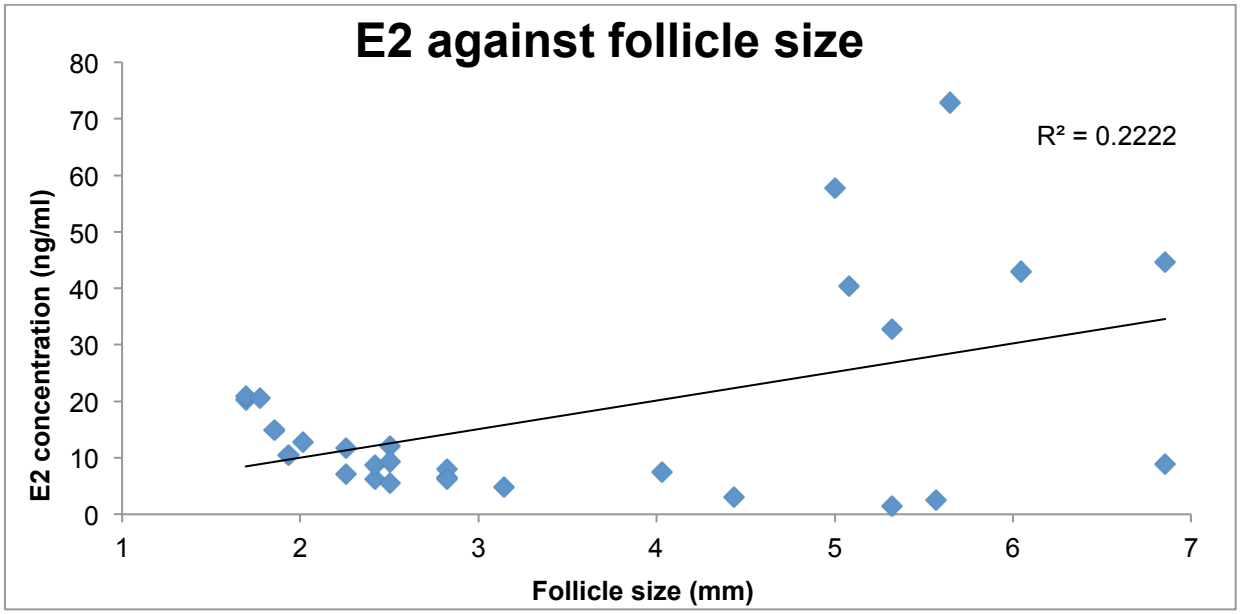

Figure 4.6. Ovine follicular fluid oestradiol concentration against follicle size

Oestradiol concentration in the follicular fluid was found to be significantly positively correlated to HIF-1α mRNA expression (P<0.001), with Pearsons correlation coefficient of 0.51 and coefficient of determination of 0.26 (figure 4.7).

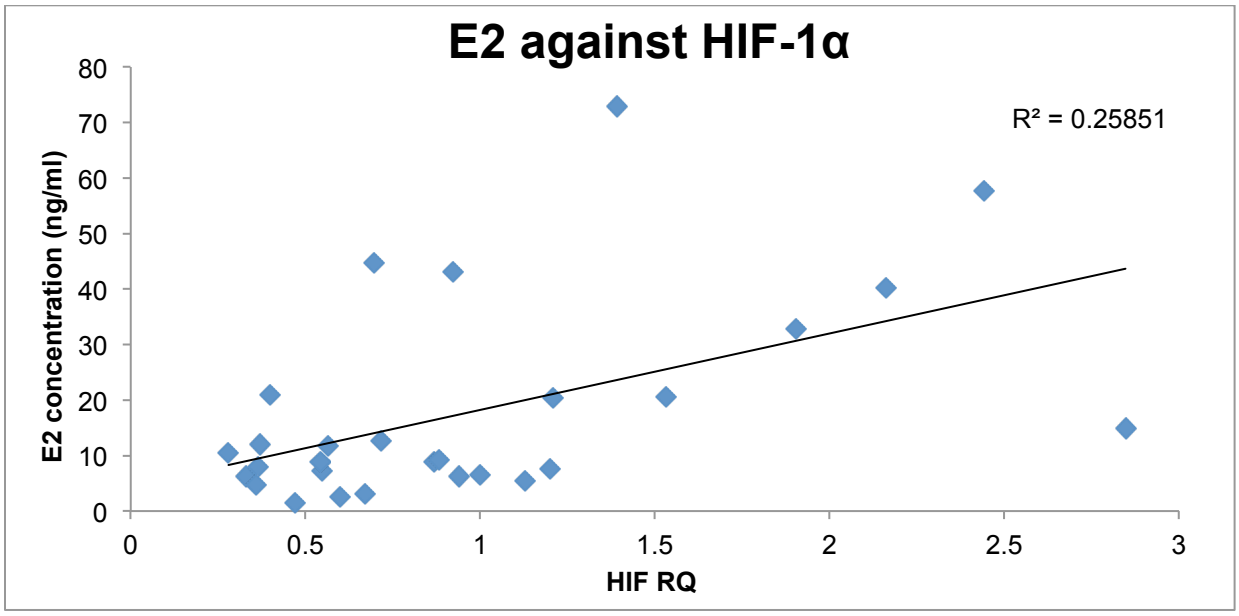

Figure 4.7. Ovine follicular fluid oestradiol concentration against granulosa cell HIF-1α expression

The follicles were grouped into oestrogenic and non-oestrogenic, based on an intrafollicular oestradiol concentration threshold of 10ng/ml. T-testing in SPSS revealed that HIF−1α expression was significantly higher in granulosa cell originating from follicles with an intrafollicular oestradiol concentration greater than 10ng/ml (figure 4.8).

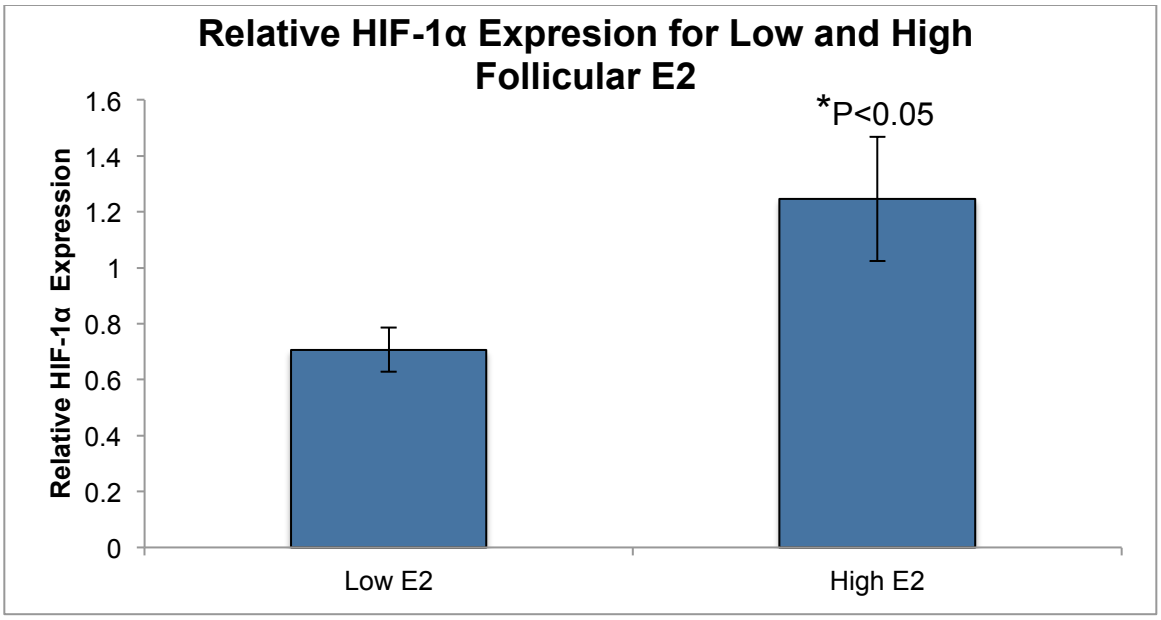

Figure 4.8. Ovine granulosa cell HIF-1α expression for follicles with low and high levels of oestradiol in the follicular fluid

Error bars denote SEM

4.4. Discussion

Unfortunately culture 1 was unable to demonstrate an effect of inducing a hypoxic state by using an increased depth of culture media for cell culture, and therefore the consequent cultures to study hypoxia were not performed based on this methodology. There was found to be no significant difference in oestradiol production between the normal and high media volumes. Furthermore, presence or absence of FSH in this experiment had no significant effect.

Increased cell seeding density was successful in inducing hypoxic conditions in *in vitro* culture. It was observed that at a much higher density, the granulosa cells formed clumps, which seem to make oxygen availability to the cells fairly limited. As a result, an increase in HIF-1α expression was observed at this very high cell density, which in turn upregulated VEGF expression by the cells in an attempt to increase thecal vascularisation and hence increase oxygen tension.

The artificial hypoxia-mimicking chemicals sodium azide and cobalt chloride had different effects on the granulosa cells in a hypoxic environment, and acted via different pathways. Sodium azide appeared to have little effect on HIF-1α and VEGF expression indicating that the mechanism of action does not

interfere with these activation pathways, at least at the concentrations that were used. In fact, this was observed to have a cytotoxic effect. Cobalt chloride however, actually induced VEGF expression, but appeared to have no effect on the expression of HIF-1α, indicating a completely different mechanism of action. We believe that reactive oxygen species (ROS) may have a role in these observations, as cobalt chloride has been shown to increase ROS generation, which in turn stabilise HIF-1α in hypoxia (Chandel *et al.*, 2000). In addition, ROS bind to the VEGF promoter sequence, leading to an increase in expression (Monaghan-Benson and Burridge, 2009).

It is clear from the results of culture 2 that steroid hormone production was significantly decreased in hypoxic conditions. Oestradiol decreased rapidly as cell density was increased, which we believe may be as a result of decreased metabolic capacity by the cells due to hypoxia and/or due to a build-up of ROS causing a depression in aromatase activity, as proposed by Winnett *et al.* (2003) in a study using breast cancer cells. Portela *et al.* (2010) also observed a decrease in oestradiol production as plating density increased, using bovine granulosa cells in a serum-free culture system, and suggested that this was as a result of stressing of the cells and increased apoptosis. Cobalt chloride had no additional depressive effect on oestradiol production, but significantly decreased cellular progesterone production rate further. It seems likely therefore that cobalt chloride has an effect on the enzymes involved in the conversion of cholesterol to pregnenolone and progesterone; cytochrome P450 and 3β-hydroxysteroid dehydrogenase.

Culture 3 showed that over a long time course, cobalt chloride suppressed the oestradiol production rate by the granulosa cells, however it took 48 hours of exposure for this effect to become inhibitory. Transfection heavily suppressed the production of oestradiol by the cells, which was not expected. Perhaps this is due to an effect on the granulosa cell membrane permeability causing a restriction in small molecule transport, however this speculative, and as a result would need to be further investigated.

HIF-1α mRNA expression was significantly correlated to follicular diameter, as seen in figure 3.28 ($P<0.001$). However, the Pearsons correlation coefficient was just 0.11, suggesting that the line is only weakly linear. In addition the coefficient of determination (r^2) was 0.0122, indicating a considerable amount of variation within the dataset. This is likely to be because of the diversity of the developmental stage of the follicles included in the experiment, as a number of the follicles would have been atretic, or

approaching atresia, which would have affected the expression levels. As a result, it is arguably more important to take into account the follicular fluid hormone measurements, which give more of an indication of the health status of the follicles.

Oestradiol was significantly positively related to follicular size, as would be expected. This is because as follicles develop to preovulatory size, the granulosa cells become increasingly oestrogenic (Scaramuzzi and Baird, 1977; McNatty *et al.*, 1985; McNeilly *et al.*, 1991; Scaramuzzi *et al.*, 2011).

Interestingly, follicles with oestradiol levels in the follicular fluid higher than 10mg/ml had significantly higher granulosa cell HIF-1α expression than those with lower oestradiol concentrations ($P<0.05$). This finding suggests that the oestrogenic follicles, which are more likely to be the healthiest, express higher levels of this transcription factor, and could also therefore have increased expression of angiogenic factors. It is possible therefore that oestrogenic follicles have increased angiogenic activity, which would suggest that the healthiest follicles with the highest oestrogenic activity may have the highest potential for vascularisation and this could contribute to the follicle acheiving dominance.

The major limitation of this study was that it is unable to answer whether oestradiol drives the production of angiogenic factors such as HIF-1α in granulosa cells, or whether the converse is true, although it has been previously demonstrated that VEGF expression increases in the presence of oestradiol using a bovine model both *in vitro* and *in vivo* (Shimizu and Miyamoto, 2007), as well as in murine endothelial cells (Morales *et al.*1995; Schnaper *et al.*, 1996). It is also thought that progesterone may also have this effect (Shimizu and Miyamoto, 2007). It may therefore be the case that higher levels of steroid hormones promote angiogenic factor expression acting through HIF-1α.

Chapter 5 – *In vivo* immuno-neutralisation study

5.1. Introduction

The two TGF-β superfamily members BMP15 and GDF9 are essential for follicular development in sheep (Juengel *et al.*, 2002a). It is thought that these two factors are able to cooperate and heterodimerize to form a more bioactive compound (Yan *et al.*, 2001; McNatty *et al.*, 2005a). Both active and passive immunization against each of these factors has previously yielded some interesting results, however thus far the BMP15 and GDF9 antisera have not been administered at the same time.

Following the effect of the combination of BMP15 and GDF9 observed in the *in vitro* cell cultures on increasing oestradiol production, in order to explore whether this phenomenon would be observed *in vivo*, an ovine infusion experiment was performed. Antisera to these two factors were used, as opposed to infusing the peptides themselves, primarily for cost reasons, as these recombinant factors are extremely expensive to produce. Consequently, the effect of blocking the action of these factors was explored, rather than the promotional effect on steroidogenesis as previously described.

5.2. Aim

The aim of this experiment was to investigate the effects of knocking down local availability of BMP15 and GDF9 in sheep, by direct infusion of a combination of antisera against both growth factors.

5.3. Method

Six Scottish black face ewes were actively immunised against BMP15 and GDF9, 6 months before the infusion study took place, so that antiserum could be collected from these animals and used for the infusion.

5.3.1. Active Immunisation

The vaccination consisted of BMP15 or GDF9 peptides specific for conserved regions of each molecule, synthesized and conjugated to keyhole limpet hemocyanin (KLH). The peptide sequences were the same as those used previously by Juengel *et al.* (2002a and 2004); KKPLVPASVNLSEYFC (GDF9) and SEVPGPSREHDGPESC (BMP15) 2ml of each conjugate emulsified at 0.1mg/ml in Fruend's incomplete adjuvant (Sigma F5506) was initially administered subcutaneously at 4 injection sites to the corresponding ewes, followed by appropriate booster injections as needed. Antibody titres were checked regularly to measure titre response. Blood was collected from these animals (6ml/kg), and the plasma extracted by centrifugation to be used in this trial. Pooled plasma samples were added to physiological saline to give antibody titres of 1:500 for both GDF9 and BMP15. This was later infused directly into the ovarian arteries of the animals used in the infusion study.

5.3.2. Oestrous synchronisation

Six mature Scottish Blackface ewes had their oestrous cycles synchronised with intravaginal progestagen (medoxy-progesterone-acetate) impregnated sponges (Dunlop, Dumfires, Scotland) approximately 10 days before the infusion study began (figure 5.1).

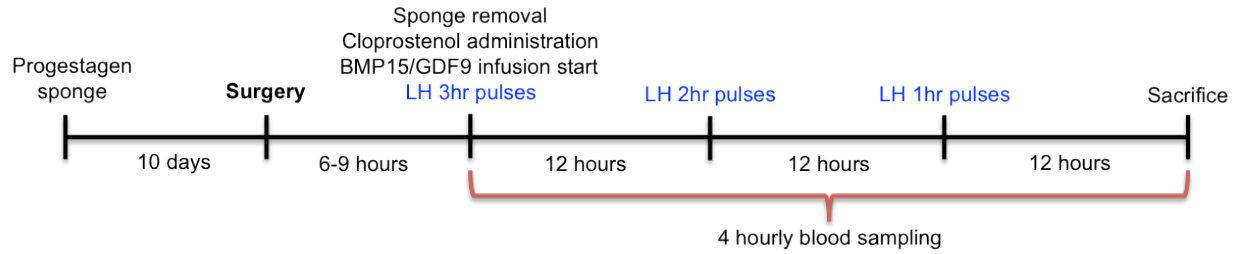

Figure 5.1. Immuno-neutralisation *in vivo* study summary diagram

5.3.3. Ovarian vascular cannulation

In order to cannulate the ovarian vasculature, the animals were first anaesthetised. General anaesthesia was induced using thiopentone, and maintained throughout surgery with isoflurane via a ventilator.

A 15cm incision was made parallel to the midline, between the umbilical scar and the udder to allow access to the abdominal cavity. A trocar was used to make an opening on either side of the skin, through to the abdominal cavity in order to pass the infusion lines through to attach to the ovarian arterial and venous cannulae. A section of 0.6mm silastic tubing was used for each ovarian vein, and a 1.02mm vascular cannula for each ovarian artery.

The reproductive tract was exteriorised and held in place so that the ovarian vasculature could be accessed, as seen in figure 5.2.

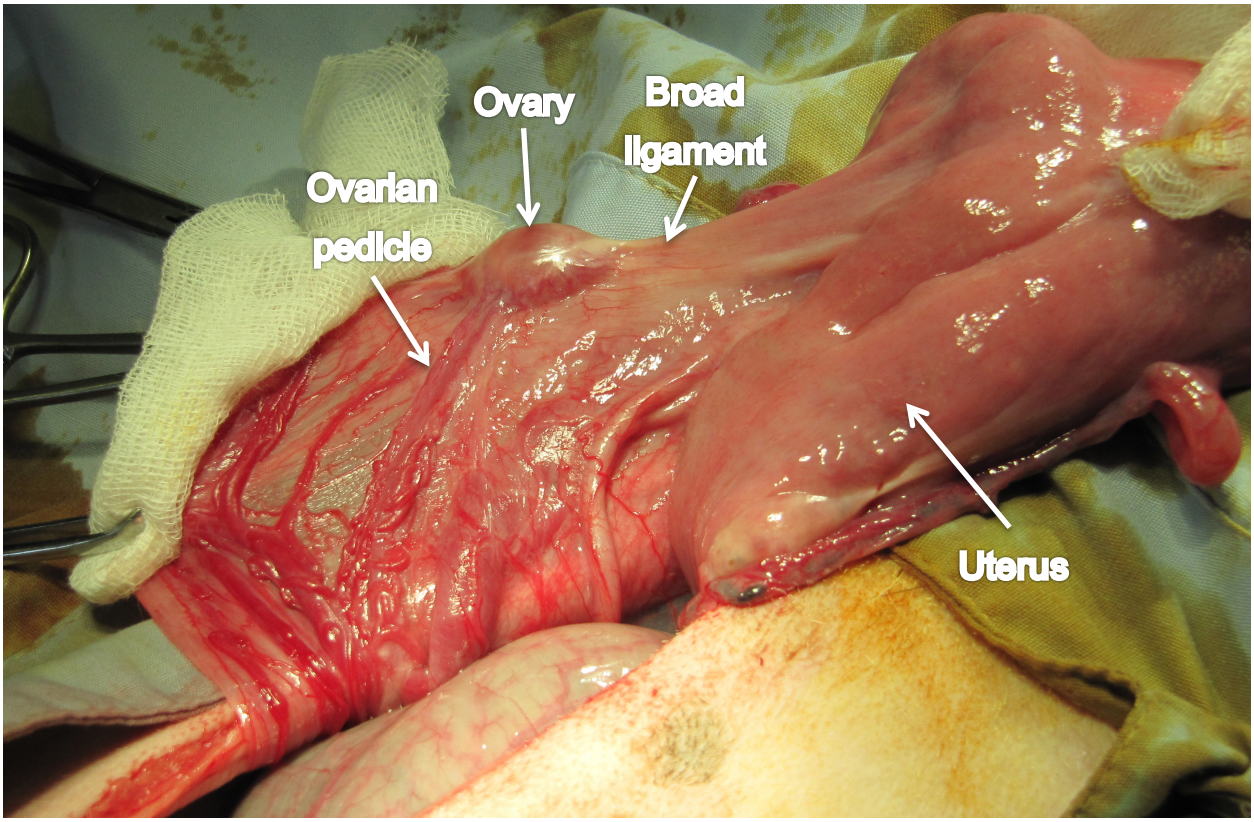

Figure 5.2. Photograph of exteriorised ovine reproductive tract with vasculature.

The utero-ovarian vein bloodflow was temporarily restricted and a small opening made in the vessel wall distal to the utero-ovarian vein bifurcation, to allow passage of the silastic tubing into the vessel lumen. The tubing was advanced until the tip lay in the body of the common utero-ovarian vein and held in place

with fine suture thread (6/0 Prolene). Blood flow was then restored following capping of the silastic tubing to prevent blood loss.

A section of a branch of the ovarian artery which supplies the oviduct was blunt dissected and the distal portion tied off (figure 5.3). A bulldog clamp was applied to the proximal end of the dissected vessel to temporarily stop arterial bloodflow, and a small incision was made in the vessel wall. A fine vascular cannula equipped with internal spring (Leaderflex©, Vygon, UK) was inserted into the lumen until it was around 10mm inside. The spring was then carefully removed, and the cannula sutured in place (5/0 Mersilk). Heparin (5000IU/ml) was passed into the tube before it was capped to prevent blood clot formation.

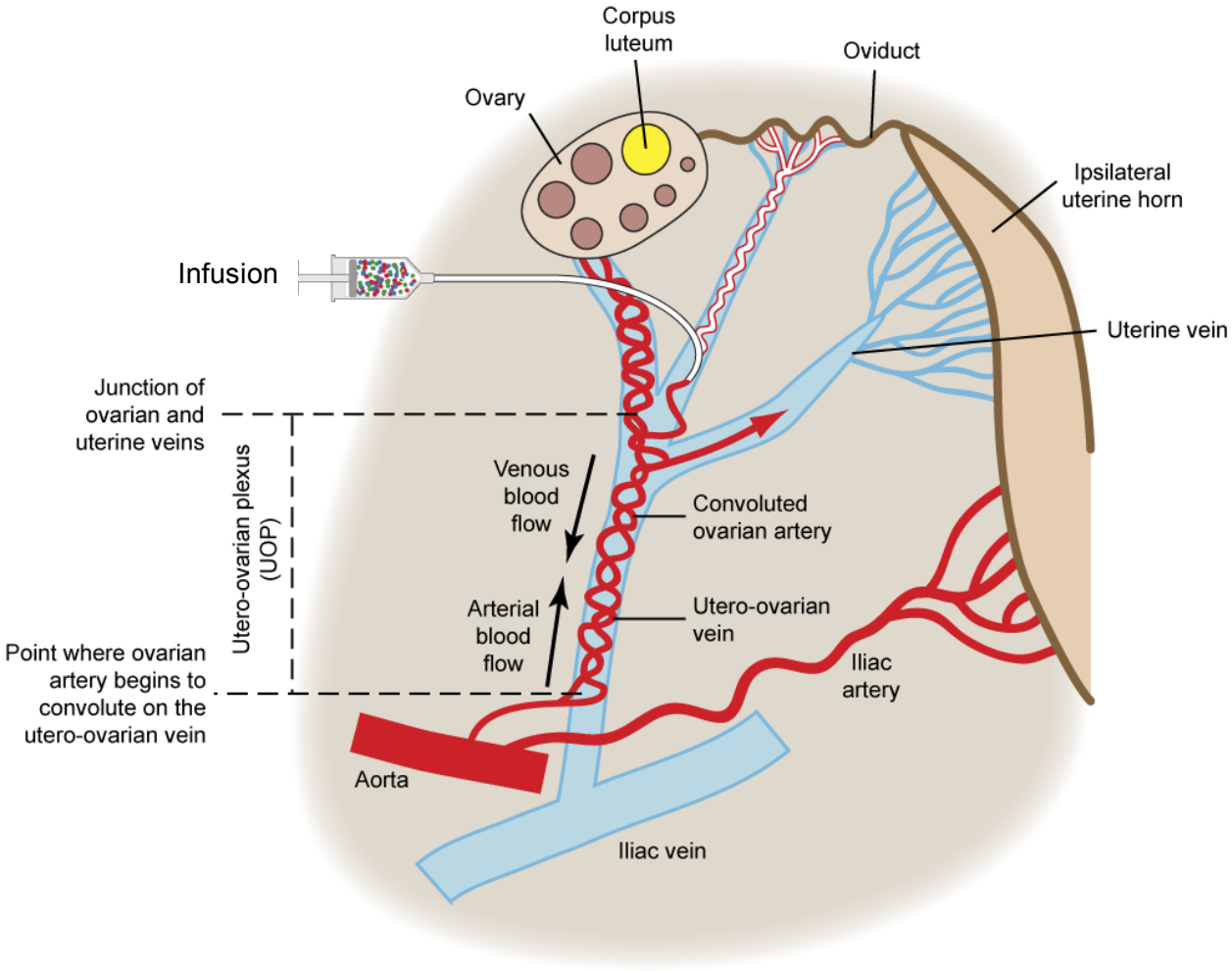

Figure 5.3. Diagram illustrating ovarian arterial cannulation in the ewe

Redrawn by McNeilly from Lee *et al.* (2010)

When the ovarian vein and artery had been cannulated on both ovaries, the arterial cannulae were clamped once again, the cannula caps removed, and the cannula tubing attached to the previously placed trans-abdominal infusion lines.

The excess tubing was pulled through and the reproductive tract repositioned back in the abdominal cavity. The midline incision was then sutured closed. The jugular veins on each side were cannulated with a 14G IV catheter (Terumo Surflo®), which were secured with a single suture. The animals were allowed to recover in individual holding crates for 6-9 hours before the infusion was started.

5.3.4. Ovarian stimulation

The progestagen sponges were removed following surgery, and then each animal was given 100µg intramuscular cloprostenol (Estrumate, Intervet, Cambridge, UK) to induce luteolysis and initiate the oestrous cycles at the time of infusion in order to synchronise the follicular phase.

At the time of laparotomy it was observed that all animals were in anoestrus, which was not expected. Following sponge withdrawal, it was decided to induce an artificial follicular phase using an established 3, 2, 1 regime (Dobson *et al.*, 1997) which involved administration of pulses of 2.5µg of LH (NIH-S26 in 0.9% w/v saline with 0.1% v/v normal sheep plasma) every 3 hours for 12 hours, every 2 hours for 12 hours, then every hour for a further 12 hours. Pulses were administered via an infusion pump connected to a timer, set to deliver 1ml/minute.

5.3.5. Ovarian infusion

The BMP15/GDF9 antiserum was administered into the ovarian artery of the treatment ovary at a rate of 2ml per hour for 24 hours, and normal sheep plasma to the ovarian artery of the control ovary. The control plasma was collected from blood taken from the ewes previous to surgery (i.e. normal sheep plasma). The infusion pumps were set up according to figure 5.4.

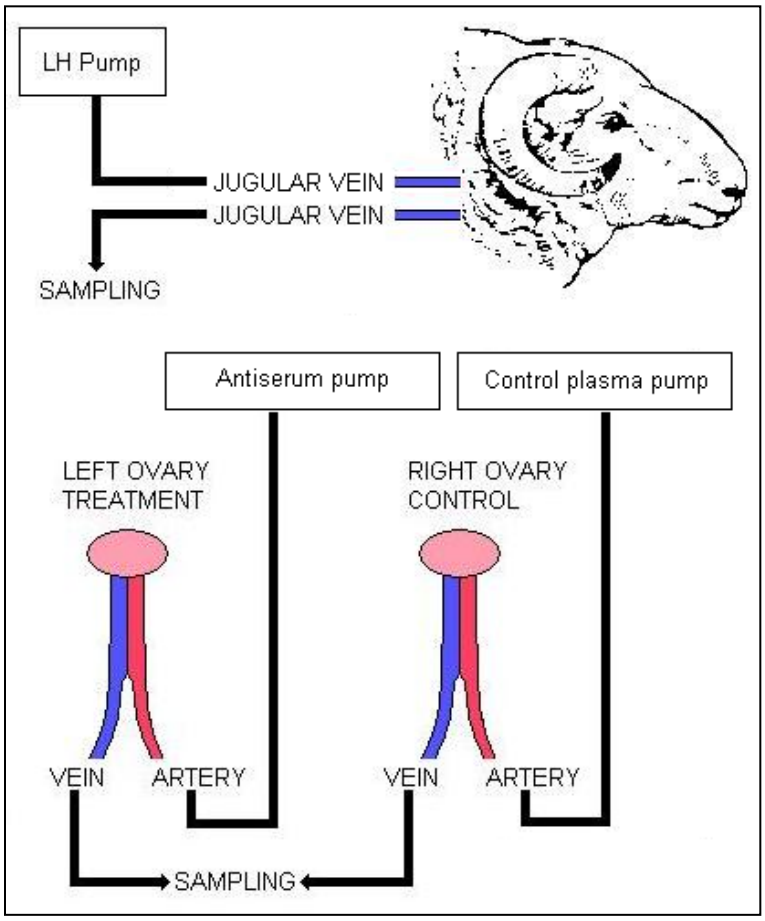

Figure 5.4. Ovine *in vivo* ovarian infusion study set-up

LH was infused directly into the jugular vein, and blood sampling taken from the other jugular vein. One ovary received direct infusion of BMP15/GDF9 antiserum and the other received control plasma in the same manner.

5.3.6. Blood sampling

Blood samples (5ml) were taken every 4 hours from each ovarian vein and the jugular vein. The blood was centrifuged with latex beads containing heparin in order to remove the red blood cells, and the plasma supernatant was transferred to 5ml tubes and stored at -20°C for later analysis.

5.3.6. Tissue collection

The animals were sacrificed approximately 36 hours after surgery, using an IV sodium pentobarbital overdose. The ovaries from each animal were collected post-mortem and fixed in Bouins fixative (appendix 3) for 24 hours, before being transferred to 70% IMS until tissue processing (section 2.3).

5.4. Results

Although all cannulations apart from one were successful, it was found that the sampling lines would intermittently become obstructed resulting in some missed data points. Problems with some of the LH infusion lines meant that in some animals, the infusion had to be continued down the sampling line. LH levels measured in these animals were therefore artificially high as a result of residual LH in the line. Consequently, LH levels in the samples taken from the ovarian venous blood were used, rather than the jugular vein. However, the overall trend of a steadily increasing LH level was observed, indicating that the infusion had been successful.

The mean ovarian LH and FSH concentrations across all animals can be seen in figure 5.5. The mean FSH across all animals was maintained at approximately 3ng/ml throughout the infusion, and mean LH increased from 0.5 to a maximum of 1.75ng/ml over the 36 hour timecourse.

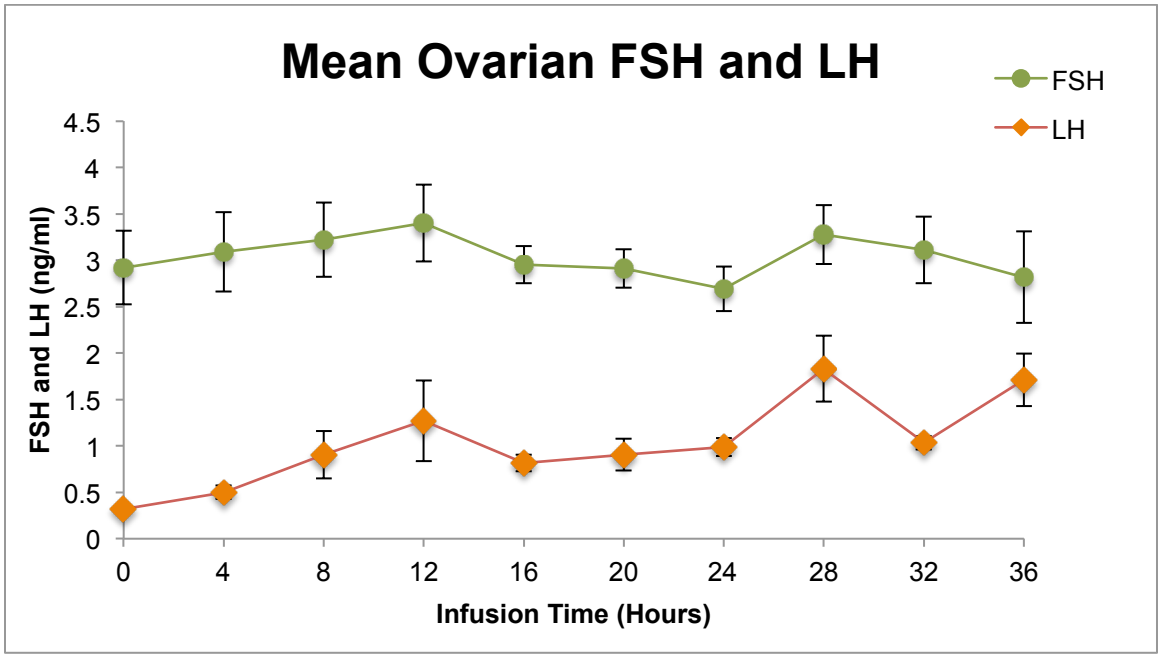

Figure 5.5. Infusion study mean ovarian FSH and LH across all animals

Error bars indicate SEM.

5.4.6. Follicle counting

Follicle counts were performed following haematoxylin & eosin staining as described in section 2.4 in order to examine any differences in the various follicle classes between the treatment and control ovaries. In particular, the effect on the larger follicles, as these were expected to show the most pronounced effect due to their high oestrogenicity.

The average counts for each follicle type for each ovary can be seen in table 5.1.

Ewe	Treatment	Primordial	Primary	Secondary	Tertiary	Pre-antral	Small antral (<3.5mm)	Large antral (>3.5mm)
1L	Treatment	46	4	1	0	3	0	6
1R	Control	118	6	2	0	3	1	3
2L	Control	9	1	2	0	1	1	6
2R	Treatment	6	1	1	0	1	1	4
3L	Treatment	9	4	1	0	1	0	4
3R	Control	14	2	0	1	0	0	1
4L	Control	21	1	1	0	2	2	4
4R	Treatment	19	1	0	1	1	4	6
5L	Treatment	65	0	1	1	0	0	4
5R	Control	85	0	0	0	0	1	1
6L	Treatment	70	0	1	0	3	0	1
6R	Control	50	1	0	0	0	1	3
Control		49.3 ± 20.1	1.7 ± 0.7	0.7 ± 0.3	0.1 ± 0.0	0.9 ± 0.4	0.7 ± 0.3	2.9 ± 1.2
Treatment		35.6 ± 11.9	1.6 ± 0.7	0.6 ± 0.2	0.3 ± 0.2	0.9 ± 0.4	0.8 ± 0.6	4.1 ± 1.0

Table 5.1. Mean follicle counts following H&E staining on ovarian sections. (L=left ovary; R=right ovary; Mean values ± SEM).

The follicle count data for treated and control ovaries was compared to see if there was any effect of the BMP15/GDF9 antiserum treatment on follicular development (table 5.1). Following within-group statistical analysis by paired T-testing, the antiserum treatment was found to have no significant effect on the number of follicles, of any size group, present in the ovary. There was found to be a greater number of large antral follicles in the treated ovaries than those of the controls, however this was not significant (P=0.177).

5.4.7. Aromatase immunohistochemistry

Aromatase immunohistological staining confirmed the presence of aromatase in the granulosa cell layer in at least one follicle of one or both ovaries in all but one of the animals (table 5.3), indicating steroidogenic activity in these follicles. The large antral follicles with positive staining were measured in order to ascertain their developmental stage. Notably, in the treatment infusion ovaries, the very large follicle of ewe 2, which was over 5mm in diameter, was seemingly unaffected in terms of staining intensity, whereas the smaller follicles of treated ovaries in other animals had lower aromatase activity.

Ewe	Ovary	Treatment	Aromatase Staining (follicle size in mm)	Staining Intensity Score (1-4)
1	Left	Treatment	Negative	1
1	Right	Control	Negative	1
2	Left	Control	Negative	1
2	Right	Treatment	Positive (5.66)	4
3	Left	Treatment	Some positive staining (2.44 and 2.34)	2
3	Right	Control	Negative	1
4	Left	Control	Positive (5.04)	3
4	Right	Treatment	Positive on one large follicle side (4.48)	2-3
5	Left	Treatment	Some slightly positive staining (4.8 and 4.52)	2
5	Right	Control	Negative	1
6	Left	Treatment	Slight positive staining (4.8)	2
6	Right	Control	Positive (4.52)	4

Table 5.2. Aromatase immunohistological staining results of ovarian sections (1=no staining, 2=light staining, 3=moderate staining and 4=heavy staining).

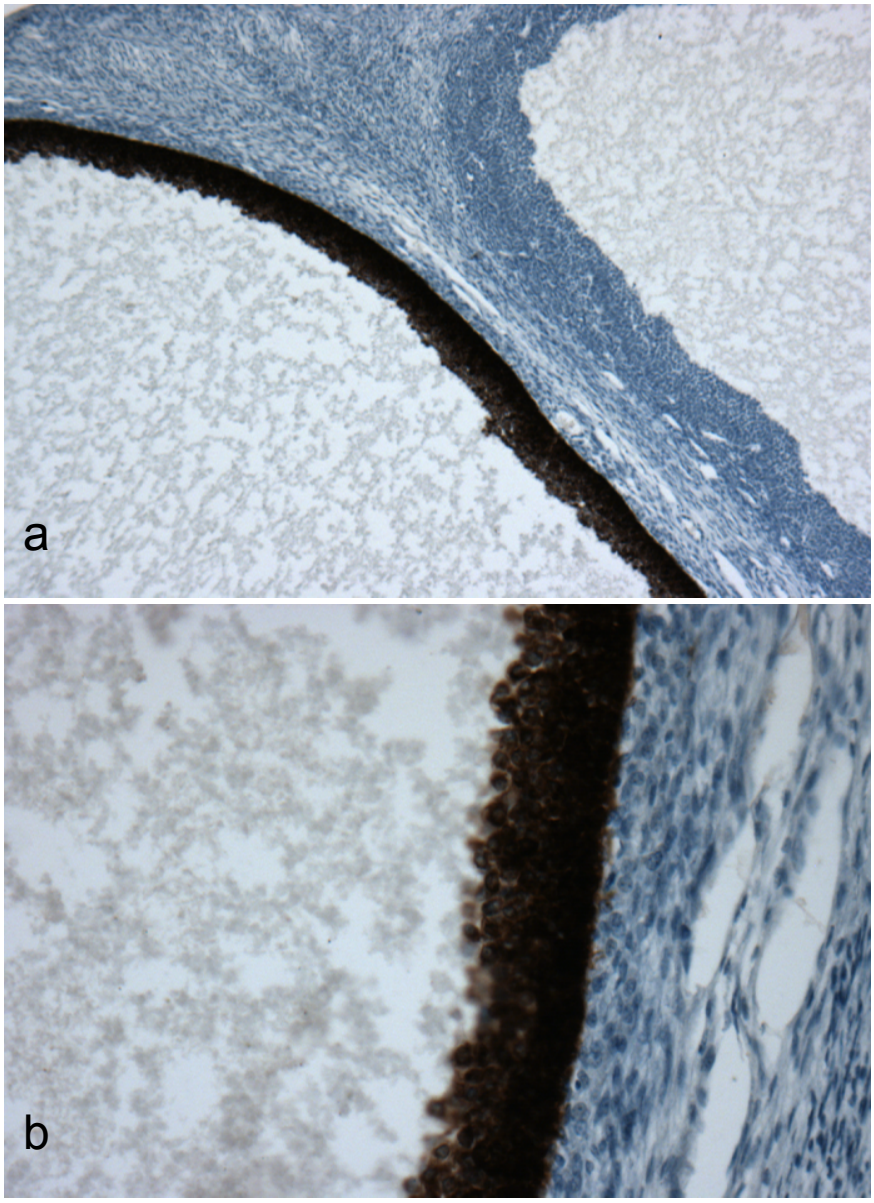

Figure 5.6. Ewe 2 positive aromatase staining of the granulosa cell layer in a large antral follicle on the treatment ovary at (a) x50 and (b) x200

(Brown staining indicates cells positive for aromatase.)

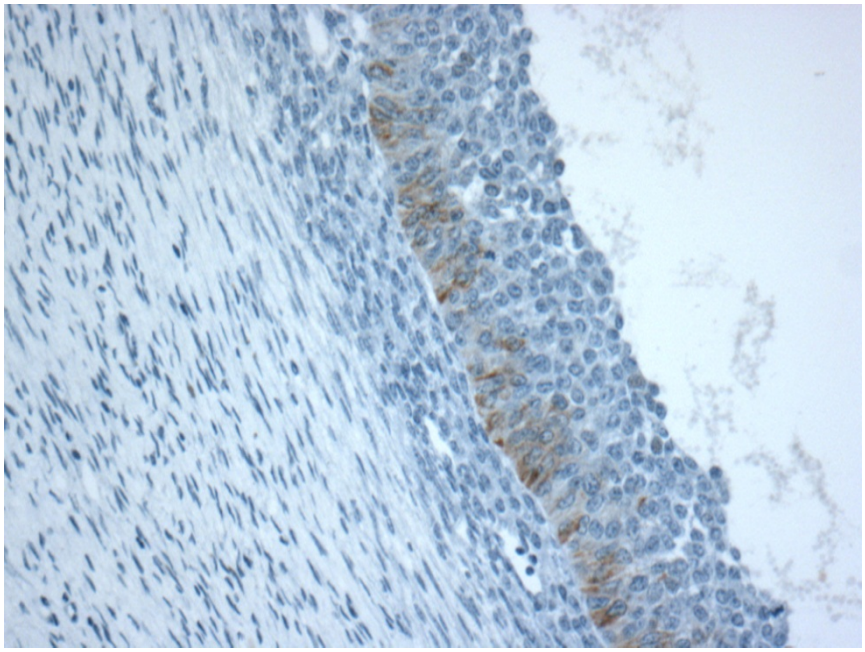

Figure 5.7. Ewe 3 treatment ovary weak positive aromatase staining of granulosa cells (x200)

Brown staining is weak but positive.

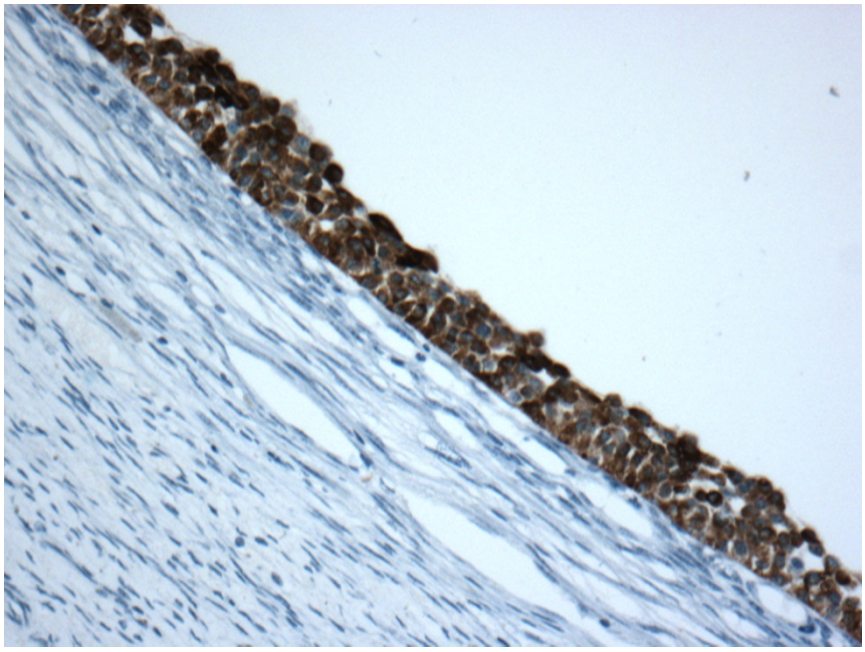

Figure 5.8. Ewe 4 control ovary positive aromatase staining of granulosa cell layer (x200)

5.4.8. Ewe 1

The right ovary of ewe 1 had ovarian vasculature that proved extremely difficult to dissect, and as a result only the vein could be cannulated. This meant that the control plasma could not be administered to the right ovary. In addition, the sampling line became blocked and so blood could not be taken after 24 hours of infusion.

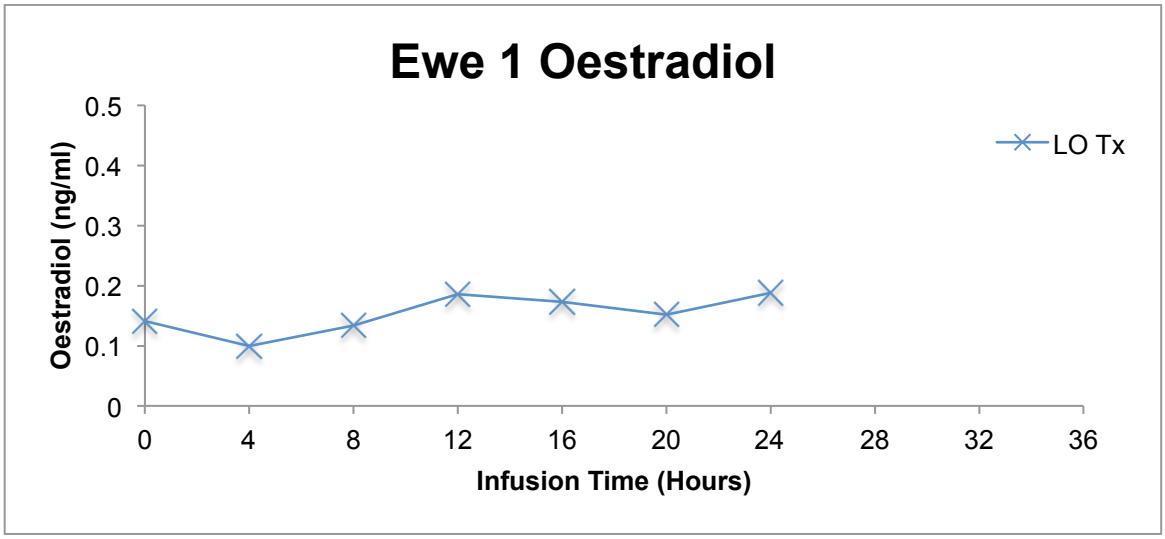

Figure 5.9. Ewe 1 oestradiol profile over 36 hours

(LO = Left ovary; Tx = Treatment)

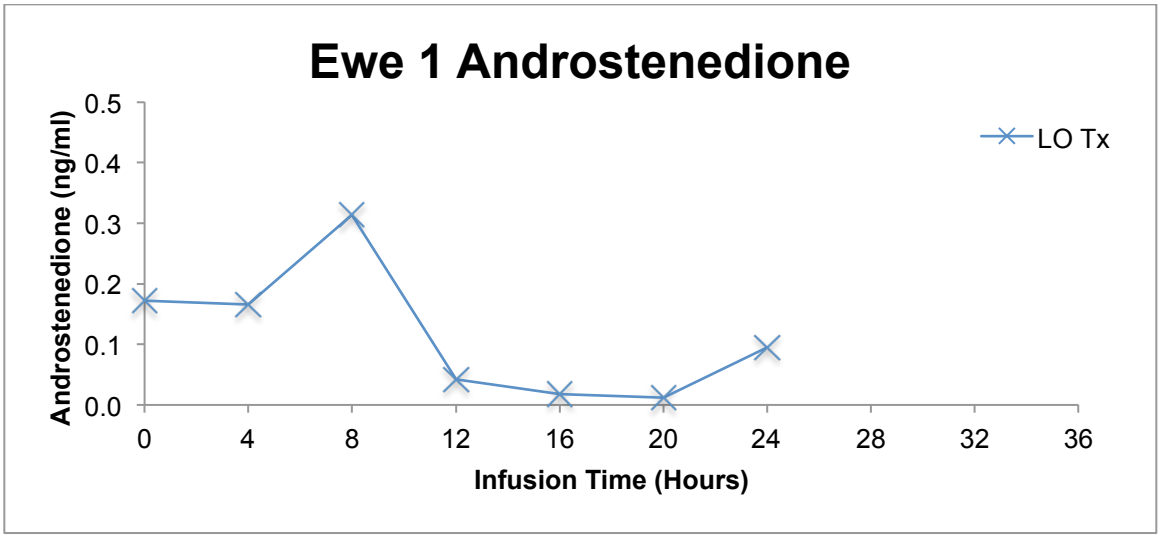

Figure 5.10. Ewe 1 androstenedione profile over 36 hours

As there were several large antral follicles on each ovary it would be expected that the granulosa cells of these follicles would be highly oestrogenic. However, immunohistological staining of ovarian sections from both ovaries revealed an absence of aromatase in the granulosa cells of these follicles, which would explain the low circulatory and ovarian venous concentrations of oestradiol. Androstenedione concentrations were also relatively low, therefore it is not certain whether the low oestrogenic capacity of the treated ovary was as a result of the antiserum treatment, or that steroidogenesis was limited by the availability of androgens to the granulosa cells.

The lack of a control ovary in this animal makes it relatively difficult to draw definitive conclusions, and the fact that the surgery for this animal had several complications associated with cannulating ovarian vasculature, however it seems that ultimately the infusion in this animal unfortunately did not work and that data derived from this animal is unreliable.

5.4.9. Ewe 2

The treatment infusion line of ewe 2, originally from the left ovary, was blocked after approximately 2 hours, and so the treatment was changed to the right side instead. Following post-mortem examination it was discovered that the line to the left ovary had detached from the cannula and as a result, this ovary was not perfused with control sera for an unknown period during the treatment infusion phase.

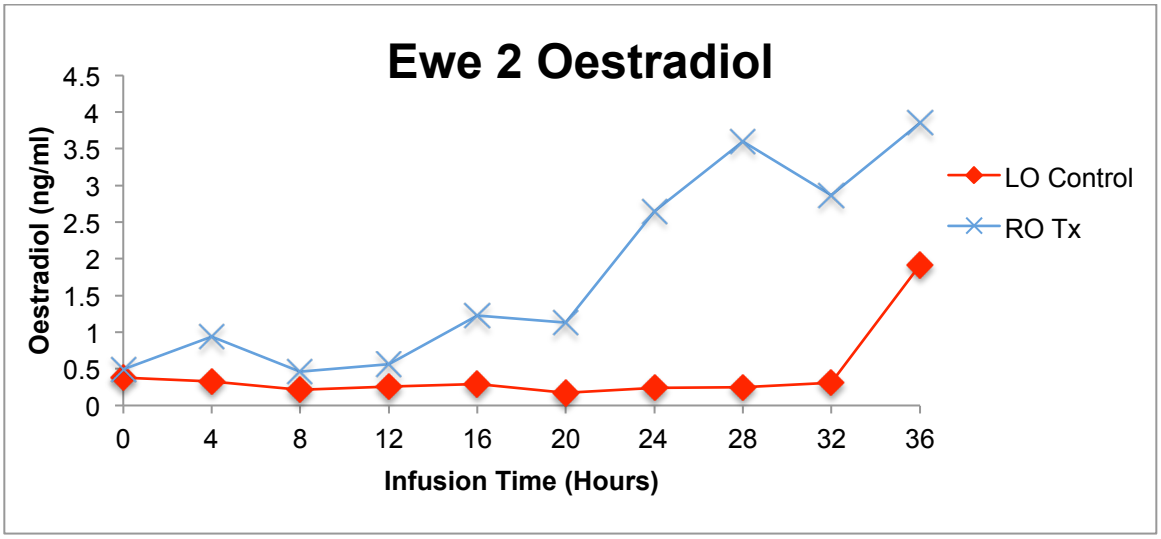

Figure 5.11. Ewe 2 oestradiol profile over 36 hours

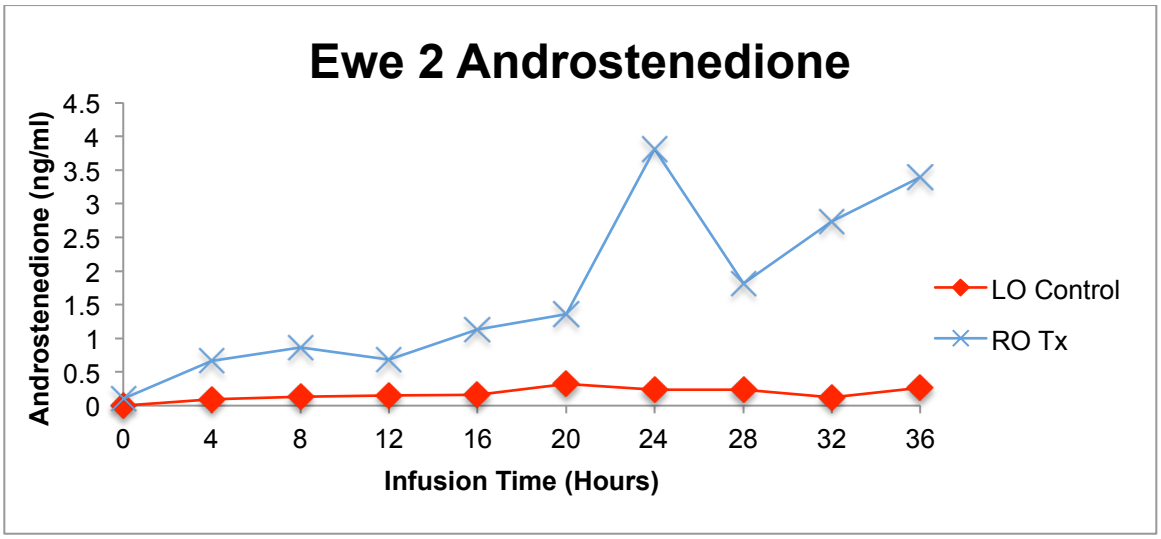

Figure 5.12. Ewe 2 androstenedione profile over 36 hours

Oestradiol concentration in the samples taken from the right ovary, which received the antiserum treatment, increased steadily over the 36 hours of sampling. The androstenedione profile also displayed this profile, which would explain the oestradiol increase.

Following immunohistochemical staining it was observed that the right ovary had a very large antral follicle with intense aromatase staining, which is consistent with the increased oestradiol concentration in the ovarian venous blood.

5.4.10. Ewe 3

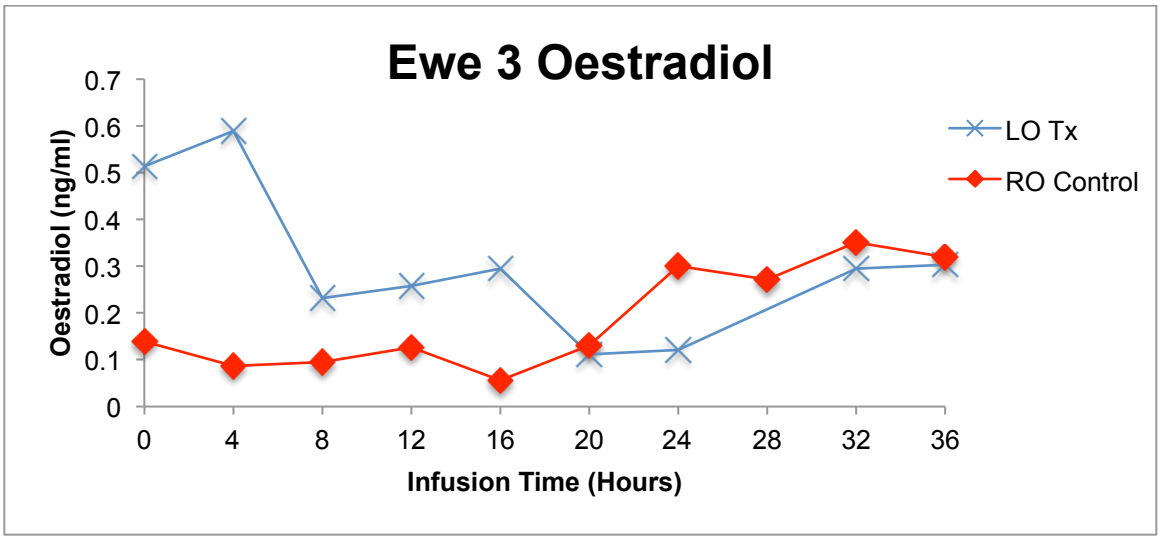

Figure 5.13. Ewe 3 oestradiol profile over 36 hours

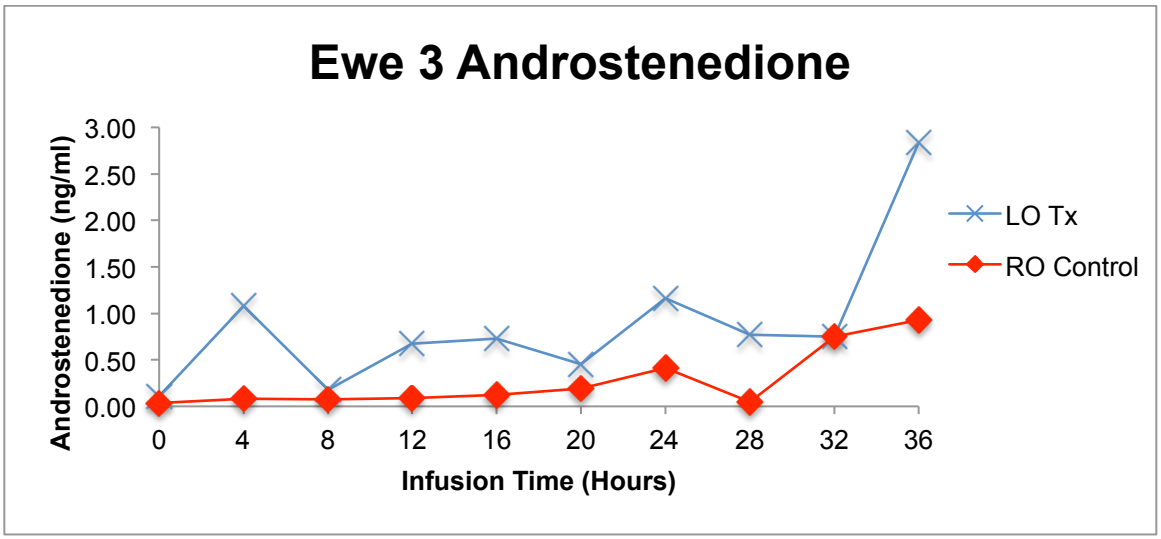

Figure 5.14. Ewe 3 androstenedione profile over 36 hours

The oestradiol profile of the treatment ovary decreased over the first 24 hours of infusion. The control ovary sample oestradiol increased gradually after 16 hours following infusion. Aromatase staining of ovarian sections was negative for the control ovary, and weakly positive for the treatment ovary.

5.4.11. Ewe 4

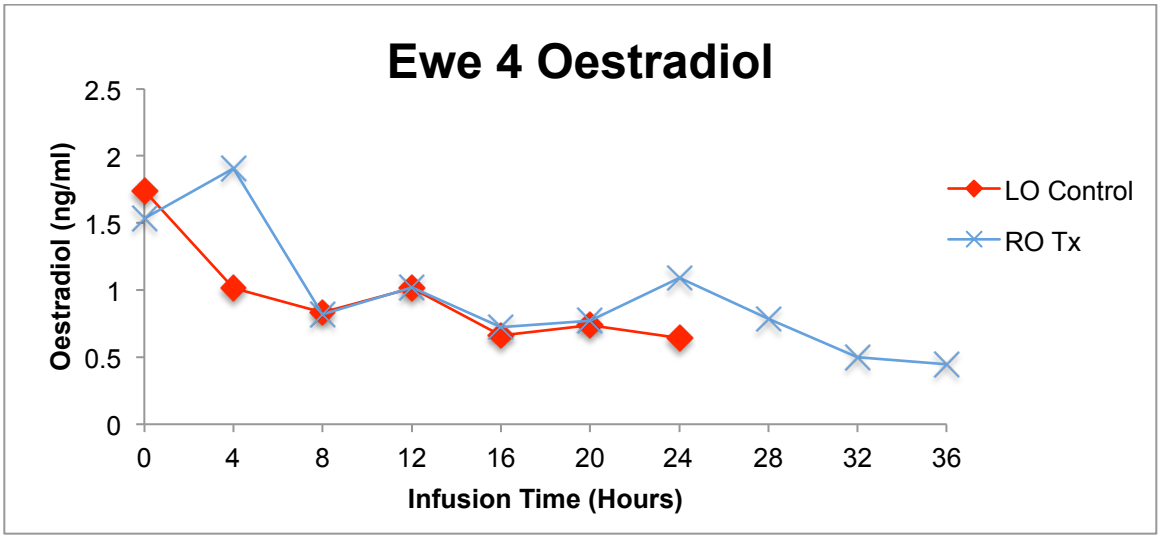

Figure 5.15. Ewe 4 oestradiol profile over 36 hours

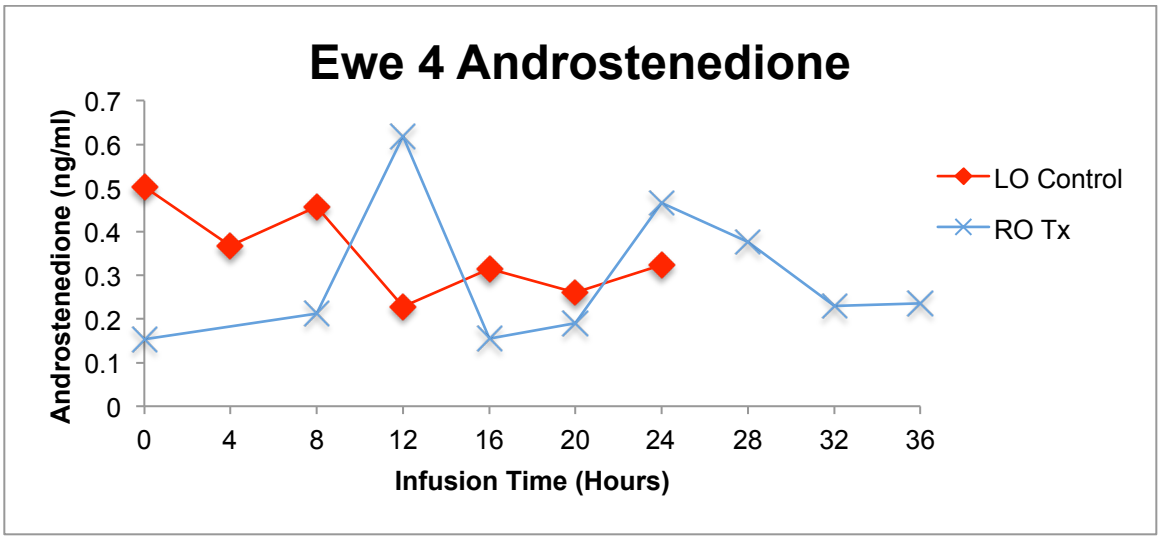

Figure 5.16. Ewe 4 androstenedione profile over 36 hours

Oestradiol levels in both ovaries decreased progressively over the infusion time course, which could have been as a result of the relatively low androstenedione concentrations.

Interestingly, although staining for aromatase was positive on large follicles from both the treatment and control ovaries, the staining on the large antral follicle in the treatment ovary was present in the granulosa cells located on one side of the follicle only. Upon closer examination of this follicle, it was observed that a very large blood vessel was present in between the theca cell layers at the opposite side to the aromatase positive cells (figure 5.17). The granulosa cells adjacent to this vessel did not stain for aromatase on any of the sections taken.

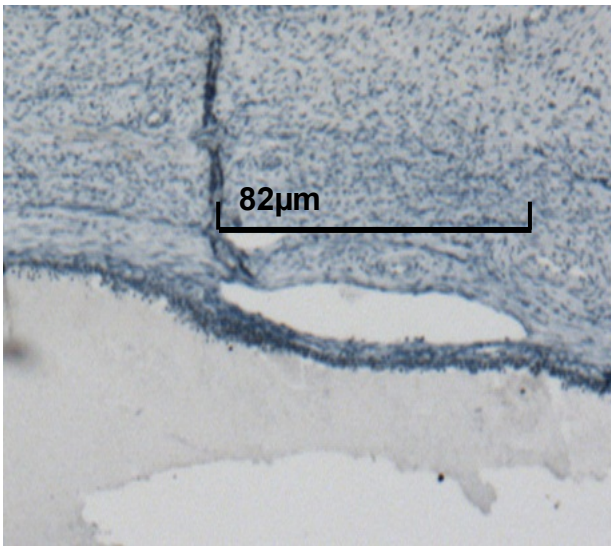

Figure 5.17. Major peri-follicular blood vessel on large antral follicle of ewe 4 treated ovary (x50)

5.4.12. Ewe 5

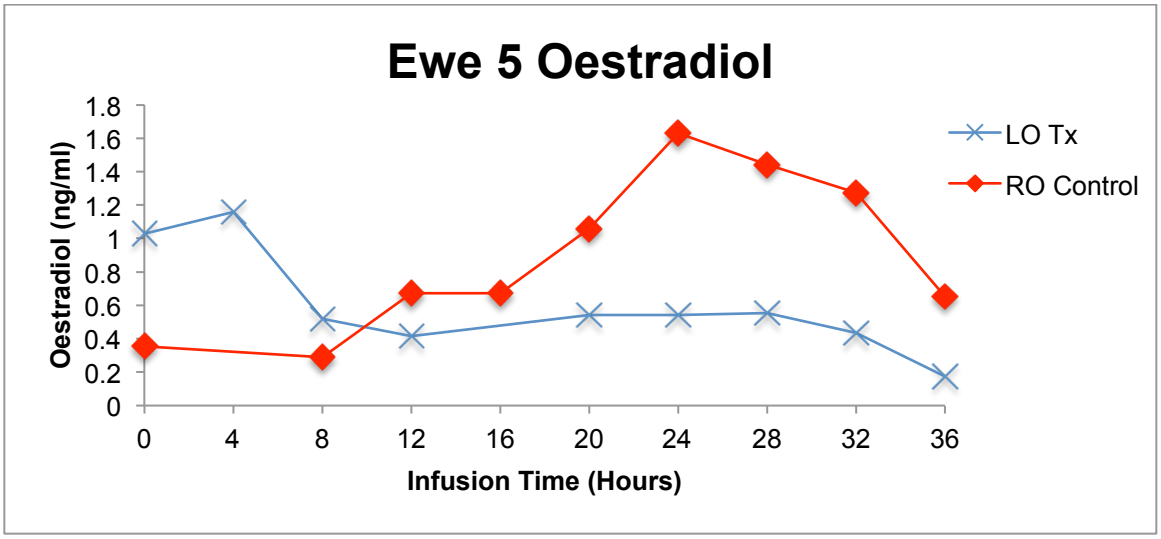

Figure 5.18. Ewe 5 oestradiol profile over 36 hours

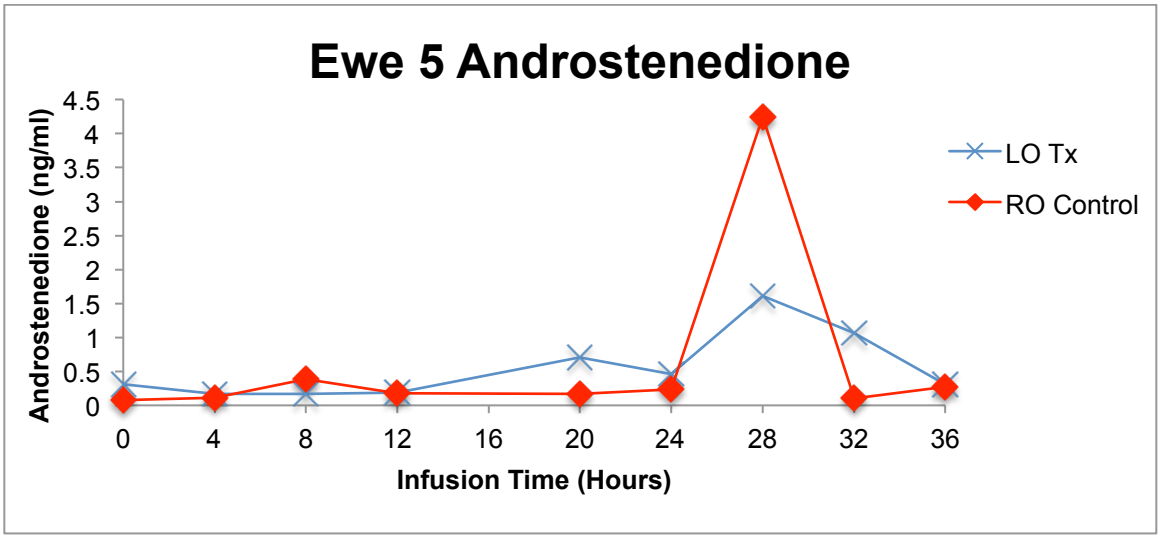

Figure 5.19. Ewe 5 androstenedione profile over 36 hours

Oestradiol concentration in the treatment ovary decreased over the first 8 hours of infusion, and remained at a low level similar to that of the main circulation, whereas the control ovary oestradiol concentration increased steadily from 8 hours of infusion.

Androstenedione concentrations were extremely low until 24 hours of infusion, when a peak in the ovarian blood samples was observed. The immunohistochemistry observations of these ovaries were somewhat conflicting, as aromatase staining was in fact negative in the control ovary, which had just one large antral follicle, whereas the treated ovary did show some slight staining on a small number of cells on a large antral follicle.

5.4.13. Ewe 6

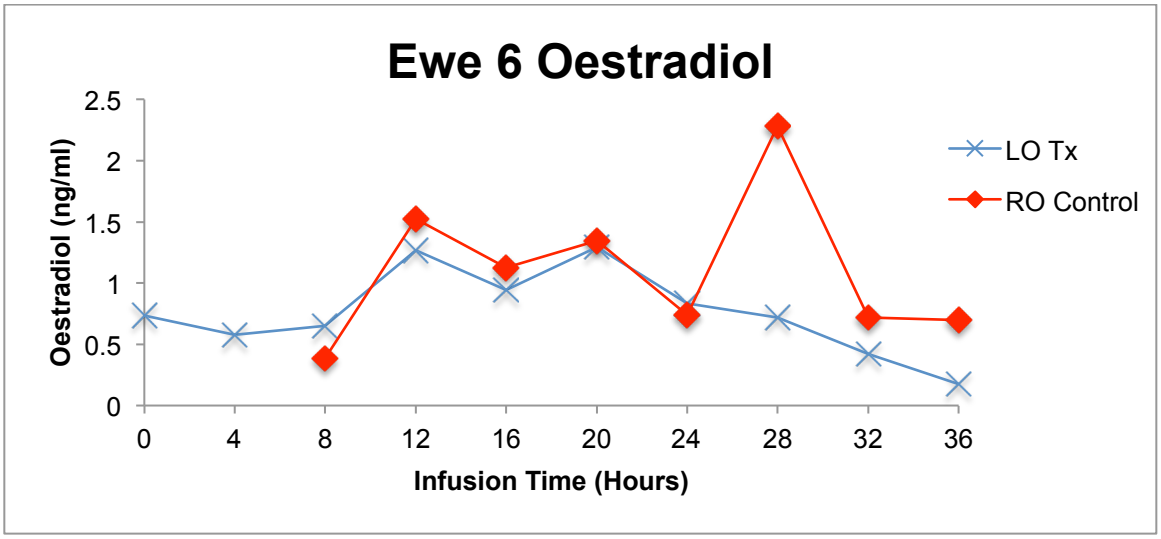

Figure 5.20. Ewe 6 oestradiol profile over 36 hours

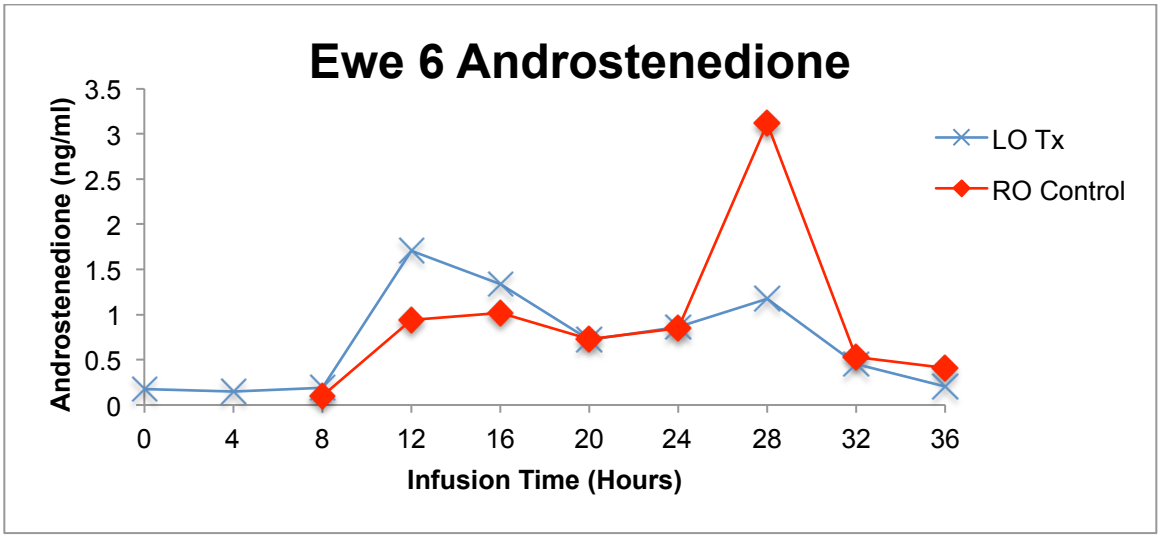

Figure 5.21. Ewe 6 androstenedione profile over 36 hours

Oestradiol production in ewe 6 was similar in the ovarian samples for the first 24 hours, after which the treated ovary production fell, while the control ovary production peaked dramatically. This result was also seen in the androstenedione secretion profile, which indicated a large peak in the control ovary at 28 hours.

Immunohistochemistry revealed positive staining for aromatase in both ovaries, although this was observed to be much weaker in the treated ovary, which had only one large follicle.

Mean data

The individual data summarised above indicates a large degree of inter-animal variation that has been somewhat compounded by technical issues with the *in situ* cannulation technique in some animals. Nevertheless, in four of the five animals in which large oestrogenic follicles were observed, there did appear to be a consistent decline in oestradiol secretion in treated ovaries (animals 3-6), which were secreting considerable amounts of oestradiol at the start of the treatment period, indicating the presence of an oestrogenic follicle at the start of the infusion in these individuals.

Figure 5.22 shows the mean ovarian oestradiol concentration for animals 3 to 6, for which oestradiol level started above 0.5ng/ml. Ewe 2 oestradiol is not included in the analysis as the converse profile of oestradiol was observed in this animal, suggesting recruitment of an oestrogenic follicle following luteal regression. Ewe 1 data was also excluded due to the lack of control.

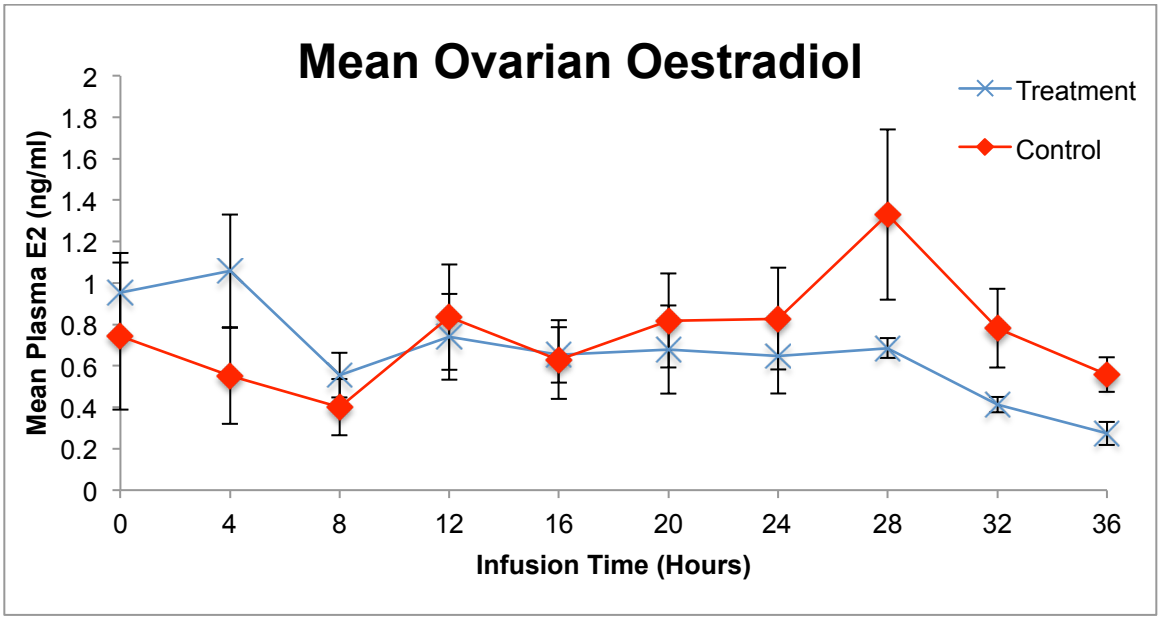

Figure 5.22. Mean ovarian oestradiol plasma concentration across all timepoints for ewes 3 to 6

Error bars indicate SEM.

The data was log transformed due to non-normality, and following repeated measured ANOVA in SPSS (Version 15, SPSS Inc), there was found to be no significant difference between mean oestradiol concentration in the ovarian venous blood in the treatment and control ovaries.

The data was then analysed in terms of change in oestradiol concentration from baseline (figure 5.23). Repeated measures ANOVA revealed a statistically significant suppressive treatment effect of BMP15/GDF9 antiserum on oestradiol production ($P<0.05$).

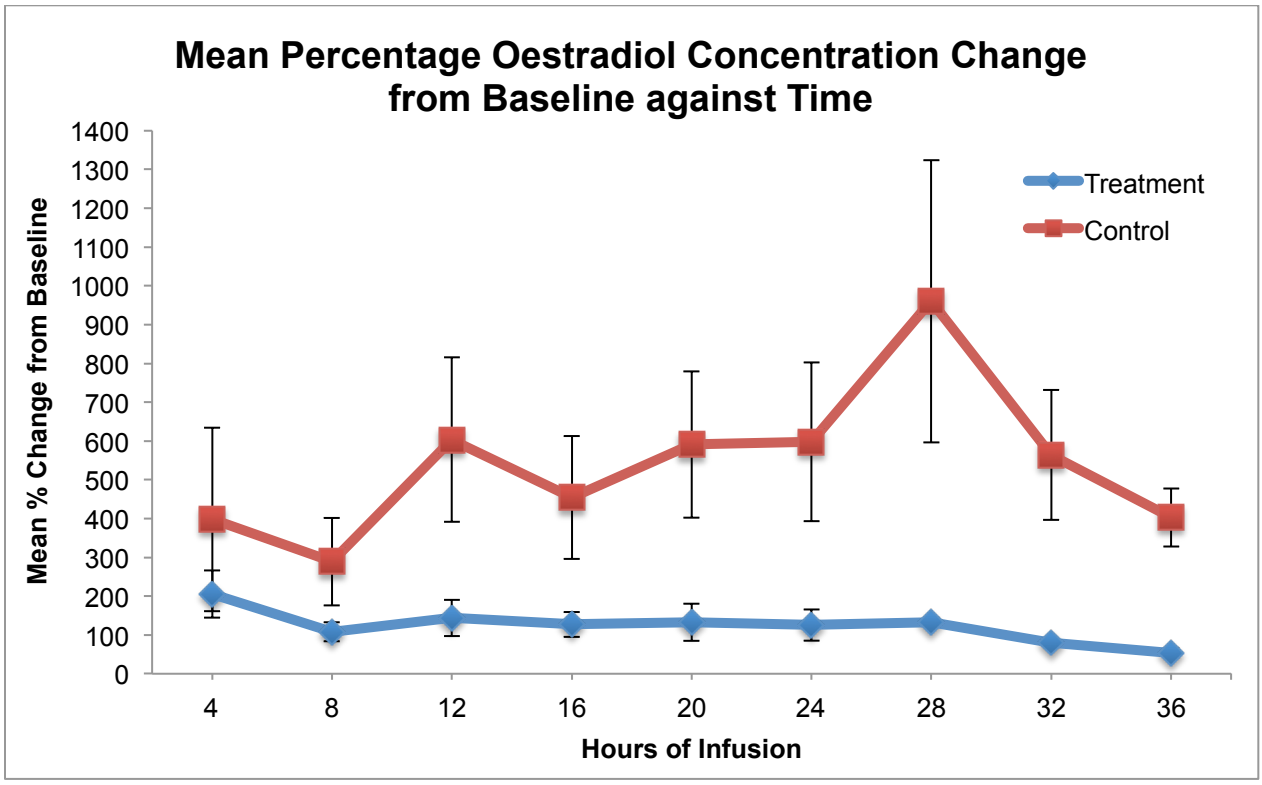

Figure 5.23. Mean percentage change in ovine serum oestradiol concentration from baseline against time in animals passively immune-neutralised against BMP15 and GDF9

Error bars indicate SEM.

5.5. Discussion

In this study it was hypothesised from the *in vitro* studies (Chapter 3) that the infusion of BMP15/GDF9 antiserum directly into the ovarian blood supply would cause a suppression of oestradiol secretion by the larger antral follicles, as the effect of these two factors secreted by the oocyte on the granulosa cells would be blocked.

Overall the hypothesised result was seen in 2 of the animals, variable results in 3 animals, and the converse effect seen in 1 animal; ewe 2 (table 5.3).

Ewe	Aromatase Staining	Endocrinology
1	Inconclusive	Positive
2	Negative	Negative
3	Negative/Ambiguous	Positive
4	Positive	Ambiguous
5	Negative/Ambiguous	Positive
6	Positive	Ambiguous

Table 5.3. Summary of aromatase staining and encrocrine profiles in relation to initial hypothesis.

Ewes 3 and 5 had clearly decreasing oestradiol levels with infusion of antiserum, suggesting that this was having a negative effect on steroidogenesis in these treated ovaries. This supports the hypothesis that passively immunization against BMP15 and GDF9 suppresses granulosa cell steroidogenesis. The endocrine results from ewe 1 concur with this, however no control was present which makes it difficult to draw definitive conclusions.

There was little difference between the treated and control ovaries of ewe 6, apart from a peak of oestradiol at 28 hours in the control, which was not observed in the treated ovary.

The results of ewe 2 were contradictory to the original hypothesis, as both oestradiol and androstenedione increased dramatically with the antiserum infusion. Furthermore, aromatase staining was only present in the treatment ovary. Although these results therefore do not support the original hypothesis, this is likely to be as a result of a follicle being recruited from the gonadotrophin-responsive follicles during the early follicular phase, as the oestradiol levels in this animal were initially low, but increased markedly during the treatment period. This scenario could be explained by the antiserum having no effect on the actual induction of aromatase, as opposed to the inhibitory effect on the maintenance of aromatase observed in other animals in which a large oestrogenic follicle was present at the time of luteal regression.

5.5.6. Follicle count

The ovaries that received BMP15/GDF9 antiserum infusion had fewer primordial follicles than those of the control ovaries, which, although not statistically significant, was observed in 5 of the 6 animals. As these two factors have previously been implicated in the initiation of primordial follicle growth (Martins *et al.*, 2008; Peng *et al.*, 2010; Kedem *et al.*, 2011), local passive immunization against these factors may have yielded this result by detrimentally affecting the primordial follicle pool. However, in a trial utilising such a short infusion period, it is unlikely that an effect on the primordial follicle population would be observed, and so this result could be simply down to coincidence.

Because follicle counts could not be performed before infusion, the use of one ovary of each animal as a control was the best comparison that could be made. Ideally a 'before and after' follicular assessment would be carried out in order to assess the effects of the infusion on the follicle population, perhaps by ultrasound, although this would, of course, be useful only in determining antral follicle numbers.

These results differ from those of an *in vivo* ovine study by Juengel *et al.* (2002a), in which passive immunization of GDF9 or BMP15 alone had marked effects on ovarian morphology. GDF9 antiserum caused alterations in follicular appearance, as the oocytes appeared much larger in size and had irregularly arranged granulosa cell layers. BMP immunization caused a dramatic reduction in antral follicle numbers, and was seen to decrease ovulation rate as a result. The study methodology, however, differed from ours in that the antisera were not used together, a large bolus of antisera was administered systemically, and the period of exposure was considerably longer. Our study used a much slower, direct infusion of combined BMP15/GDF9 antiserum, and the data was collected after 36 hours of treatment, and so major differences in results could be expected. Perhaps if our study was continued for a longer period of time then these effects may have been observed.

In a more recent *in vivo* study by Juengel *et al.* (2009) in cattle, active immunization again decreased antral follicle numbers and resulted in abnormally large oocytes. However, again the study period took place over a number of weeks, and so the effects could be observed over a longer timecourse.

5.5.7. Aromatase and oestradiol

As previously mentioned, the use of the second ovary as a comparative control was not ideal, as the physiological status of the ovaries would not necessarily have been the same before the infusion treatment began. This effect was most noticeable in the aromatase immunohistochemistry results, as due to the nature of the study, it was unclear which follicles had been previously oestrogenic or atretic and so determining the effect of the antiserum treatment is relatively difficult.

The expected result of negative (or weakly positive) antiserum-treated ovary staining and positive control staining was only seen in 2 of the animals; ewes 4 and 6. Interestingly, a large antral follicle in the treated ovary of ewe 4 had aromatase staining on only one side of the follicle wall, opposite a large blood vessel. This finding may indicate that this large peri-follicular blood vessel was delivering the blood supply to the follicle in such a way that the antiserum infusate had a local effect on suppressing aromatase activity on the granulosa cell layer adjacent to the vessel. A similar suppressive effect was seen in the ovaries of ewe 6, as only some slight staining was observed in granulosa cells of a follicle in the treatment ovary, compared to considerable positive staining in the control.

As before, ewe 2 exhibited a contradictory result to the proposed hypothesis, as the treatment ovary contained positively stained granulosa cells, whereas there was no staining present in the control ovary. It is possible that the very large oestrogenic follicle on the treatment ovary in this animal had become so dominant that any negative effect of antiserum treatment was unable to overcome steroidogenesis at the levels given. Also the infusate may not have been able to directly reach this large antral follicle, and if the hypothesis that the antiserum can only have a local effect is indeed true, the blood supply to this follicle may not have been directly from the canulated vessel, and so the exposure to infusate would have been minimal.

The aromatase results of ewes 1, 3 and 4 were not indicative of an effect of the antiserum infusion on aromatase expression, as in these animals the control ovaries did not show any staining for aromatase. Some staining was present in follicles from treated ovaries of ewes 3 and 5, however this was fairly weak and so a definitive conclusion is difficult to draw.

In summary, it seems that the hypothesised result of an antiserum knockdown of aromatase staining was observed in just 2 of the 6 animals. With regard to the other animals, aromatase staining was either inconclusive, or in one case, contradictory to the hypothesis.

The endocrine profiles of 3 of the animals supported the proposed hypothesis of a negative effect of the antiserum on oestradiol production. Ewes 1, 3 and 5 had decreasing oestradiol levels in the treated ovary (though ewe 1 did not have a control for comparison), indicating that the antiserum had a suppressive effect on steroidogenesis in these animals. There was no treatment effect observed in the endocrine profiles of ewes 4 and 6.

Ewe 2 was the only animal in which the hypothesis was not supported in both the aromatase immunohistochemistry and the oestradiol profile. As previously mentioned, this animal had a very low oestradiol level at the start of infusion, which increased steadily throughout the infusion period. This suggests that the antiserum may have been effective only in follicles which had cells already expressing aromatase, and that it would not have an inhibitory effect on aromatase expression induction.

Overall, the infusion was observed to have a significant treatment effect on ovarian oestradiol production, but due to the small number of animals further replication would be needed to confirm this effect, not least because an even smaller number of animals exhibited the expected endocrine response profile.

5.5.8. Limitations of the study

During the surgical procedures, it was noticed that none of the animals were found to have corpora lutea present on the ovaries, indicating that the animals were unexpectedly still seasonally anoestrous. Although the study took place at the end of January, in Edinburgh, it was probably as a result of being kept indoors these animals had not exhibited reproductive cycling. Clearly this was not ideal in terms of using these animals to determine the effect of the growth factors on reproductive physiology, but the impact of this complication was minimised by the imposition of a standard LH pulsatile stimulation regime designed to mimic the normal follicular phase that has been previously shown to be highly effective. Some conclusions can therefore still be drawn from the study.

If this study were to be repeated, it might yield more consistent results if down regulation with GnRH agonist/antagonist and ovarian stimulation were to be used; this would allow complete control over the animals' reproductive cycle, thereby bypassing the issues associated with seasonality in this breed.

The experimental design employed was designed to allow each animal to be used as its own control, but one drawback of this design is that the control ovary used in each case may not necessarily be the the same physiologically as the treatment ovary. For example, larger, more oestrogenic follicles may be present on one ovary than the other, which would have affected the endocrine profiles obtained from the ovarian blood samples. This problem is more likely to arise in breeds of sheep with lower ovulation rates, and the only way to overcome this issue in the Scottish Blackface animals used in this study would have been to use more animals, and clearly this study needs to be repeated to increase the sample number.

In conclusion, this pilot study has provided some preliminary data to suggest that BMP15/GDF9 may positively regulate aromatase activity *in vivo*, as infusion of BMP15/GDF9 antiserum had a knockdown effect on aromatase and oestradiol production, however it is evident that further experimentation is required to confirm and investigate this finding in more detail.

Chapter 6 – Ultrasound phantom studies

6.1. Introduction

Technology for evaluating blood flow to and around the ovary has progressed rapidly over recent years, which has proven extremely valuable both in terms of research on ovarian blood flow from a scientific point of view, and also improving patient treatment regime by facilitating ovarian monitoring by the clinician.

This new technology has enabled measurement of blood flow around individual follicles, as the technique is highly sensitive, however the indices given by the ultrasound machine are in arbitrary units that have not yet been correlated to known parameters such as speed of blood flow (Deane, 2001). As a result, the relevance of these measurements is not yet fully understood. It seemed appropriate, therefore, to utilise a physiologically relevant phantom model in order to clarify the relevance and also the accuracy of these measurements, thereby qualifying its use as both a research and clinical tool.

The basis for the model is that bovine ovaries can be cannulated and blood passed through the ovarian artery at a determined flow rate. The ultrasound scan Doppler information can then be compared to the blood flow data in order to determine quantification of the vascular indices given by the machine.

6.2. Blood flow phantom models

The combined utilisation of three-dimensional (3D) ultrasound alongside power Doppler enables the quantification of blood movement within a defined area of interest (Pairleitner *et al.*, 1999), and hence is a valuable tool for studying blood flow in the ovary. The technique is completely non-invasive and safe as there is no requirement for contrast agents or radiation exposure (Raine-Fenning *et al.*, 2008b).

Three indices of vascularity and blood flow are given by power Doppler analysis; Flow index (FI) represents the mean power Doppler signal intensity from all of the coloured voxels, vascular index (VI) is the ratio of coloured voxels to total voxels in the volume of interest (expressed as a percentage), and vascular flow index (VFI) is a combination of the two multiplied by 100 (Pairleitner *et al.*, 1999). FI is thought to signify the intensity of the blood flow and VI the number of vessels within the volume of interest, and by combining them an overall indication of vascularity and blood flow can be observed.

These vascular indices given by the software are arbitrary in terms of units, and hence their relationship to actual blood flow and vascularisation is not currently known. If the technique is to be used effectively in a clinical setting then this issue must be addressed (Raine-Fenning *et al.*, 2008b). This study was designed to investigate this relationship, based on a phantom blood flow model using bovine reproductive tracts. Phantom models to investigate ultrasound measurement have been performed a number of times before (Rubin *et al.*, 1995; Guo and Fenster, 1996; Nilsson *et al.*, 1997; Cloutier *et al.*, 2000; Raine-Fenning *et al.*, 2008b), however these have all been based on utilisation of submerged tubing, with relatively high flow rates. It is somewhat difficult to set up a model with physiologically low flow rates due to the sensitivity to attenuation, which causes high amounts of artifact. Schulten-Wijman *et al.* (2008) even went as far as to say that power Doppler cannot currently be used for measuring blood flow in small blood vessels due to poor sensitivity causing overestimation. However, this group used a blood-mimicking fluid pumped through polyurethane tubing, and scanning was performed through a liquid barrier, making the whole set-up somewhat artificial. By using a more physiological approach, we hoped to investigate the power Doppler measurement of blood flow in a phantom model.

6.2.1. Aim

To investigate the relationship between power Doppler ovarian vascular indices measured in clinical IVF, and true flow parameters using an *in vitro* phantom model by perfusing bovine ovaries with blood under differing flow rates. It is not currently known how these vascular indices presented by power Doppler analysis actually relate to blood flow, and so this experiment was designed to mimic ovarian vascular dynamics under controlled conditions. A bovine model was chosen to due ovarian morphological similarities to the human.

6.2.2. Method

Bovine reproductive tracts were collected from the abattoir and those with ovaries containing a large follicle and a corpus luteum were selected for use, as these would be expected to have a suitable degree of vasculature to allow detectable blood flow.

The ovarian artery on each side of the reproductive tract was cannulated and secured in place with suturing thread.

Human red blood cells were obtained from the blood transfusion department of Clinical Pathology at the Queens Medical Centre. This solution was centrifuged and resuspended in PBS to obtain a physiological packed cell volume (PCV) of approximately 50%. As it is the red blood cell portion of the blood that is detected by power Doppler ultrasound (Missaridis and Shung, 1999), this would serve as an accurate representation of whole blood for ultrasonic measurement of blood flow indices.

After a small number of replications of the experiment it was found that using human blood in the phantom model was unsustainable due to variable availability from the transfusion unit, and therefore the source was consequently changed to bovine whole blood, as this was more readily available. Analysis was performed separately to avoid possible discrepancies between blood types.

Excess whole bovine blood was obtained from a bovine pathology laboratory (NUVetNA Diagnostic Services, Nottingham), and used at a PCV of approximately 50%. Normal human parameters for packed cell volume are between 36% and 54% (Billett, 1990) so 50% PCV was chosen for simplicity.

The blood was perfused at various flow velocities into the ovaries using a syringe attached to a syringe pump (Precidor by Infors-AG, Basel, Switzerland), as shown in figure 6.1. The ovaries were covered with a tissue barrier of bovine uterus in order to move the ultrasound probe off the surface of the ovary, and then scanned in 3D using a transvaginal probe on a Voluson E8 Expert ultrasound machine (GE Medical Systems, Kretz, Austria). The VOCAL tool on the 4D software was used as previously described, with 15° rotations drawn in the B plane.

Statistical analysis was performed using SPSS (Version 15, SPSS Inc). R^2 values were used to assess the data goodness of fit and P values to determine the correlation between the perfusion flow rates and the ultrasound vascular indices.

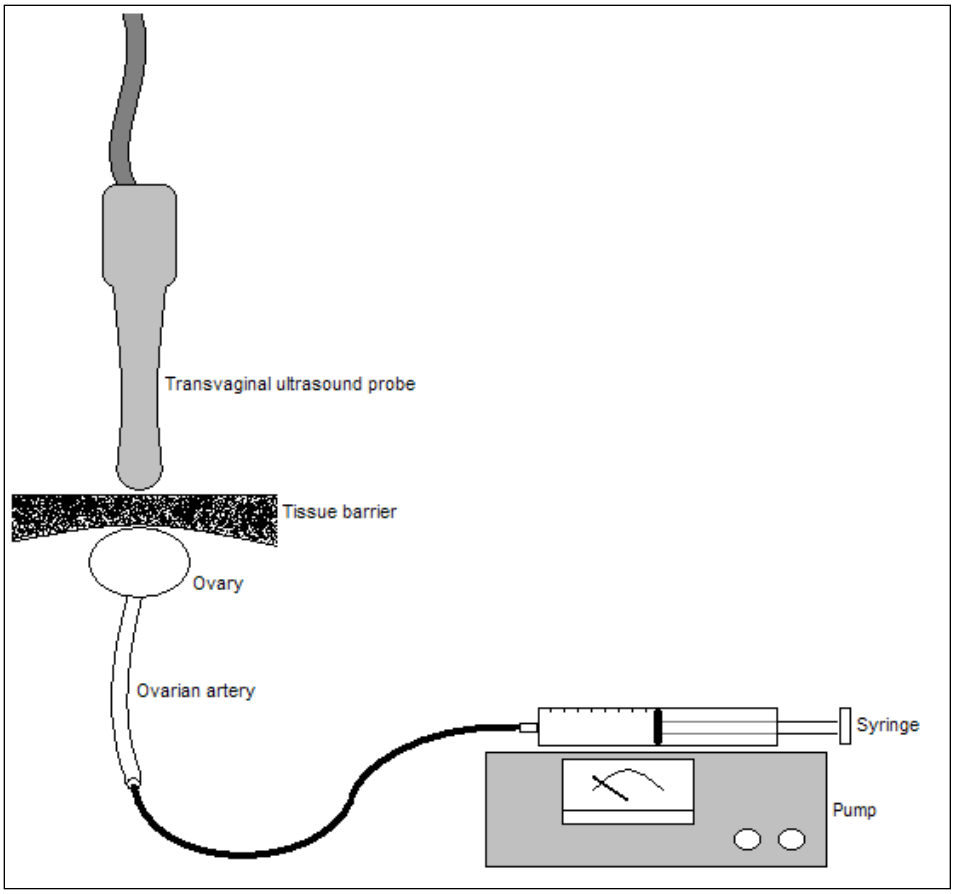

Figure 6.1. Ultrasound set-up for the measurement of blood flow in a bovine ovary at different flow rates

6.2.3. Results

Blood Type	Infusion Rate (ml/min)	n	FI ± SEM	VI ± SEM	VFI ± SEM
Human	0.75	3	19.588 ± 0.40	0.056 ± 0.04	0.011 ± 0.01
	1.5	5	25.123 ± 2.84	0.186 ± 0.06	0.054 ± 0.02
	2.25	2	20.222 ± 1.25	0.782 ± 0.71	0.167 ± 0.15
	3	4	27.320 ± 2.76	0.257 ± 0.12	0.080 ± 0.04
	3.75	1	22.483 ± 0.00	0.05 ± 0.00	0.011 ± 0.00
	4.5	4	34.008 ± 4.21	0.642 ± 0.29	0.253 ± 0.13
	5.25	1	36.258 ± 0.00	0.191 ± 0.00	0.069 ± 0.00
	6	3	30.434 ± 1.54	0.595 ± 0.31	0.300 ± 0.06
Bovine	0.75	11	20.464 ± 1.10	0.475 ± 0.19	0.095 ± 0.04
	1.5	11	21.259 ± 1.17	0.495 ± 0.20	0.124 ± 0.05
	2.25	9	23.627 ± 1.43	0.658 ± 0.32	0.179 ± 0.10
	3	8	23.239 ± 1.49	1.65 ± 0.43	0.410 ± 0.11
	3.75	9	26.451 ± 1.36	1.81 ± 0.61	0.485 ± 0.18
	4.5	10	26.886 ± 1.26	1.963 ± 0.45	0.537 ± 0.14
	5.25	9	26.992 ± 1.58	3.935 ± 1.26	1.124 ± 0.38
	6	9	26.379 ± 1.83	4.633 ± 1.21	1.148 ± 0.32

Table 6.1. Flow index, vascular index and vascular flow index for 8 defined infusion rates using human and bovine blood.

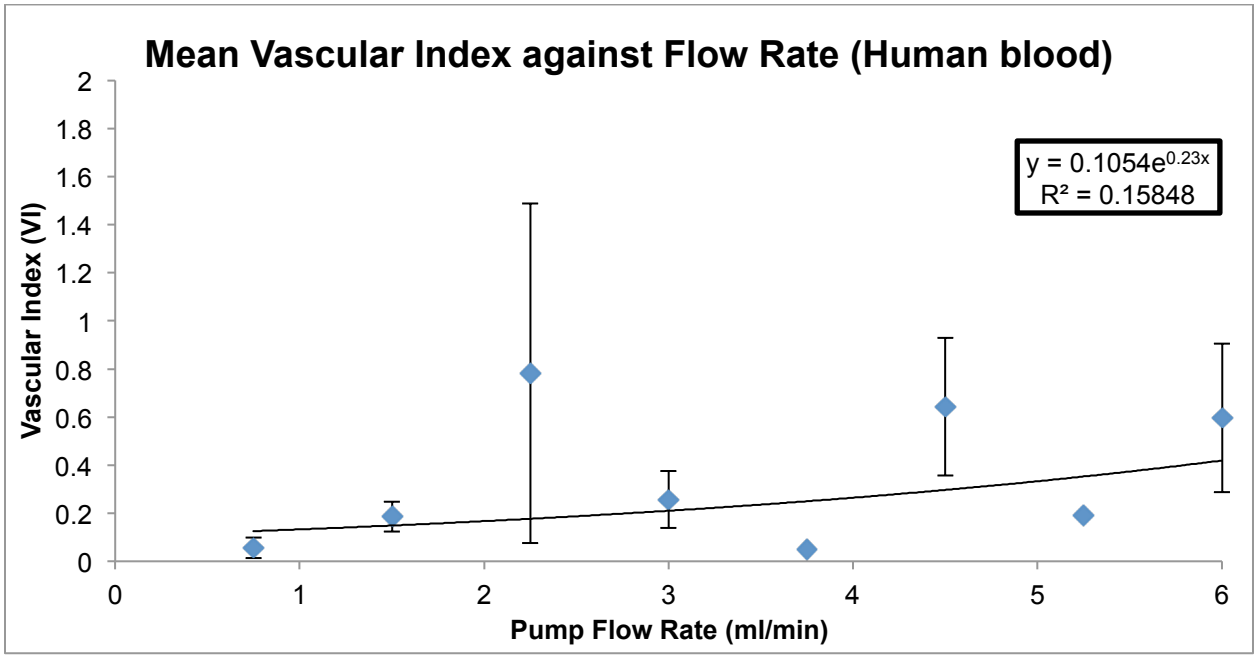

Figure 6.1. Mean vascular index against flow rate using human blood

Error bars indicate SEM

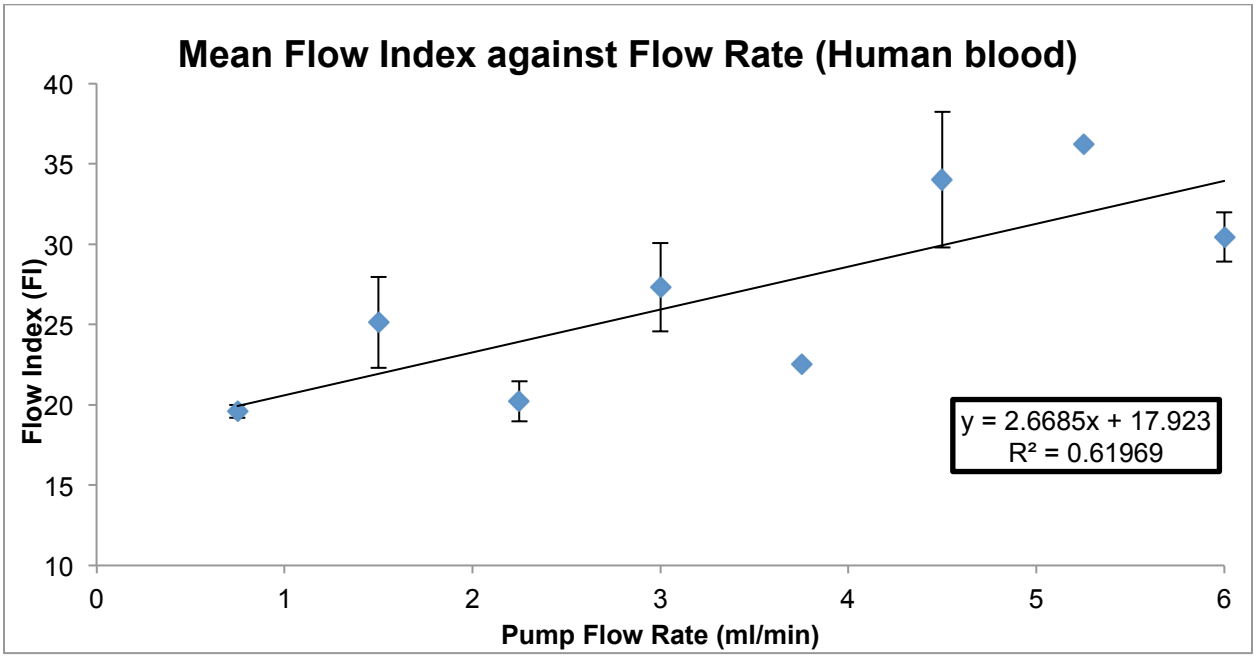

Figure 6.2. Mean flow index against flow rate using human blood

Error bars indicate SEM

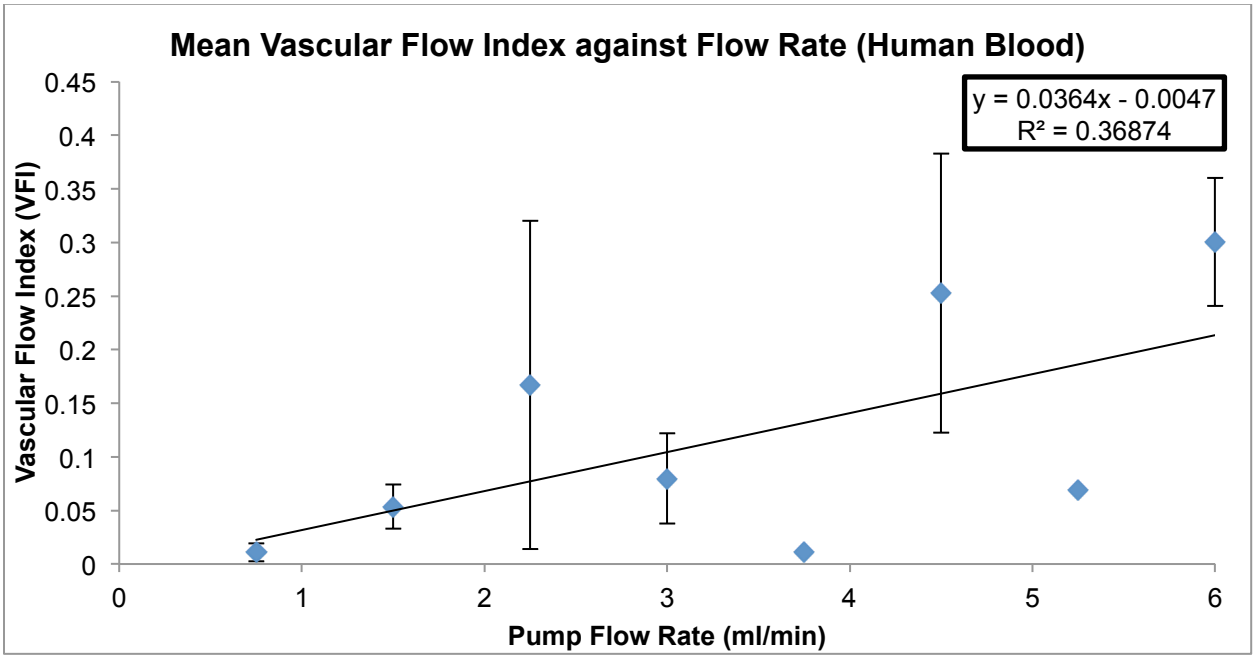

Figure 6.3. Mean vascular flow index against flow rate using human blood

Error bars indicate SEM

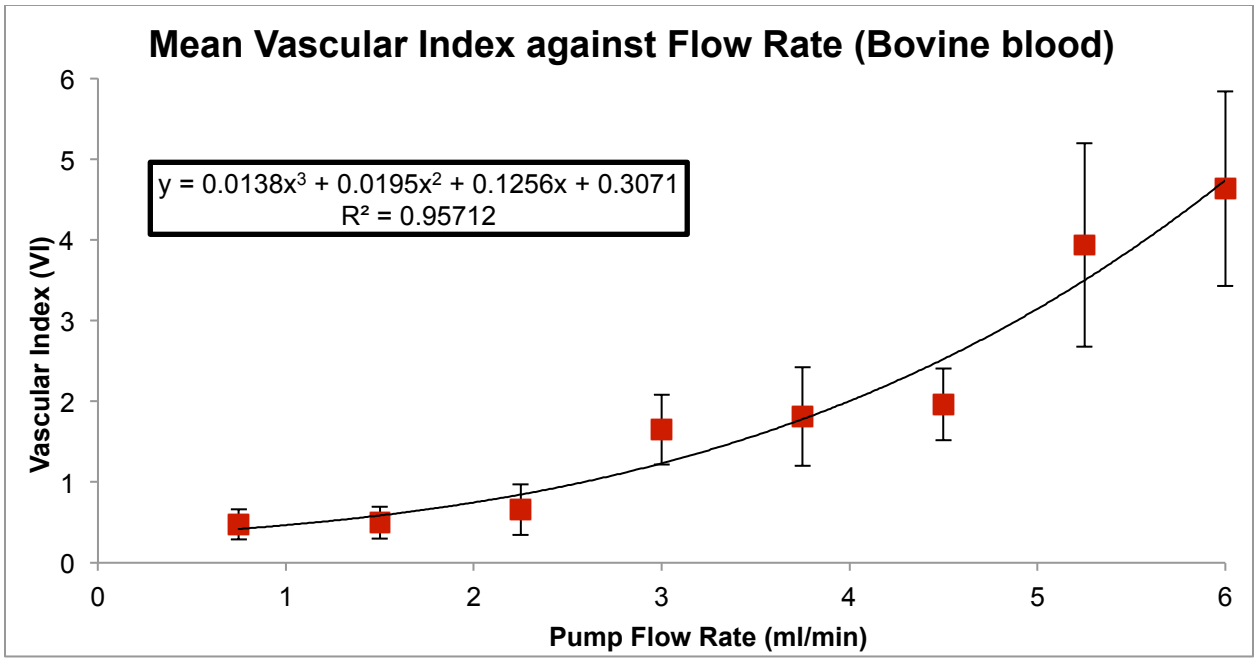

Figure 6.4. Mean vascular index against flow rate using bovine blood

Error bars indicate SEM

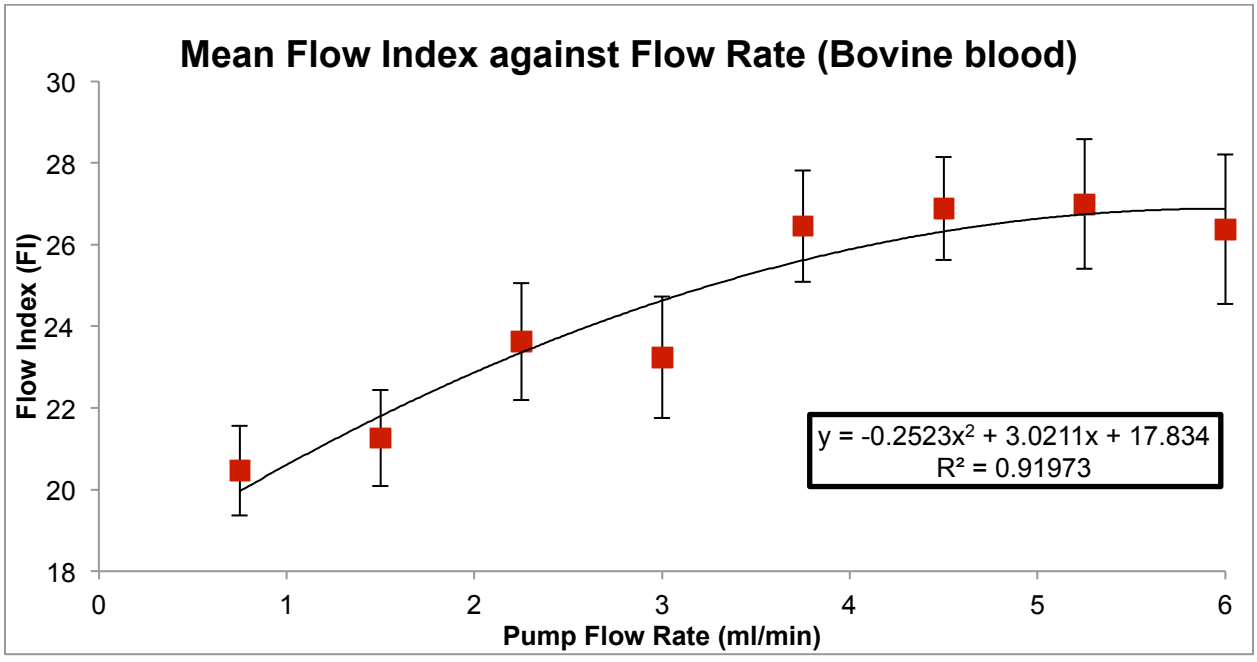

Figure 6.5. Mean flow index against flow rate using bovine blood

Error bars indicate SEM

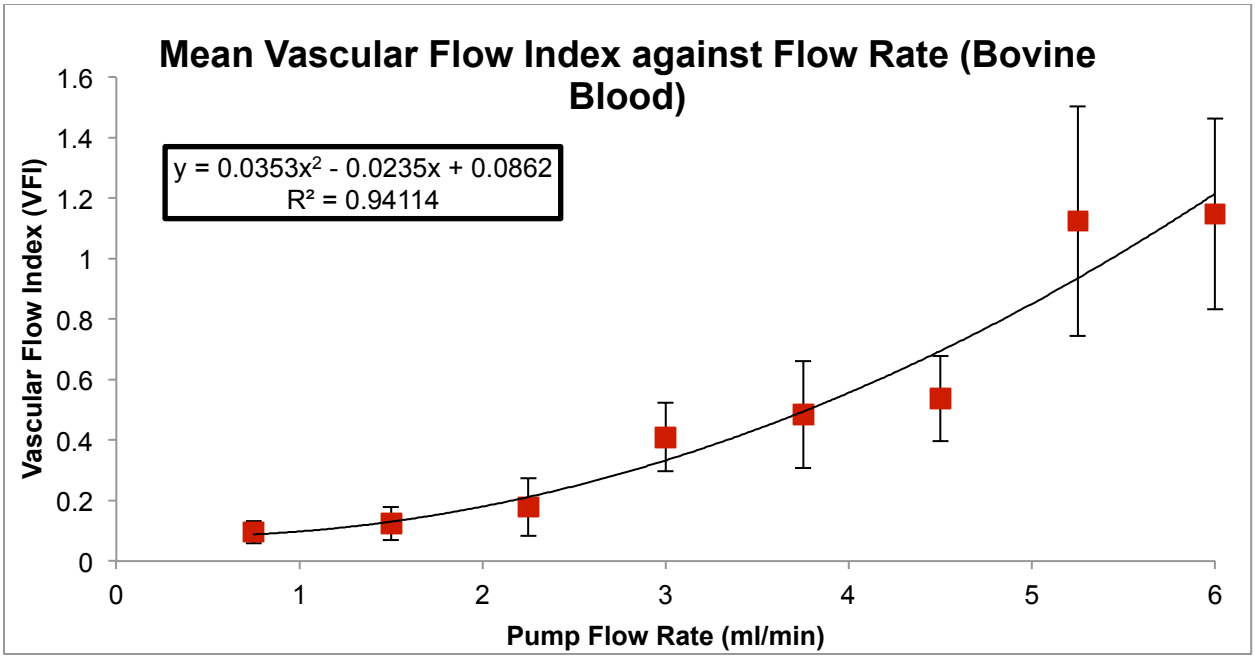

Figure 6.6. Mean vascular flow index against flow rate using bovine blood

Error bars indicate SEM

Following statistical analysis a significantly positive correlation was found between infusion rate and the ultrasound measured vascular indices VI, FI and VFI ($P<0.001$).

6.2.4. Discussion

This study revealed complex relationships between the controlled flow rate and the flow index and vascular index using a bovine blood phantom model.

Analysis of the vascular index against flow rate indicated a best fit obtained with a third order polynomial relationship ($P<0.01$). The VI is thought to represent the number of vessels in the volume of interest (Guimarães Filho *et al.*, 2008), however as VI increased with flow rate, this cannot be the case, as the number of vessels did not change. One possible explanation is that as the flow rate increased, the increment in blood pressure could have caused the blood vessels to dilate and hence smaller vessels became detectable at higher flow rates. This is unlikely to be the primary cause, however, as another study also found an increase in VI with flow rate using tubing rather than physiological tissue vessels (Raine-Fenning *et al.*, 2008b).

The observed complex third order polynomial (cubic) relationship between VI and flow rate differs from that of Jones *et al.* (2009), who conducted a phantom study based on a placental model, which found flow rates to relate to VI in a linear relationship. However, the study included flow rates up to 10ml/min, and used haematocrits of 0.5% and 5%, which may explain the discrepancies between our studies. Power Doppler indices have been shown to be significantly affected under haematocrits of 26%, and so these low haematocrits used will differ to normal physiological conditions, and indeed the results presented in our study (Wu and Shung, 1999). In addition it is important to note that human haematocrit is 36-48% in women and 40.7-54% in men (Dacie and Lewis, 1995), and so it could be argued that our study was more physiologically applicable.

In a study published by Hope *et al.* (2009), the mean VI observed was 12, mean FI 39 and mean VFI 5.1 (n=368), compared to the ultrasound parameters measured in this experiment peaking at 4.5, 27 and 1.2 respectively. This indicates that the perfused flow rates used in our particular set-up could have been somewhat lower than those *in vivo*, and therefore this experiment may not have strictly replicated *in vivo* ovarian blood flow in the human. Unfortunately the nature of this set-up was that the maximum flow rates that could have been used were performed with the equipment available. Furthermore, the perfusion cannulae in the ovaries would not have been able to sustain the flow rates that would have been required to mimic human *in vivo* conditions, as although the ovaries were perfused as quickly as possible, inevitably there would have been some blocking of ovarian blood vessels. However, even though the flow rates had to be restricted in this experiment, the relationship between flow rate and ultrasound parameters of blood flow could be demonstrated.

The best fit relationship between measured flow index and flow rate was found to be a second order polynomial (quadtratic) regression ($P<0.01$), which appeared to reach a plateau at a flow rate of approximately 5ml/min. As a result, it seems that flow rate is a relatively poor indicator of blood flow, which has also been the conclusion reached by some other studies (Dubiel *et al.*, 2006; Jones *et al.*, 2009).

The VFI, obtained by multiplying the VI and FI together, was also related to flow rate in a non-linear correlation, with the line of best fit following a quadratic regression ($P<0.01$). This would be expected as it

would be likely that the combination of the VI and FI would result in a similar relationship to the components, and hence this does not elucidate any additional information concerning flow rate.

Overall it seems that vascular index is the measure most reflective of flow rate, although the relationship is relatively complex and non-linear, and also that flow index has limited as a flow rate indicator, and as a result should be interpreted with caution. It would seem pertinent to interchange the names of these parameters, as in actual fact, in this study at least, VI reflects blood flow, and FI may better reflect the vascularity.

On a number of the ultrasound images there was a significant degree of power Doppler artifact at analysis, which rendered the scans unusable as it affects the blood flow measurement considerably, and cannot be removed post-scanning. This artifact appears as striated areas of colour, perpendicular to the ultrasound probe, which is interpreted by the software as being blood movement.

Power Doppler artifact was particularly noticeable on the scans on ovaries with a dense corpus luteum (figure 6.7). This is likely to be as a result of the additional artifact from the ultrasound itself, as a result of attenuation by the very dense tissue, causing the whole dataset to become subject to misinterpretation by the software. Because of the relatively small volume of interest required to analyse blood flow in the ovary, even minor differences in tissue density, probe depth and tissue barrier characteristics can significantly affect the ultrasound image acquired, and hence the consequent analysis.

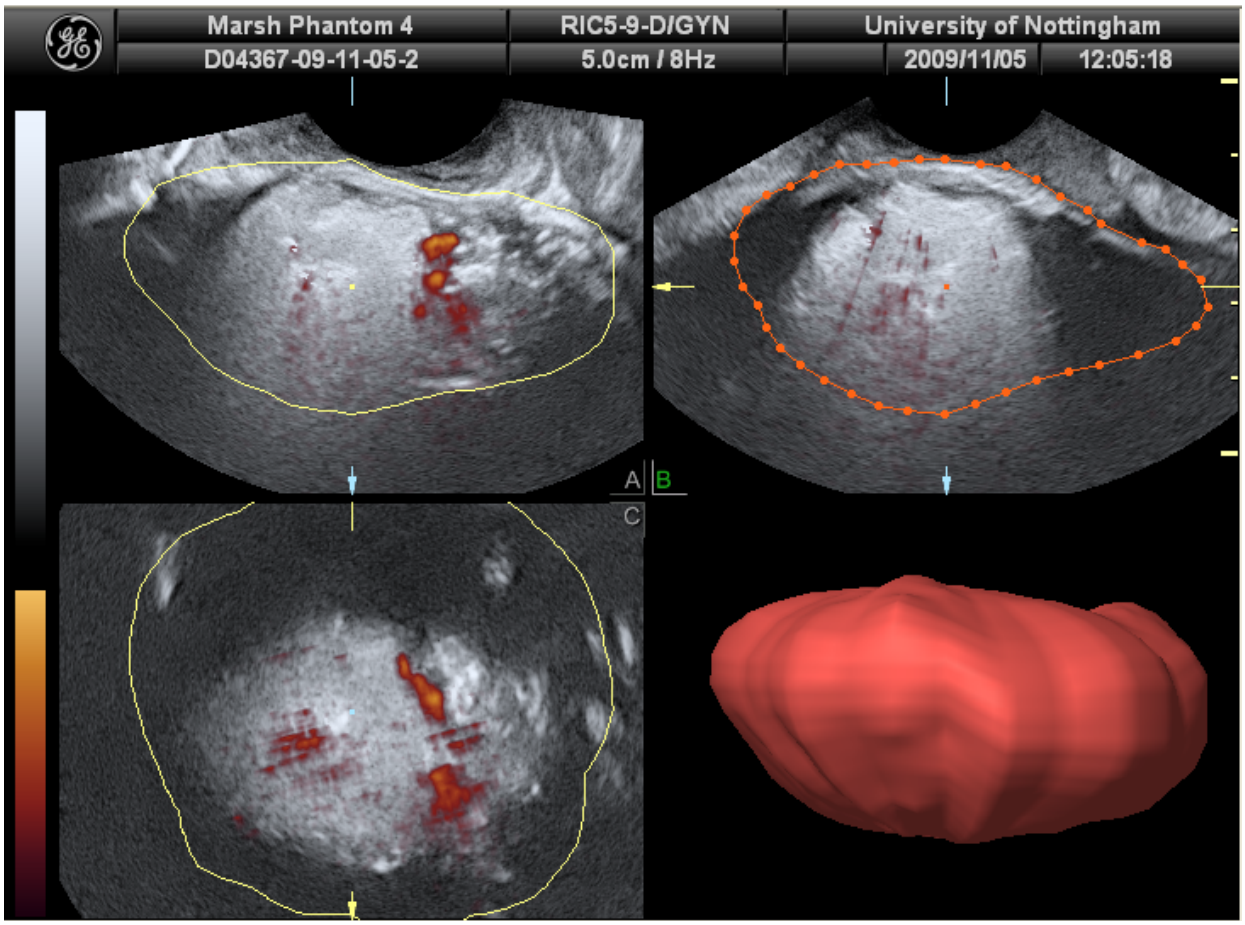

Figure 6.7. Visible artefact on the ultrasound scan image

The artefact can be seen in planes B and C on the image as red striated areas, which cannot be excluded from the image for analysis once the scan has been performed.

In addition it is important to note that due to the sensitivity of the technique, any movement of the probe during image acquisition, however slight, will cause a certain degree of attenuation of the ultrasound signal, potentially resulting in image artefact. A clinician performed this study by hand, and although the individual is extremely experienced in the field, it is physically impossible to hold the transducer absolutely stationary for the duration of these procedures. It was decided to utilise human input for holding the probe because the study needed to be as physiologically relevant as possible, and as in a clinical setting ultrasound scanning is performed by clinicians, it was deemed suitable to mimic these conditions.

Although a certain amount of artifact is expected, and considered a normal part of the scanning process in human patients, only scans with a very small amount of artifact were included in the study in order to determine the relationship to true flow.

One drawback of the phantom method used in this experiment was that the pump used produced a continuous flow, which obviously is not a truly accurate reflection of physiological blood flow *in vivo*. Pumps that mimic circulatory pulsatility are available, and could be used for *in vitro* phantom systems (Raine-Fenning *et al.*, 2008b), however it was decided that a simple continuous flow would be more appropriate for the purpose of ascertaining the correlation of blood flow to ultrasound vascular indices.

In addition, whilst this study was able to show a relationship between flow rate and the ultrasound generated vascular indices, the results cannot necessarily be directly extrapolated to an *in vivo* setting, as additional external factors such as depth of the volume of interest come into effect (Jones *et al.*, 2009).

6.3. SonoAVC™

SonoAVC™ software (sonographic automated volume count) (GE Medical Systems, Kretz, Austria), as the name suggests, is designed to automatically count and measure ovarian follicles from a 3D ultrasound scan. Ultrasound ovarian follicle counting and developmental monitoring is a well established and useful tool used to indicate ovarian reserve in addition to monitoring ovarian response to stimulation (Ritchie, 1985), and the novel 3D technique is proving to be an important development in the process, due to increased reliability and a decreased time requirement (Murtinger *et al.*, 2009; Raine-Fenning *et al.*, 2009b). Follicular volume and diameter can also be measured using this technique, which is of value as follicle size is considered to be a key indicator of oocyte development and maturity (Raine-Fenning *et al.*, 2008a).

Follicles appear as dark areas on the ultrasound image, as they are fluid-filled and hence low echoic regions that are fairly easy to distinguish in the ovarian tissue. As a result, the technique is reported to be very easy to perform, with good reproducibility and reliability (Salama *et al.*, 2009).

It is thought that because of the way in which SonoAVC™ measures volume in objects of interest in ultrasound scanning, it may systematically underestimate the volume of the object in question (Rousian *et al.*, 2008; Raine-Fenning *et al.*, 2009a). This is because it is only able to draw around the inside of the hypoechoic space, thereby excluding the ovarian follicle wall. As a result, the extent of this underestimation was examined.

6.3.1. SonoAVC measurement of follicular size

Previously, ultrasound assessment of ovarian follicles for the purpose of monitoring ovarian stimulation during ovulation induction or IVF treatment has been performed using two-dimensional ultrasound, with the aim of measuring follicular size and development (Hackeloer *et al.*, 1979). This method was based on the mean of two linear measures of follicular diameter, as described by Haadsma *et al.* (2007), however due to the irregularly-shaped nature of ovarian follicles (Ritchie, 1985; Rodrígues-Fuentes *et al.*, 2010), this measurement is relatively difficult to perform and hence relatively inaccurate, with poor reproducibility (Forman *et al.*, 1991). Inaccuracies in follicular measurement can result in suboptimal timing of ovulation induction, thereby reducing the success of IVF (Wittmack *et al.*, 1994, Deutch *et al.*, 2009). In addition, current 2D ultrasound is unable to resolve structures less than 2mm in diameter (Baerwald *et al.*, 2009), making small follicle identification for antral follicle count relatively inaccurate.

With the advent of three-dimensional (3D) ultrasound along with novel computing software, the technique has developed and improved considerably. Sonography-based Automated Volume Count (SonoAVC™: GE Healthcare Ultrasound, Zipf, Austria) allows improved, automated identification and measurement of ovarian antral follicles (Raine-Fenning *et al.*, 2008a; Deutch *et al.*, 2009) which decreases the time needed for ultrasound follicular assessment to take place (Raine-Fenning *et al.*, 2007). The software identifies hypoechogenic regions on scan, and calculates the size and volume of these structures by extrapolating from the number of voxels in the space (Lamazou *et al.*, 2010).

A number of studies have validated SonoAVC™ against a manual rotational method of follicular assessment from 3D ultrasound; Virtual Organ Computer-aided Analysis (VOCAL). Salama *et al.* (2008) and Lamazou *et al.* (2010) found SonoAVC™ to correlate strongly with VOCAL results, and previously our own research group concluded that SonoAVC™ measurement of follicular volume and diameter may

even be more valid than the 2D and VOCAL methods (Raine-Fenning *et al*, 2008a and 2009a), although follicular diameter was systematically underestimated slightly by SonoAVC™. Deutch *et al.* (2009) utilised an artificial phantom model to examine validity of SonoAVC™, and found it to be accurate in measuring both diameter and volume of the ultrasound phantom, as well as in comparison to manual measurement from a 3D scan. Conversely, Rodríguez-Fuentes *et al.* (2010) found that 3D measurement of ovarian follicles has poor correlation with 2D measurement. However, previous work in this area has attempted to validate the SonoAVC™ technique compared to other ultrasound-based methods only, and not to 'true' measurements.

6.3.1.1. Aim

The objective of this study was to examine the accuracy of the SonoAVC™ technique of measuring follicular diameter compared to true diameter utilising a physiologically relevant animal model.

6.3.1.2. Method

24 Bovine ovaries from the abattoir, which are structurally similar to that of the human, were scanned in 3D using the Voluson E8 Expert ultrasound (GE Medical Systems, Zipf, Austria), with a 5-9MHz transvaginal probe. So that the whole organ could be seen in all planes, a bovine uterine tissue barrier was used to create a 30-50mm space between the ovary and the ultrasound probe, by placing the ovary beneath a non-pregnant uterus. The 3D image was acquired and then analysed offline using SonoAVC™ on 4D View software (GE Healthcare Ultrasound), using the method similar to Deb *et al.* (2010). The follicles appear as dark areas on the ultrasound scan due to their hypoechogenicic characteristic.

The software calculates the relaxed sphere diameter from the follicular volume measured (d[V]), and the mean follicle diameter is also calculated, from the average measurement in the x,y,z planes [m-d]. The d[V] was calculated in duplicate and the m-d from the second replicate.

The follicles were then blunt dissected from the ovaries and measured by comparison to a linear scale while submerged, in order to obtain a 'true' relaxed sphere diameter. This was performed by firstly cutting away the tough hilus from the edge of the ovary with scissors, and then proceeding to carefully tear the ovarian stroma with forceps so as to expose individual follicles. The follicles could be seen relatively

easily in the tissue as the ovaries were dissected on a light box, so that they stood out from the surrounding tissue. The exposed follicles could then be removed gently from the ovary without bursting them and each was measured by comparison to a linear scale while submerged in phosphate buffered saline, in order to obtain an accurate 'true' relaxed sphere diameter, as seen in figure 6.8.

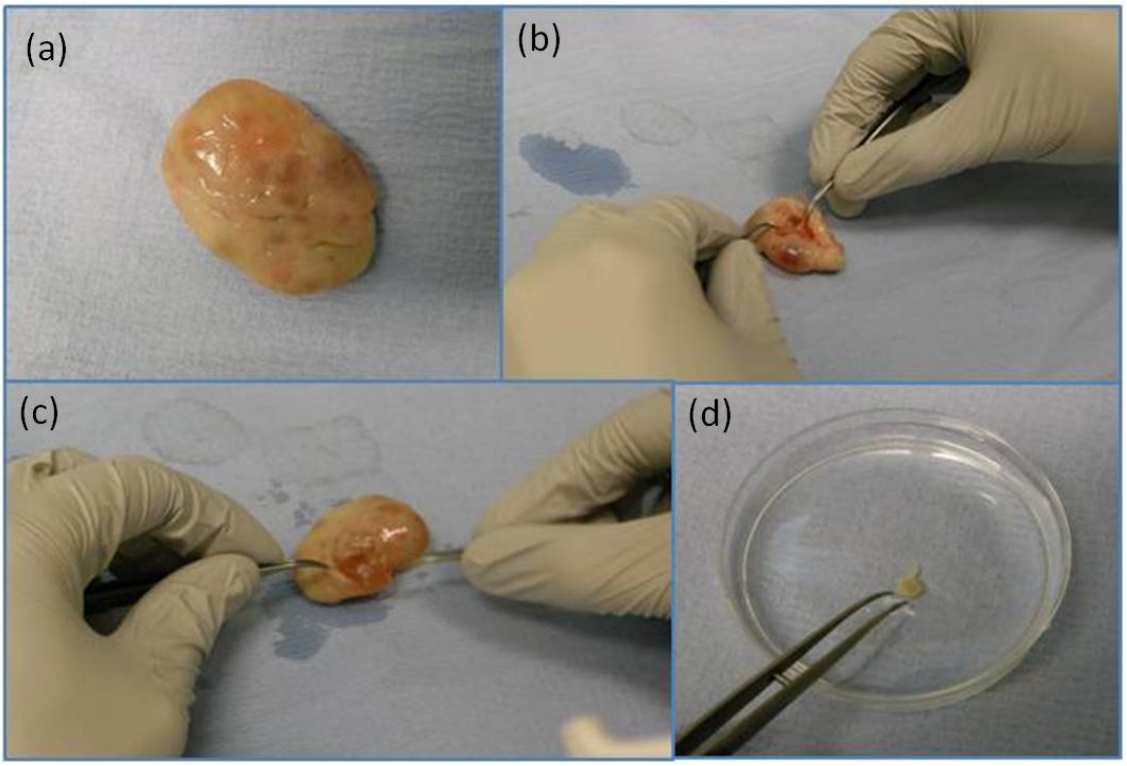

Figure 6.8. Manual dissection of a bovine ovary

Bovine ovaries (a) were dissected manually using forceps (b and c), and the follicles removed and measured compared to a linear scale, while submerged (d).

Follicles larger than 10mm in diameter were included in the study, as these were easily individually identifiable on the ultrasound scan. In addition, follicles of more than 10mm are the most relevant for oocyte retrieval in clinical IVF.

The 'true' relaxed sphere diameter was compared to the d(V) and m-d values calculated by the software. Data was statistically analysed using paired student t-testing, with significance set at $P<0.05$.

6.3.1.3. Results

Dissection Diameter (mm)	SonoAVC Diameter (mm)			m-d (2nd rep)	Diameter Difference (%)	m-d Difference (%)
	1	2	Average			
10	7	6.2	6.6	7	30.00	30.00
11	8	8.8	8.4	9.3	27.27	15.45
11	6.8	6.8	6.8	7.1	38.18	35.45
11	9.6	10.3	9.95	10.8	12.73	1.82
11	8.9	8.3	8.6	9.1	19.09	17.27
11	9.5	9.7	9.6	10.8	13.64	1.82
12	8.4	8.4	8.4	9	30.00	25.00
12	9.8	9.8	9.8	10.8	18.33	10.00
12	13.3	13.3	13.3	14.1	-10.83	-17.50
12	11.2	11.4	11.3	12	6.67	0.00
12	10.7	10.1	10.4	10.8	10.83	10.00
12	10	10.6	10.3	11.7	16.67	2.50
12	10.4	11	10.7	11.6	13.33	3.33
13	12.4	13.1	12.75	14.4	4.62	-10.77
13	10.8	10.8	10.8	11.2	16.92	13.85
13	11.5	10.9	11.2	11.5	11.54	11.54
14	10.3	11.1	10.7	11.9	26.43	15.00
14	8.1	10.1	9.1	10.6	42.14	24.29
14.5	13.3	14.1	13.7	15	8.28	-3.45
15	13.4	14	13.7	14.4	10.67	4.00
15	11.5	11.2	11.35	12.4	23.33	17.33
15	13.5	13.5	13.5	14.1	10.00	6.00
15.5	13.6	12.5	13.05	15.6	12.26	-0.65
16	14.3	14	14.15	14.6	10.63	8.75
17	13.1	13.9	13.5	14.9	22.94	12.35
17.5	15.8	15.5	15.65	17	9.71	2.86
18	13.8	13.9	13.85	14.3	23.33	20.56
18	17	17.3	17.15	18	5.56	0.00
18.5	9	10.2	9.6	10.7	51.35	42.16
18.5	15	14.4	14.7	15.6	18.92	15.68
19	13.9	15.5	14.7	16.2	26.84	14.74
19	13.2	13.8	13.5	14.7	30.53	22.63
				Average	**18.50**	**11.00**

Table 6.2. Follicle measurements taken at dissection and by SonoAVC software.

The relaxed sphere diameter (d[V]) and the mean follicular diameter (m-d) measured by SonoAVC™ was significantly different from that of the manual follicle dissection. The software detected 100% of follicles larger than 10mm in diameter, however when calculating the follicular diameter from the volume, SonoAVC™ significantly underestimated the d[V] by 2.56mm (SD=1.88), or 20.40% (SD=14.77)

(P<0.001). The software also significantly underestimated follicular diameter when calculating from the mean follicular diameter (m-d) by 1.6mm (SD=1.91), or 12.6% (SD=14.76) (P<0.001).

As seen in figures 6.10 and 6.11, SonoAVC™ and dissection diameter measurements had a strong linear correlation, with Pearsons correlation coefficients (r) of 0.76 and 0.77 respectively, although the coefficients of determination values (r^2) of 0.58 and 0.59 respectively indicate a considerable amount of variation in the data.

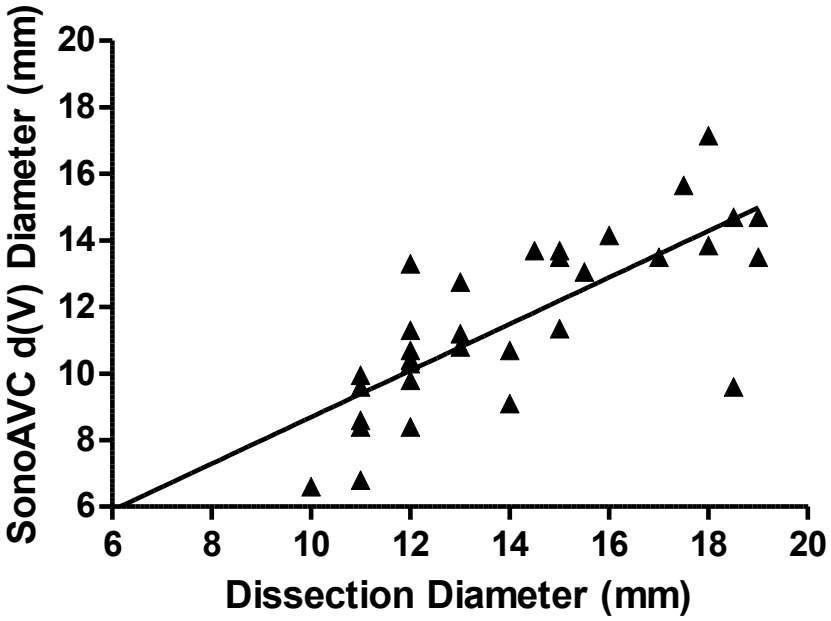

Figure 6.10. SonoAVC™ d(V) measurement of follicular diameter against the dissection diameter measured manually following dissection

Equation of the line of best fit y=0.6997+1.6934, r^2=0.5775.

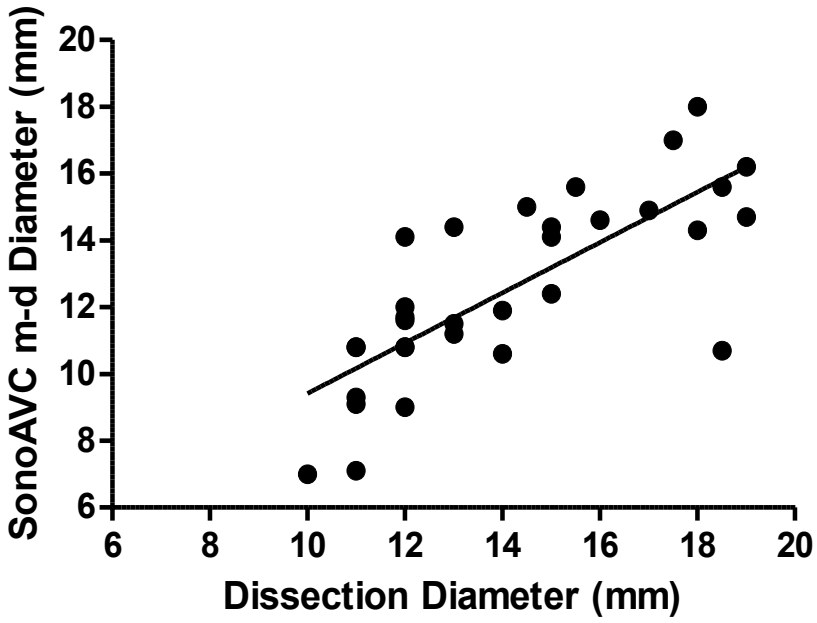

Figure 6.11. SonoAVC™ m-d measurement of follicular diameter against the dissection diameter measured manually following dissection

Equation of the line of best fit y=0.754+1.8761, r^2=0.5871.

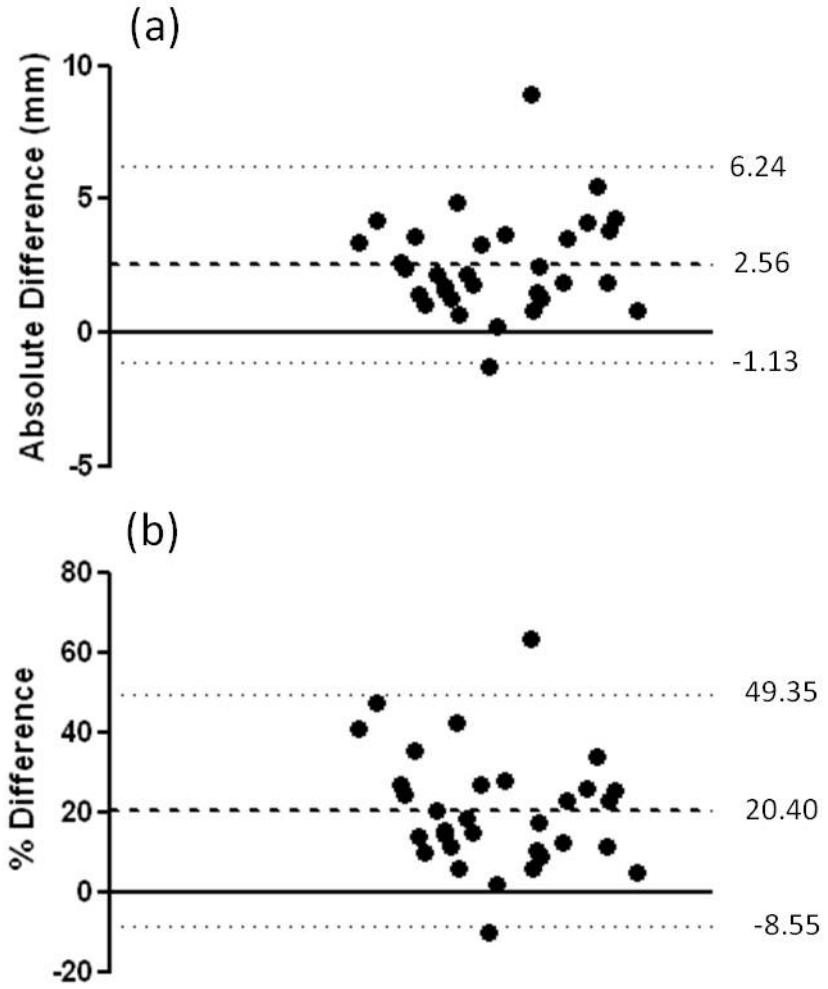

Figure 6.12. Bland-Altman plots of dissection diameter minus the measured SonoAVC™ diameter by volume d(V) as (a) absolute difference and (b) percentage difference.

The central dashed line indicates the mean, and dotted lines are the upper and lower 95% confidence intervals. Overall, SonoAVC™ underestimated follicular diameter by an average of 2.56mm, which equates to 20.4%.

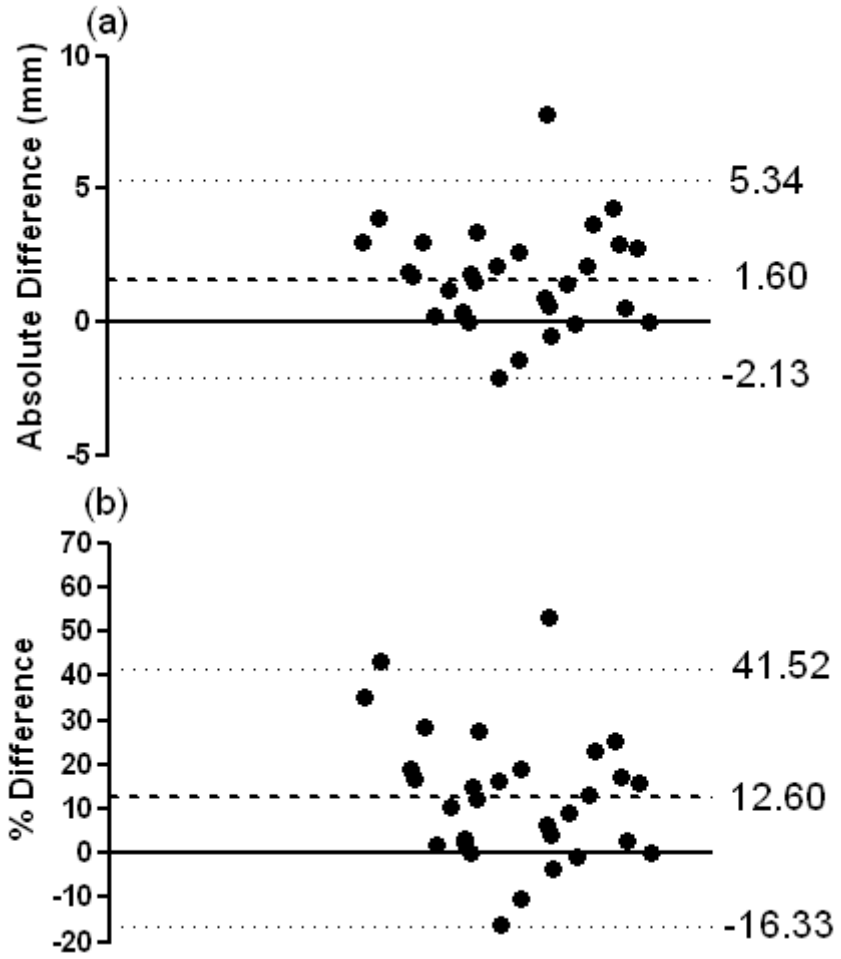

Figure 6.13. Bland-Altman plots of dissection diameter minus the measured SonoAVC™ mean diameter m-d as (a) absolute difference and (b) percentage difference

The central dashed line indicates the mean, and dotted lines are the upper and lower 95% confidence intervals. The m-d calculated by SonoAVC™ underestimated follicular diameter by an average of 1.6mm, which equates to 12.6%.

In this study we found that we were unable to analyse a small number of scans confidently due to poor image quality, and as a result these were not included in the analysis (n=2; 7.7%). Although the resolution of ultrasound has been improved over recent years, the technique continues to require development in order to increase image resolution and hence improve scan quality. Once the 3D image has been acquired there is no way of improving the image quality in order to facilitate analysis any further, therefore it is very important to acquire an image of sufficient quality before examination.

6.3.1.4. Discussion

This is the first study to compare the accuracy of the SonoAVC™ technique of three-dimensional ultrasound compared to large follicles manually dissected from ovaries. In our study it was found that SonoAVC™ significantly underestimated the follicular diameter when calculating from the volume and from the mean diameter ($P<0.001$) and as this measurement is commonly used by clinicians to determine the timing of hCG ovulation induction during fertility treatment this finding has clinical relevance.

In IVF treatment it is important to aspirate follicles of an optimal size in order to optimise success (Wittmaack *et al.*, 1994), which is normally decided based on the size of the lead follicle (Rosen *et al.*, 2008). As a result, underestimating follicular size could lead to prolonged ovarian stimulation that extends the growth of follicles to the detriment of oocyte quality due to post-maturation (Arnot *et al.*, 1995; Rosen *et al.*, 2008).

Comparable studies examining efficacy of SonoAVC™ have analysed the software in relation to 2D ultrasound techniques (Deb *et al.*, 2010; Rodríguez-Fuentes *et al.*, 2010), other software such as VOCAL (Salama *et al.*, 2009; Lamazou *et al.*, 2010) and artificial phantoms (Deutch *et al.*, 2009). One explanation for the discrepancy is that due to the nature of our study, the manual dissection of follicles from the ovaries included the follicle wall in the measurement of the follicular diameter. However, the wall thickness of follicles of this size are between 40 and 120μm (Singh and Adams, 2000), therefore the wall thickness of the follicles in this study would not have affected the results to any significant degree.

Due to the linear correlation between SonoAVC™ and dissection diameter, it would seem that applying a correction factor to the software results could be a viable means to convert measurements made by SonoAVC™ to true diameter. In addition, the mean diameter m-d measurement appears to be more accurate than that of the SonoAVC™ diameter by volume d(V), suggesting that this may be a more appropriate measure to consider.

The quality of the acquired 3D ultrasound image is key when analysing the ovary using SonoAVC™ (Rodríguez-Fuentes *et al.*, 2010). This is of particular importance because once the image has been acquired, little can be done in order to improve image quality to facilitate analysis.

In conclusion, SonoAVC™ appears to underestimate follicular size by approximately 20% following 3D ultrasound scan, therefore clinicians should take care when interpreting the results from the software, or

apply a correction factor to bring the diameter in line with current clinical practice, in order to avoid prolonged ovarian stimulation.

6.3.2. SonoAVC measurement of ovarian reserve

Three-dimensional (3D) ultrasound offers an alternative method for estimation of the number of antral follicles with improved intra- and interobserver reliability than the conventional 2D technique (Deb *et al.*, 2009). Manual measures of follicle size are still time consuming and liable to error both in terms of the number and size of the follicles. Sonography-based automated volume count (SonoAVC™: General Electric Healthcare, Zipf, Austria) is a new software which automatically identifies and quantifies fluid-filled, hypoechoic areas within a three-dimensional datatset (Raine-Fenning *et al.*, 2008). Whilst it was developed for larger follicles, and follicle tracking in particular, it may be applied to the unstimulated ovary and be used to identify and measure antral follicles (Deb *et al.*, 2009; 2010). Previous work from our research group has demonstrated that the software is more reliable (Deb *et al.*, 2010) and valid (Jayaprakasan *et al.*, 2008) than 2D equivalent techniques (Raine-Fenning *et al.*, 2007; 2008; 2009), however in relation to actual follicle number the validity of SonoAVC has not yet been determined.

6.3.2.1. Aim

The objective of this study was to examine the validity of SonoAVC for the identification and quantification of antral follicles by comparing the automated ultrasound measures with actual measures derived by dissection in a physiologically relevant animal model.

6.3.2.2. Method

23 bovine ovaries, which are morphologically similar to those of the human (Adams and Pierson, 1995), were obtained from an abattoir. The ovaries were surrounded by a bovine uterus to create a tissue barrier of 3-5cm in order to distance the ultrasound transducer a set distance from the ovary and create a physiological interface comparable to that encountered in the clinical situation. The ovaries were

visualised by applying a 7.5MHz transvaginal ultrasound transducer (Voluson E8 Expert: GE Medical Systems, Zipf, Austria) manually held in place to locate and demonstrate the ovary in 2D.

A 3D acquisition was then performed using the high quality mode and wide angle. The resultant image was checked to ensure the whole ovary had been included and then saved to the ultrasound machine, before being transferred to disc for later analysis. The ultrasound settings were standardised and kept identical for all of the ovarian scans.

All of the follicles were then manually dissected from the ovaries using forceps, under a magnifier lamp using the same method previously described for the follicular measurement study.

All data were analysed offline using 4D View software (version 7.0, GE Medical Systems) using the method described in the methods section 2.4.1.

Deb *et al.* (2009) showed that the software is not capable of detecting all of the visible follicles from the scan automatically, and so post-processing was used so that any follicles that had been missed by SonoAVC could be added manually to the dataset by clicking on the dark areas of the scan. This process simply adds follicles to the list from the automated analysis, so the automated measurement is also still performed on the manually selected follicles. This does negate some of the advantages of using automated software, however post-processing was necessary to ensure that all visible follicles were included in the antral follicle count. The total number of follicles detected both automatically and manually was considered to be the antral follicle count for each scan dataset.

The smallest structures identifiable on ultrasound are around 2mm in diameter (Broekmans *et al.*, 2009) and this was considered, therefore, to be the lower detection limit for the study. Follicles measuring up to 10mm in diameter were included in the analysis as these are the largest follicles used for the assessment of antral follicle count; larger follicles being considered more likely to be atretic (Khairy *et al.*, 2008). The follicles were grouped into sizes based on their mean diameter measured at dissection, into those of interest in current literature; 2-5mm (Chang *et al.*, 1998; Pellicer *et al.*, 1998; van Rooij *et al.*, 2002), 2-6mm (Haadsma *et al.*, 2007; Deb *et al.*, 2009a; Jayaprakasan *et al.*, 2010a), 2-8mm (Weenen *et al.*, 2004), 2-9mm; which is the Rotterdam criteria for polycystic ovary syndrome diagnosis, defined in 2003 (Scheffer *et al.*, 2003; Ng *et al.*, 2005; Jayaprakasan *et al.*, 2008) and 2-10mm (Broekmans *et al.*, 2009).

Data distribution was tested for normality using the D'Agostino and Pearson omnibus test in GraphPad Prism (version 5.03), and then the software was used to statistically analyse the data using chi-squared testing. Statistical significance was set at 0.05.

6.3.2.3. Results

Following D'Agostino and Pearson omnibus testing, the data was found to be normally distributed. In this study the SonoAVC software significantly underestimated the antral follicle count for follicle ranging from 2 to 10mm in diameter ($P<0.001$), by an average of 13.96 (SD±15.05) follicles per scan, equating to 38.64% (SD±24.19). Table 6.3 below shows the dissection and SonoAVC antral follicle counts.

Ovary	Dissection AFC	SonoAVC AFC	Follicles missed by software
1	31	24	7
2	14	10	4
3	9	8	1
4	21	13	8
5	16	8	8
6	31	25	6
7	8	1	7
8	68	30	38
9	61	38	23
10	64	21	43
11	5	4	1
12	54	17	37
13	39	24	15
14	18	9	9
15	16	14	2
16	73	22	51
17	5	5	0
18	14	8	6
19	19	6	13
20	14	13	1
21	55	29	26
22	33	19	14
23	31	30	1
		Mean	13.96
		SD	15.05

Table 6.3. Antral follicle counts performed following ovarian dissection and using SonoAVC software.

Figure 6.14 shows SonoAVC antral follicle counts plotted against the follicle count at dissection. Notably, all of the SonoAVC data points lie below the expected direct linear relationship dashed line, and so the line of best fit falls lower than expected, indicating that the software persistently underestimates the follicle count. In addition it seems that the number of follicles missed increases as the antral follicle count increases.

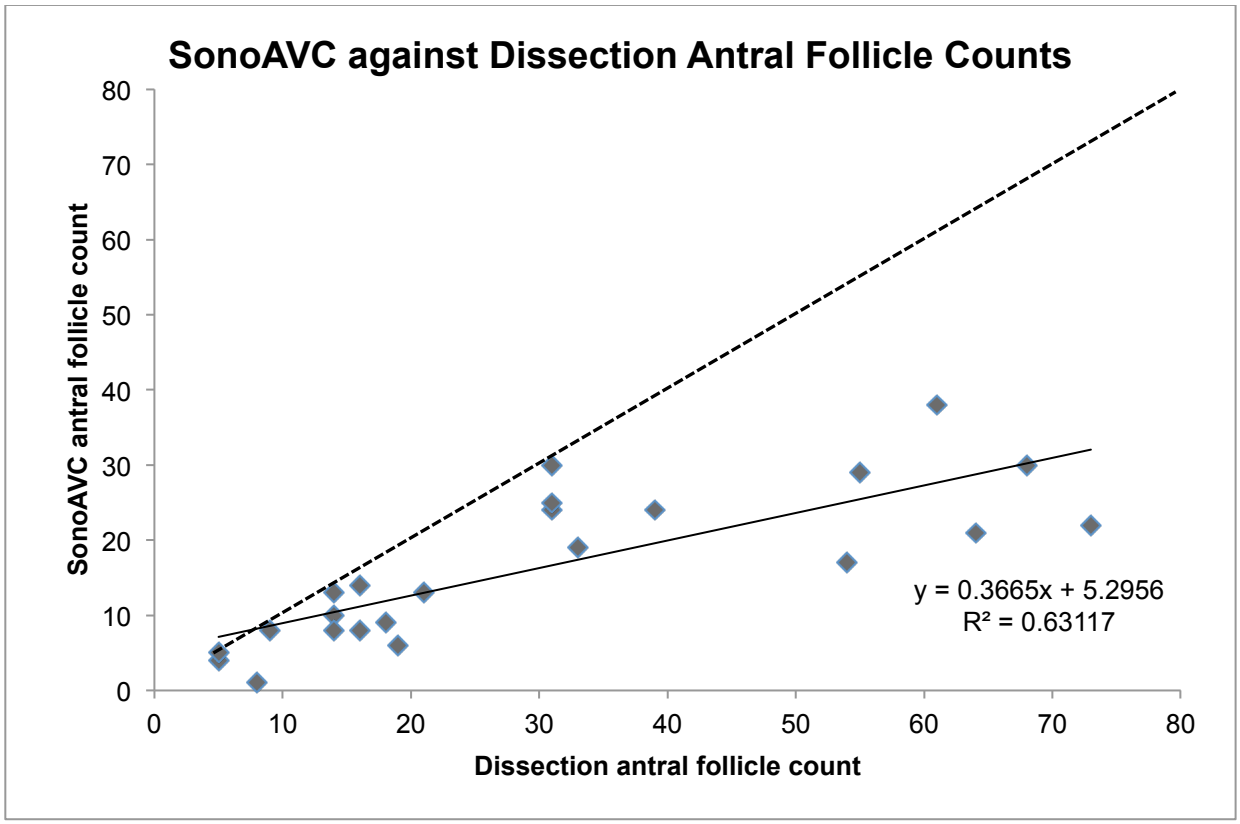

Figure 6.14. SonoAVC antral follicle count against follicle count at dissection.

The solid black line of best fit (y=0.3665x + 5.2956) has a coefficient of determination of 0.63, and correlation coefficient of 0.79. The dashed line indicates a linear relationship y=x, which would be the line of best fit if the two method measurements were identical.

Figure 6.15 shows the average number of follicles missed by SonoAVC, per scan, grouped according to the number of follicles counted at dissection. This further demonstrates that as the number of follicles in the ovary increases, the number missed by the software also increases.

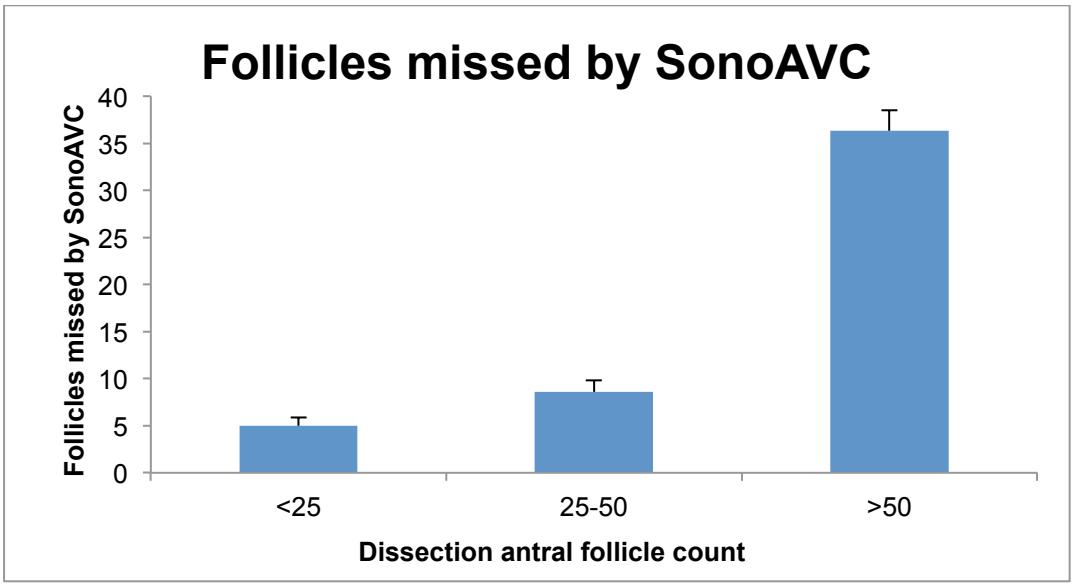

Figure 6.15. Average number of follicles missed by SonoAVC compared to the count at dissection, grouped into 3 groups of low (<25), medium (25-50) and high (>50) follicle numbers

Error bars indicate SEM.

Follicle visibility was calculated as the percentage of follicles detected by SonoAVC compared with the count at dissection. It was found that this increased with follicular size, as seen in table 5.4 below. The follicles were grouped into sizes into equal or smaller than 2mm, 2-3mm, 3-4mm, 4-5mm, 5-10mm and greater than 10mm. In this study we found that 100% of follicles larger than 6mm in diameter were successfully detected by the software.

	≤2mm	2-3mm	3-4mm	4-5mm	5-10mm	>10mm
Dissection AFC	227	184	159	52	46	31
SonoAVC AFC	123	109	55	28	42	31
% visibility	54.19	59.24	34.59	53.85	91.30	100.00

Table 6.4. Antral follicle counts at dissection and by SonoAVC with percentage visibility for follicles in different size groups.

Follicles were also grouped in to small, medium and large diameter groups; 2-4mm, 4-6mm and 6-10mm respectively. The smaller follicles had the poorest rate of detection with only 47.4% of follicles measuring 2-4mm in diameter being detected by SonoAVC. Follicle detection increased with follicular size; 60.56% of follicles 4-6mm in diameter were detected, and the larger follicles 6-10mm in diameter were all detected by the software when post-processing was applied (figure 6.16).

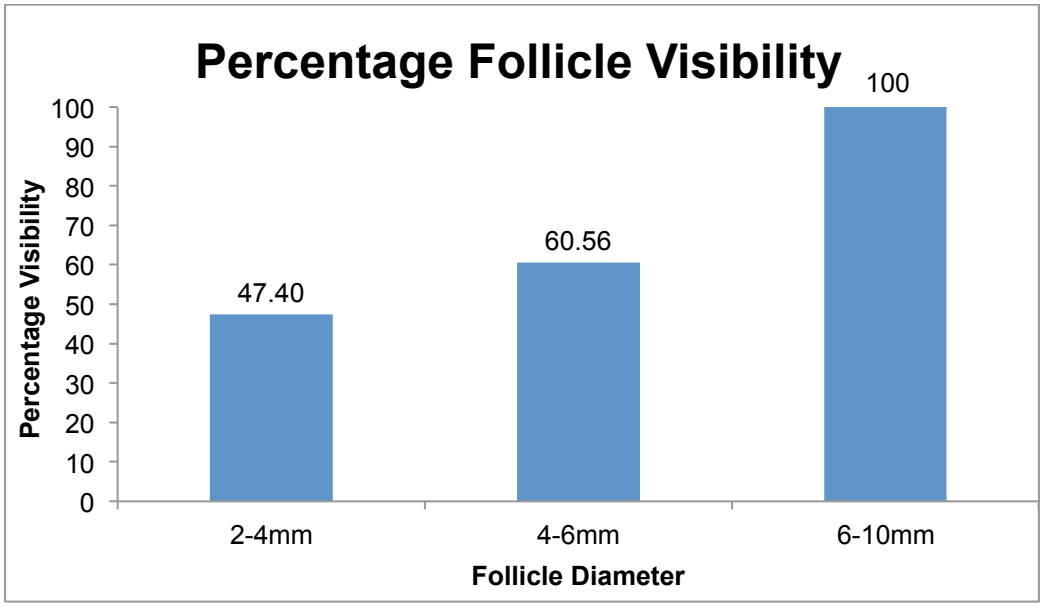

Figure 6.16. Overall SonoAVC percentage follicle visibility in three major follicle size classes

The results were analysed both in terms of overall follicle visibility and also on a per scan basis, as seen in table 6.5 which shows the percentage follicle visibility grouped into relevant follicle sizes classes according to the literature (see method section) as well as mean follicle visibility per scan.

	2≤5mm	2≤6mm	2≤8mm	2≤9mm	2≤10mm
Dissection Count	395	417	433	440	441
Sono AVC	192	207	223	228	234
% Visibility	48.61	49.64	51.50	51.82	53.06
Average % Visibility per Scan	56.04 ± 31.26	54.48 ± 27.79	58.29 ± 29.95	58.00 ± 29.99	54.85 ± 30.53

Table 6.5. Overall visibility of antral follicles detected by SonoAVC software in major AFC size classes used in the literature, and mean percentage follicle visibility per scan (±SD).

6.3.2.4. Discussion

To date this is the first study to examine the validity of SonoAVC for the identification and quantification of antral follicle number and size compared to a physiological phantom model. Our results show that SonoAVC significantly underestimates the number of antral follicles compared to the manual count obtained through dissection of the ovary, although the degree of underestimation depended on both follicle size as well as the number of follicles present in the ovary.

For follicles 6-10mm in diameter SonoAVC detected all follicles, but this high visibility declined dramatically to less than 50% for small antral follicles measuring 2-4mm in diameter. As small antral follicles are more numerous, collectively antral follicles visibility was low when total AFC from 2-10mm was calculated (table 2).

The software underestimated the antral follicle count by an average of 38.64% per scan, equating to 13.96 follicles, indicating that SonoAVC may not be an accurate method for estimating ovarian reserve. Antral follicle visibility ranged from 48.6% for follicles 2-5mm in diameter, to 53% for follicles 2-10mm in diameter, and therefore it is worth noting that follicle visibility differs slightly depending on the follicle size inclusion criteria for antral follicle count (table 6.5).

As illustrated by figure 5.15, as the total number of follicles in the ovary increased, the number of follicles missed by the software also increased. This suggests that the proportion of follicles missed by the software increases exponentially; therefore error is highest in the ovaries containing the highest numbers of follicles. From a clinical standpoint, this may mean that the discrepancy between estimated and actual AFC may not be of major significance as very few human patients for IVF have AFCs greater than 25 (Almog *et al.*, 2010). Conversely, for those women who do have unusually high numbers of small antral follicles, SonoAVC AFC determination may lead to patients being administered higher doses of gonadotrophin that would be considered appropriate.

The software is capable of successfully identifying all of the follicles greater than 6mm in diameter, indicating that the resolution of 3D ultrasound is sufficient for the software to identify these follicles with relative ease. The software was able to identify these large follicles automatically, without the need for adding them through post-processing, however detection of smaller follicles required additional processing in order to be detected.

Follicles in the cohort larger than 6mm in diameter are not considered to contribute to the so-called 'functional' ovarian reserve (Gougeon and Lefèvre, 1983; Webb and Campbell, 2007). Recently our research group found that it is the antral follicles measuring between 2 and 4mm in diameter that may be the most predictive of pregnancy following assisted reproductive treatment (Deb *et al.*, 2009b). In addition, the number of follicles 2-4mm in diameter was significantly predictive of fertilisation and embryo cleavage rates, supporting the theory that the smaller follicles are a more reliable representation of the true ovarian potential.

The results indicate that the program may be inaccurate in detecting small antral follicles, 2-6mm in diameter, within a three-dimensional ultrasound dataset. The small follicles had the poorest visibility, which is of particular significance as it seems probable that it is the smaller sized follicles that form the functional ovarian reserve, bear the closest correlation to serum AMH levels and are also form the best predictor of ovarian response in assisted reproductive treatments (Weenen *et al.*, 2004; Haadsma *et al.*, 2007; Jayaprakasan *et al.*, 2009). The software appears to miss a large proportion of these follicles, which is almost certainly as a result of the current resolution limit of ultrasound, as it was found that these small follicles were relatively difficult to distinguish on the ultrasound image.

It is important for the clinician to consider that when estimating the ovarian reserve using SonoAVC, the technique does not detect a significant proportion of small antral follicles that form the functional ovarian reserve. Therefore it would be advisable to utilise additional methods in order to provide a more robust estimation of ovarian reserve estimation, such as serum AMH, which has a good correlation to ovarian reserve (Jayaprakasan *et al.*, 2009). Utilising these methods side-by-side would be likely to offer a reliable indication of ovarian responsiveness to stimulation during fertility treatment (de Vet *et al.*, 2002; van Rooij *et al.*, 2002; Hansen *et al.*, 2011; Majumder *et al.*, 2010).

In conclusion, SonoAVC is a novel and promising technique for automated large antral follicle tracking as it is capable of detecting all large antral follicles without the need for post-processing and editing. The use of this software for antral follicle counting does however have some limitations, as a considerable amount of operator processing and editing is required, and it is unable to detect a significant proportion of small antral follicles from the growing cohort. These limitations should therefore be considered when utilising

this technology at the present time. Currently the SonoAVC is more suited to be used as a research tool rather than a routine clinical method of AFC assessment (Deb *et al.*, 2009a).

Chapter 7 - Clinical Trial: Peri-follicular blood flow, molecular markers and follicular fluid constituents as predictors of oocyte and embryo quality

7.1. Introduction

Despite 30 years of development and extensive research, the success rate of *in* vitro fertilization (IVF) is still relatively poor, and as a result there is a continuous drive to improve the technique for the benefit of both practitioners and patients (Pennings and Ombelet, 2007). Relatively recently, the focus has shifted toward single embryo transfer (SET), to avoid the negative financial and health implications associated with multiple gestations (Fauser *et al.*, 2006). As a result, it has become even more important to select individual embryos for transfer with the highest chance of producing a pregnancy (Assou *et al.*, 2008).

Oocyte quality is a major limiting factor in female fertility, and currently there is a lack of objective and reliable predictors of oocyte developmental competence (Li *et al.*, 2008). Morphological parameters, which at present form the basis for oocyte selection in the clinical setting, are based on appearance of cytoplasm, polar body and cumulus cells, making the process highly subjective and as a result, inaccurate (Balaban *et al.*, 1998; Nicholas *et al.*, 2005; Wang *et al.*, 2007). As a result, a key goal of improving IVF outcome is to define objective and non-invasive markers of oocyte quality that are capable of predicting oocyte developmental competence (Li *et al.*, 2008).

One source of material for analysis of oocyte developmental competence is the somatic cells to which the oocyte is associated in the follicle, as it is thought that the health status of the oocyte is directly related to that of the surrounding cells (Gilchrist *et al.*, 2008). Recently, research using cumulus cells has shown gene expression to reflect oocyte and embryo quality, and even pregnancy outcome (Assou *et al.*, 2008; Feuerstein *et al.*, 2007). Granulosa cells, which line the inside of the ovarian follicle, are thought to harbour the same potential.

The follicular fluid in which the oocyte resides can be considered as a 'biological window', which reflects the metabolic and hormonal status of the oocyte microenvironment prior to ovulation or egg retrieval, and consequently is an additional potential target for analysis relating to oocyte quality and consequent IVF outcome (Wiener-Megnazi *et al.*, 2004). The follicular fluid of each individual follicle is unique, and is also different to that of the circulatory system, and its composition changes continuously throughout folliculogenesis, both in terms of steroid hormones (Andersen, 1993) and growth factors such as endothelial growth factor (Westergaard *et al.*, 1990). Measurement of growth factors secreted by the oocyte (for example BMP15 and GDF9), angiogenic growth factors from somatic cells (for example VEGF and HIF), and also constituents of the follicular fluid such as steroid hormones (for example oestradiol and progesterone) could potentially be used as indicators of oocyte quality.

Assessment of blood flow to the follicle by 3 dimensional power Doppler ultrasonography is a tool that could also be used for the purpose of assessing follicular status. Peri-follicular blood flow has a considerable influence on the development of the oocyte within. As the method is objective and non-invasive, and closely related to follicular development, it could serve as a good predictor of oocyte competence and embryo development. The measurement of peri-follicular blood flow is more likely to directly relate to the quality of the oocyte within than quantifying the blood flow to the whole ovary itself, as the microenvironment of the individual follicle is directly affected by its locally available vasculature. Although ovaries with high blood flow are associated with improved response to stimulation (Pan *et al.*, 2004), measurement of stromal flow is not predictive of oocyte quality or IVF outcome (Ng *et al.*, 2005; Ng *et al.*, 2006). Therefore measuring peri-follicular blood flow would seem like an ideal candidate to relate to oocyte developmental competence.

Hypothesis

The follicular fluid and follicular somatic cells are discarded during clinical IVF and ICSI, as they currently serve no further purpose, however we hypothesise that analysis can be performed on these by-products in order to relate peri-follicular blood flow with oocyte and embryo developmental competence, and later pregnancy rates. Molecular and gene expression analyses are techniques that can be applied to the follicular fluid and somatic cells that are normally discarded during the procedures involved in IVF. The

results of such analyses could be related to the non-invasive assessment of follicle health by 3D power Doppler ultrasound and the developmental competence of the embryo, so as to develop novel quality assessment techniques to be used alongside the morphology-based method in place. This would ensure that the embryo selected for transfer has the best possible chance of producing a pregnancy, and hence improve IVF outcome.

7.2. Aim

The principle aims of the study are to determine whether follicular growth rates and peri-follicular blood flow are related to levels of expression of local factors that regulate angiogenesis and metabolism within ovarian follicles which, in turn, provide information on the developmental competence of oocytes within these follicles. We intend to do this by carrying out the following objectives:

1. To measure follicular size and rate of growth by 3D ultrasound in patients undergoing ovarian stimulation.
2. To measure peri-follicular and ovarian blood flow by 3D Power Doppler ultrasound in patients undergoing ovarian stimulation.
3. To track developmental progress of embryos from fertilization until clinical pregnancy.
4. To measure antral fluid concentrations of sex steroid hormones such as oestradiol and progesterone, oocyte secreted factors such as BMP15 and GDF9 and ovarian somatic cell factors such as AMH.
5. To measure levels of mRNA and protein expression of markers of angiogenesis such as VEGF and HIF-1α in granulosa and cumulus cells.

7.3. Study outline

Participants are recruited from the cohort of female patients undergoing infertility treatment using ICSI in the NURTURE fertility clinic, as the oocytes and embryos in this treatment regime can be tracked through development with relative ease. In addition the cumulus cells can be collected from around the oocytes as part of this technique.

Initially, a pilot study was performed in approximately 100 patients, after which the distribution of the data was analysed. Following appropriate statistical analysis it was then be determined how many patients are needed to power a study suitably.

The NURTURE fertility clinic has around 600 patients per year, half of which have ICSI treatment. This would indicate a potential sample size of 300 patients, with approximately half of these patients having single embryo transfer under the new NHS funding system.

Due to the study requiring human participants, ethical approval had to be sought from a local NHS research ethics committee through the Integrated Research Application System (IRAS). The study received full ethical approval from the Derbyshire NHS Research Ethics Committee (Project reference number 09/H0401/81), following peer review from Professor Helen Picton of Leeds University.

Screening (Visit 1)

The screening visit takes place a maximum of 2 months prior to commencement of fertility treatment.

- The patients attend a consultation and antral follicle count by 3D ultrasound scan, between day 2 and day 5 of the menstrual cycle, as per normal protocol. Eligible patients who are expected to have ICSI are invited to participate in the study, provided that the inclusion/exclusion criteria are met.
- The study is explained in detail and the participant given the patient information sheet and written consent form, which the patient is given a minimum of 24 hours to read and sign.

Trial period

Participants are managed as per the normal clinical practice for ICSI treatment protocol with some alterations;

- At ovulation induction, the patient undergoes 3 dimensional ultrasound to identify follicles that are likely to be aspirated. This does not require an additional visit, but adds a maximum of 15 minutes onto the normal time required.
- Before oocyte retrieval, the participant undergoes 3 dimensional transvaginal ultrasonography and around 6 individual follicles identified for aspiration. Follicles of 10-26mm in diameter are chosen at

random and scanned using power Doppler ultrasound to examine their peri-follicular blood flow. The selected follicles are then aspirated and the fluid and cells used for analysis in the study.

- This additional scan adds no more than 10 minutes onto the normal protocol, which currently requires 30 minutes. A 45 minute window is allowed for the whole procedure so as not to delay the patients which follow.
- As per the normal ultrasound scanning procedure, the 4D scans obtained are stored on the scanning machine computer and may be used for future research in retrospect, with signed permission from the participant.
- Following ICSI on the selected individual oocytes, fertilized zygotes are cultured individually in microdrops in order to allow tracking and identification. This is not considered to be detrimental to the development of the embryo.
- Embryo transfer of 1 or 2 embryos is performed at the discretion of the patient and clinician, at day 2-3 or day 5 for blastocyst transfer. During embryo transfer, 3D ultrasound is used instead of 2D in order to examine the status of the endometrium. This is not expected to result in any significant increase in the amount of time taken for the procedure. Post-implantation data is used only for the participants undergoing single embryo transfer, as tracking of individual embryos is not possible for double embryo transfer patients.

The number of embryos to be transferred is entirely at the discretion of the patient and clinician, and unaffected by the study.

Post-transfer data from patients undergoing single embryo transfer (SET) is kept separate from those having two embryos transferred, so that individual follicle data can be tracked through to pregnancy for SET patients. Data from double embryo transfer patients is also recorded and may be used, in a separate analysis, as even though individual embryo tracking is lost, the final outcomes can still be related back to some pre-transfer data.

SCHEMATIC DIAGRAM OF POEM STUDY DESIGN

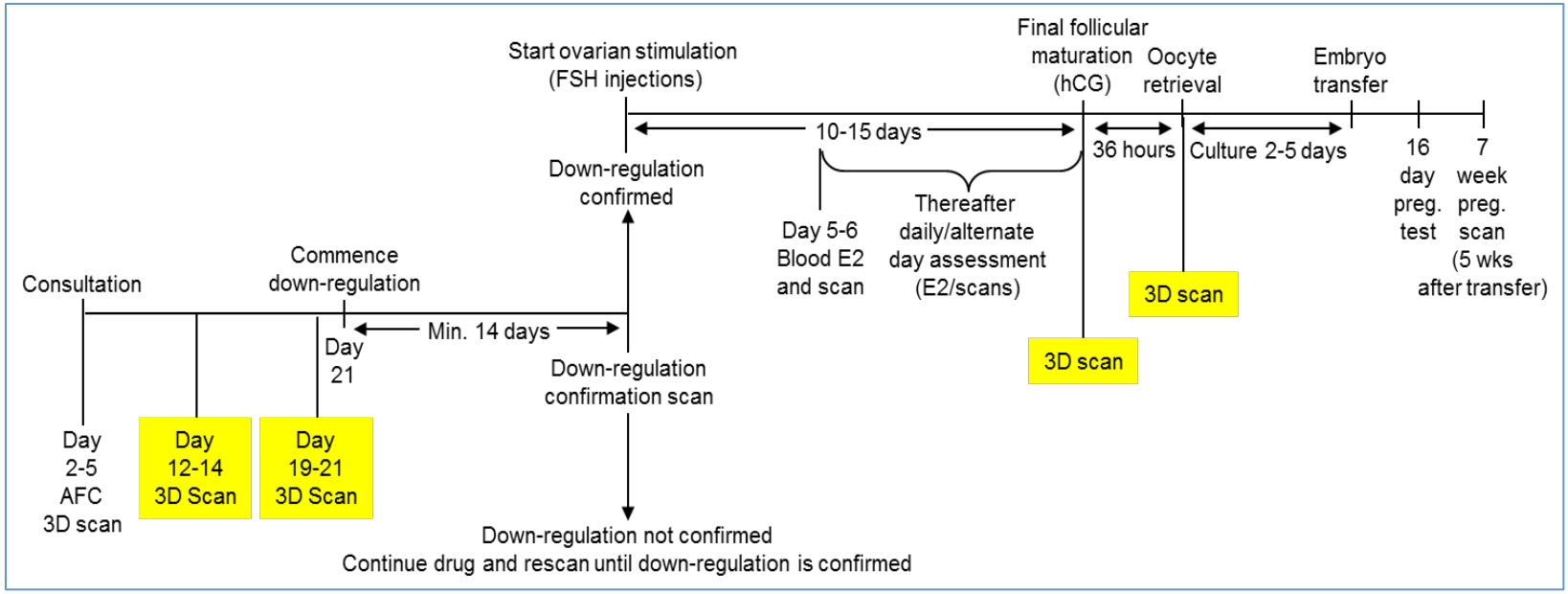

Figure 7.1. Schematic diagram of POEM study design

Yellow boxes indicate the additional 3D ultrasound scans required for the POEM study. The first two yellow boxes indicate study scans to be used in another study, the data from which are not utilised for the purposes of the POEM study.

7.4. Data Collection

7.4.1. Transvaginal ultrasonography

All data was acquired using the same ultrasound machine and a 5-8 MHz transvaginal probe for all patients. Scans were performed in the standard manner with the patient in a supine position with knees flexed and hips abducted. The individual conducting each ultrasound scan was recorded, and the ultrasound settings used for every scan were kept constant for every patient.

3D Power Doppler and pulse wave Doppler analysis of and ovarian blood flow was carried out as per normal treatment protocol, with an additional scan at the time of oocyte retrieval. Vascularisation Index (VI), Flow Index (FI) and Vascular Flow Index (VFI) around individual follicles was generated from the Power Doppler analysis, as well as follicle volume using shell imaging (Jayaprakasan *et al.*, 2009).

7.4.2. Blood sampling

Blood samples were taken as per the regular treatment protocol, to determine levels of serum gonadotrophins (FSH, LH) and sex steroids (oestrogen, progesterone), as part of the normal treatment regime.

7.4.3. Ovarian cells and follicular fluid

Follicular fluid was collected following aspiration of individually identified follicles. If the follicles require aspiration to find the oocyte, the aspirate fluid was also collected from these flushes. Once the oocyte had been retrieved from the follicular aspirate by the embryologist, the remaining follicular fluid with cells was collected and processed.

All follicular fluids and aspirates were collected in separate tubes and labeled with patient initials, date of birth, patient ID number, follicle number, and F or A (for follicular fluid or aspirate). The oocyte associated with the follicular fluid was given a patient ID number, and the same follicle number as its follicular fluid.

Oocytes were kept separate from each other in order to maintain individual identity throughout.

Following maturation, the cumulus cells were stripped from the oocyte before fertilization, as per the normal ICSI protocol, and the cumulus cells collected into labelled tubes. Cumulus cell tubes were labelled with patient initials, date of birth, patient ID number and follicle number, followed by a C (for cumulus cells). Identifiers were kept identical on all study documents.

Example of complete dataset from an aspirated follicle from one patient;

JB-050475-102-1-F	Josephine Bloggs, D.O.B., Patient 102, Follicle 1, Follicular fluid
JB-050475-102-1-A1	Josephine Bloggs, D.O.B., Patient 102, Follicle 1, First aspirate
JB-050475-102-1-A2	Josephine Bloggs, D.O.B., Patient 102, Follicle 1, Second aspirate
JB-050475-102-1-C	Josephine Bloggs, D.O.B., Patient 102, Follicle 1, Cumulus cells
JB-050475-102-1-G	Josephine Bloggs, D.O.B., Patient 102, Follicle 1, Granulosa cells*

*(Following separation from follicular fluid and aspirates by centrifugation)

7.4.4. Granulosa cell and follicular fluid collection

Follicular aspirates from egg collection were transferred from petri dishes to 1.5ml Eppendorf tubes under a stereo microscope to ensure all cells had been collected. Tubes were labelled with the participant ID and the aspirate type (follicular fluid or flush), and then centrifuged at 10,000rpm for 3 minutes.

The supernatant (follicular fluid) was transferred into 1.8ml cryotubes and snap frozen in liquid nitrogen before being moved into a -80°C freezer for storage. The cell pellets were resuspended in PBS and centrifuged at 10,000rpm for 3 minutes to wash the cells. The supernatant was discarded, and 350µl RLT lysis buffer (Qiagen 79216) with β-mercaptoethanol (β-ME) at 0.01% added to lyse the cells. The cell suspension was then transferred to cryotubes and snap frozen in liquid nitrogen and stored at -80°C.

7.4.5. Cumulus cell collection

Cumulus cells were stripped as part of the normal ICSI procedure in hyaluronidase. The cells were transferred to 1.5ml Eppendorf tubes under a stereo microscope, and labelled with participant ID and

'CC', before being centrifuged at 10,000rpm for 3 minutes. The supernatant was discarded, and the cells taken through a wash step of 200µl PBS. The cell pellet was resuspended, and then then centrifuged at 10,000rpm for 3 minutes once again. The supernatant was discarded, and 200µl RLT lysis buffer with β-mercaptoethanol (Qiagen, UK) was added to lyse the cells. The cell suspension was then transferred to cryotubes and snap frozen in liquid nitrogen and stored at -80°C.

7.4.6. Molecular analysis

Follicular fluid and aspirates were thawed centrifuged in order to separate out the granulosa cells, as well as any other contaminants such as erythrocytes.

Sex steroid hormones were measured in follicular fluid using antibody-based quantification techniques; ELISA and radioimmunoassay. In addition, oocyte-secreted growth factors (BMP15, GDF9) and somatic cell factors (AMH) were later quantified using specific ELISAs as per the procotol supplied with the kits (chapter 2).

In the future when 2 samples are able to be collected from a single participant, the granulosa and cumulus cells will analysed for gene expression of growth factors, including the angiogenic growth factors VEGF and HIF, as well as a range of RNA and protein expression profiles, by extracting the mRNA and synthesizing cDNA, followed by real-time PCR. A proportion of the cumulus cells will also be collected to be used for staining to indicate their redox state and hence oxidative stress, which has been shown to relate to oocyte and embryo quality (Tarín, 1996; Tamura *et al.*, 2008).

7.5. Results

The amount of time required for the planning, ethical approval and implementation of the clinical trial meant that the number of patients successfully recruited and completed was much lower than anticipated. A summary of the study participant outcomes can be seen in figure 7.2 below.

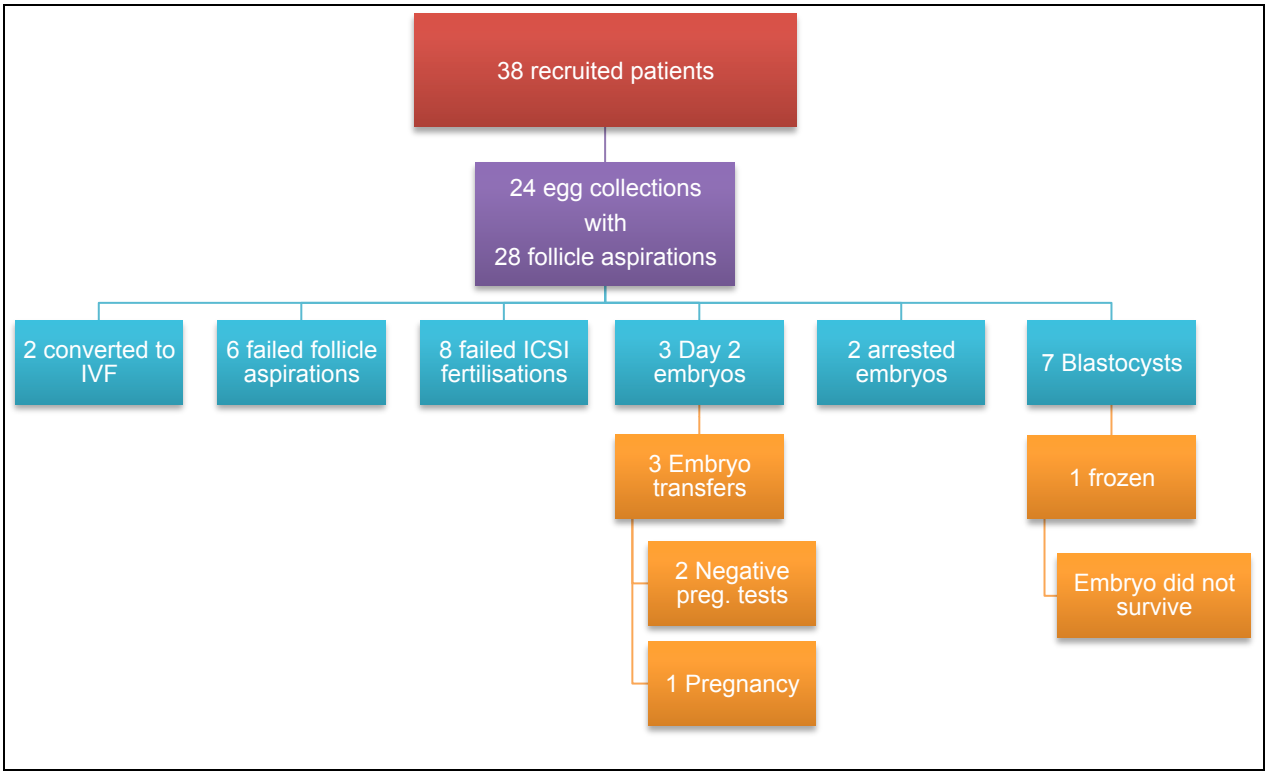

Figure 7.2. POEM study patient outcome flow diagram

A total of 38 patients were recruited during the 6 months that the trial ran for, and 24 egg collections were performed. Of the 24 patients that underwent egg collection, 2 converted to IVF treatment due to an improved ejaculate sample being received on the day of collection. As a result, these patients were excluded from the embryo tracking part of the study, but their study scans were retained for analysis.

- Twenty eight follicles were aspirated in total due to 4 patients having both right and left ovaries aspirated for a selected oocyte. This was performed if an oocyte was not found in the aspirate of the first ovary. Twenty two oocytes were retrieved in total, all of which were assessed as being metaphase II and therefore suitable for fertilization. Six of the aspirated follicles did not contain a retrievable oocyte, and so no embryological data could be collected from these individuals.

- Eight of the oocytes retrieved did not fertilise successfully following intracytoplasmic sperm injection. Of the oocytes that did fertilise, 3 embryos were transferred on day 2 after egg collection, with 2 failed embryo implantations, and 1 confirmed positive pregnancy by foetal heartbeat on ultrasound.
- Two embryos arrested on day 5 at 4 cell and 8 cells, however 7 embryos continued to develop to blastocyst stage. None of these blastocysts were selected for transfer, however one was frozen to be used in a later frozen embryo cycle. Unfortunately this embryo did not survive the thawing process and so was not able to be transferred.

Initially the addition of the power Doppler scan at egg collection caused the procedure to take slightly longer than usual. As a result it was decided to aspirate only one follicle from each patient, so that the increase in time required for egg collection would not cause damage to the oocyte. When an oocyte was not retrieved from a selected follicle and sufficient time was available, the aspiration needle was replaced and a follicle on the other ovary was scanned and aspirated. Although no oocyte was found in these follicles, the scans were kept and analysed, and the follicular fluid and granulosa was collected.

Ovarian Stimulation

Patients were down-regulated from day 21 of the cycle by buserelin subcutaneous injection or nasal spray, or nafarelin nasal spray. Following this ovarian stimulation was administered using Menopur™ (Ferring Pharmaceuticals, Berkshire, UK) (n=17), Pergoveris™ (n=1) or Gonal-F™ (both Merck Serono, Geneva, Switzerland) (n=6). Ovulation was induced using Ovitrelle® or Pregnyl® (Merck Serono, Geneva, Switzerland).

7.5.1. Case Report

One patient recruited for the POEM study successfully became pregnant, following ICSI treatment with frozen semen obtained by electroejaculation. This 32 year old patient originally had what would be considered to be a relatively poor prognosis, due to this cycle being her third and her partner suffering from ejaculatory dysfunction.

At the hCG scan, the patient had 18 visible follicles on both ovaries, with an endometrial thickness of approximately 14mm, and so was administered hCG 36 hours before egg collection.

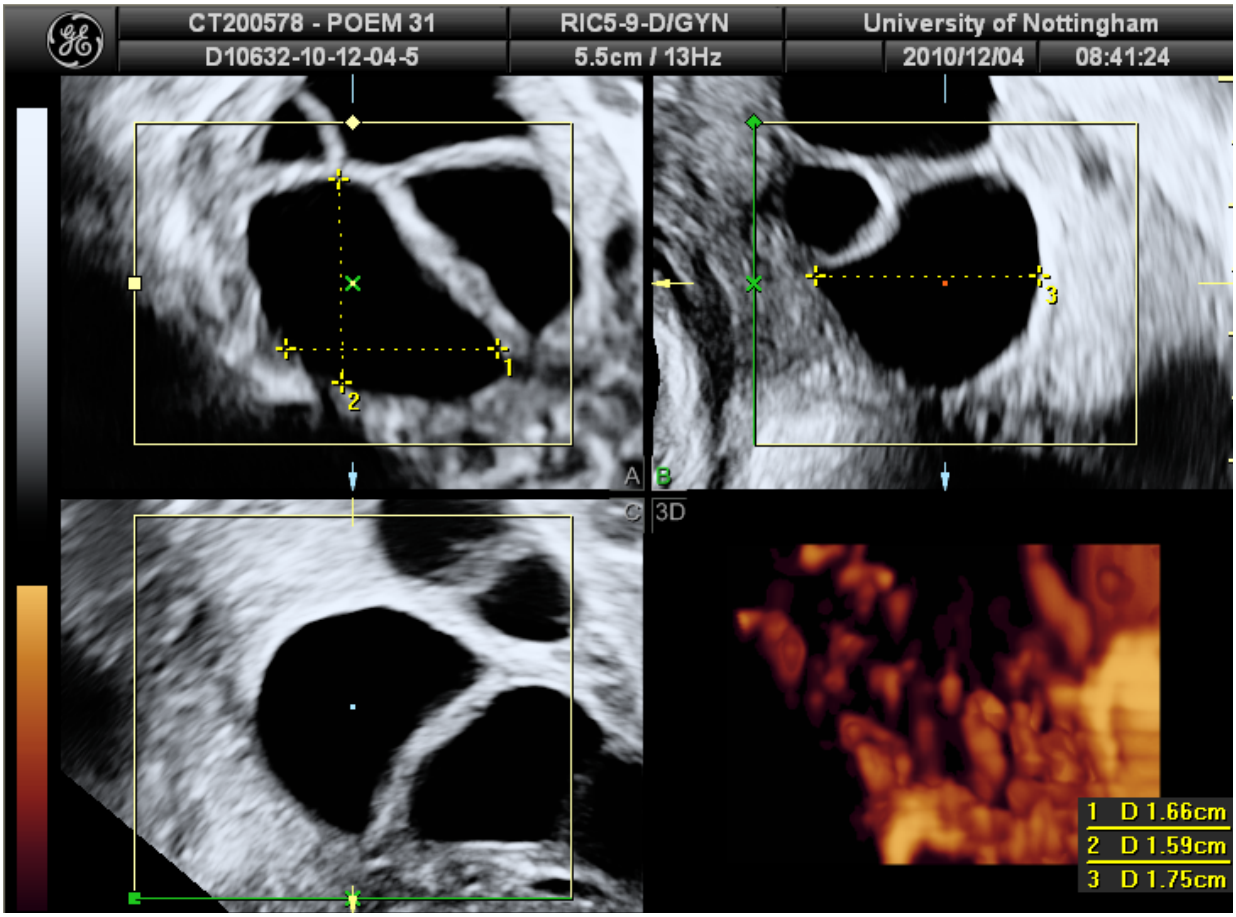

Figure 7.3. 3D power Doppler ultrasound scan of the ovarian follicle at hCG scan, later selected for aspiration

The measured follicular dimensions can be seen in the lower right hand corner. Mean follicular diameter was 1.66cm, and volume 2.59cm³.

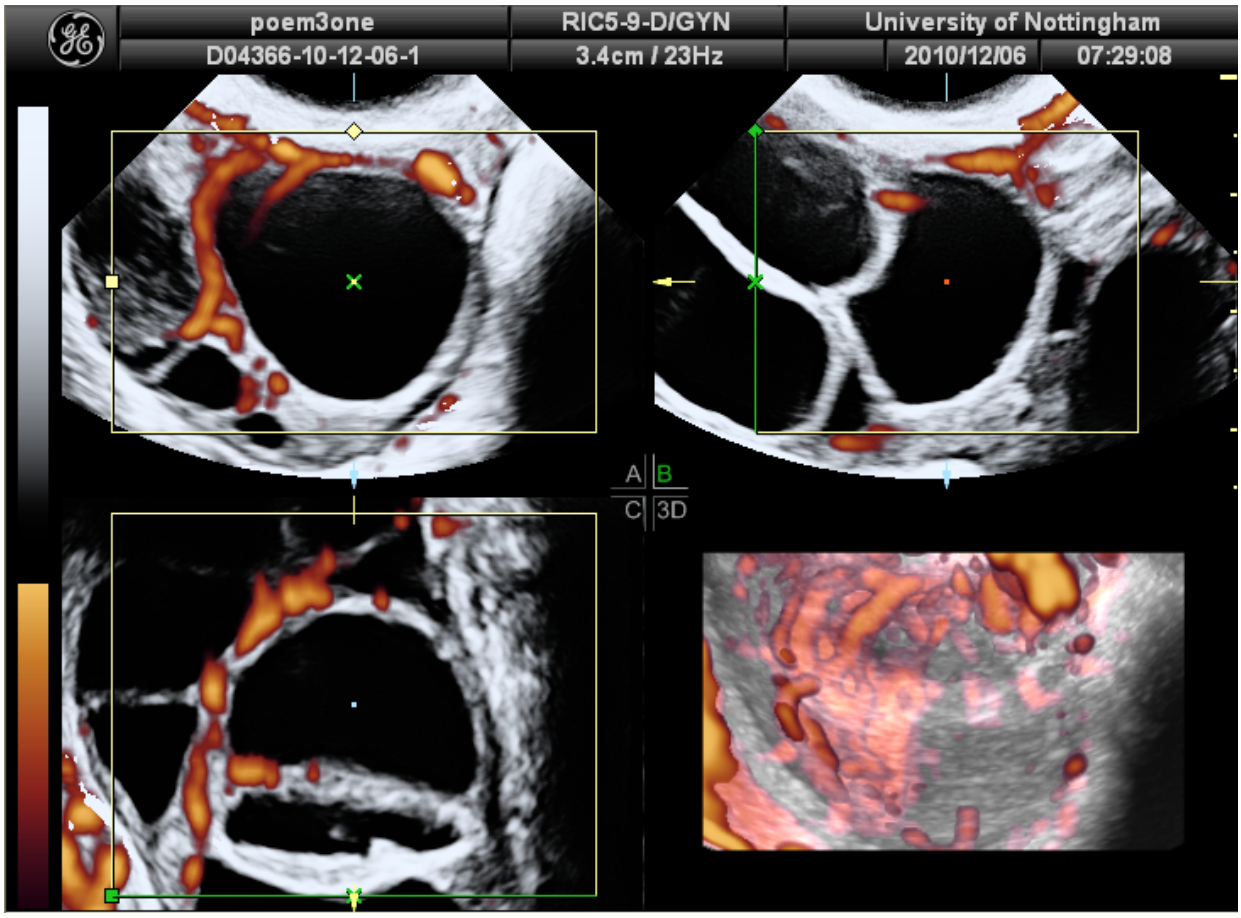

Figure 7.4. 3D power Doppler ultrasound scan of the ovarian follicle selected for aspiration at egg collection

The follicle had a mean diameter of 1.83cm and a volume of 3.32cm³.

A total of 9 oocytes were retrieved at egg collection, 6 of which were found to be metaphase II and suitable for ICSI. Just 1 of these oocytes fertilized successfully, and developed to a 4 cell, grade I embryo by day 2 of culture. This embryo was transferred, and a biochemical pregnancy test performed after approximately 7 days, the result of which was positive. The patient then returned to the clinic for an early pregnancy scan after 8 weeks, which revealed the foetal heartbeat of a singleton uterine pregnancy, with a crown rump length of 17mm, which is due to be born on 27th August of this year. This is the first reported case of successful tracking of a human pregnancy from hCG scan through to 8 weeks gestation.

7.5.2. Power Doppler Ultrasound

The data from prehCG and egg collection ultrasound measured vascular indices and follicular measurements are shown in table 7.1 below.

PrehCG						
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Oocyte retrieved	13	8.270 ± 1.90	30.177 ± 1.99	2.886 ± 0.74	19.56 ± 1.28	5.35 ± 1.46
Oocyte not retrieved	4	7.039 ± 2.88	30.473 ± 3.24	2.404 ± 1.21	19.70 ± 2.59	5.39 ± 2.44
T-test P value		0.38	0.47	0.38	0.48	0.5
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Fertilisation	7	10.984 ± 2.56	34.297 ± 2.24	4.037 ± 1.06	18.43 ± 0.43	3.89 ± 0.41
No fertilisation	6	5.102 ± 2.42	25.370 ± 2.23*	1.543 ± 0.75*	20.88 ± 2.74	7.06 ± 3.13
T-test P value		0.06	***0.01**	***0.05**	0.18	0.15
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Transferred	2	7.989 ± 5.56	31.725 ± 3.50	2.733 ± 2.08	17.42 ± 0.85	2.96 ± 0.38
Not transferred	19	8.608 ± 1.62	30.680 ± 1.59	3.002 ± 0.62	19.52 ± 1.08	5.26 ± 1.17

Egg Collection						
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Oocyte retrieved	20	11.031 ± 1.44	35.055 ± 1.09	4.080 ± 0.63	22.33 ± 1.11	7.53 ± 1.60
Oocyte not retrieved	6	11.725 ± 2.43	38.939 ± 3.27	4.693 ± 1.07	20.74 ± 1.93	6.33 ± 2.08
T-test P value		0.41	0.08	0.32	0.25	0.35
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Fertilisation	12	9.645 ± 1.46	35.464 ± 0.84	3.495 ± 0.55	21.63 ± 1.15	6.18 ± 0.88
No fertilisation	8	12.935 ± 2.80	34.493 ± 2.44	4.885 ± 1.30	23.38 ± 2.23	9.55 ± 3.82
T-test P value		0.34	0.15	0.12	0.23	0.14
	n	Mean VI	Mean FI	Mean VFI	Mean D	Mean vol
Transferred	3	8.329 ± 3.32	35.771 ± 2.51	3.080 ± 1.33	23.96 ± 3.21	7.81 ± 2.94
Not transferred	25	10.724 ± 1.19	35.997 ± 1.08	4.036 ± 0.52	21.97 ± 0.97	7.36 ± 1.25

Table 7.1. Power Doppler ultrasound and follicular size parameters measured at prehCG scan and egg collection, grouped according to oocyte retrieval, fertilisation and embryo transfer (± SEM). n = Number of replicates, VI = Vascular Index, FI = Flow Index, VFI = Vascular Flow Index, D = Mean follicular diameter in mm and vol = Follicular volume in cm^3.

7.5.3. Oocyte Retrieval

The relationships between follicular size and ultrasound vascular indices with oocyte retrieval were examined as seen in figures 6.5 to 6.9 below. None of the measures of follicular size or peri-follicular blood flow at prehCG or egg collection were significantly different in follicles from which an oocyte was retrieved.

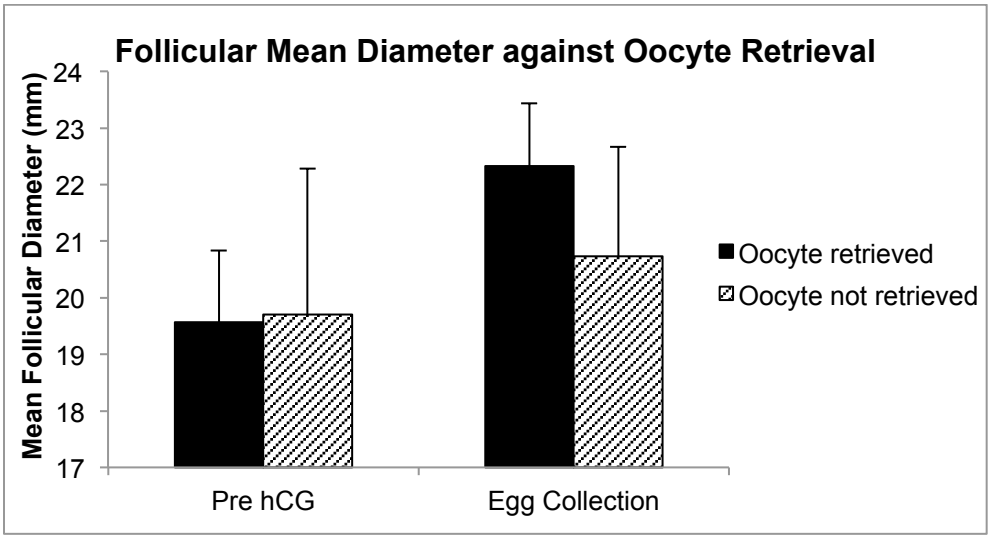

Figure 7.5. Mean follicular diameter against oocyte retrieval

Error bars indicate SEM.

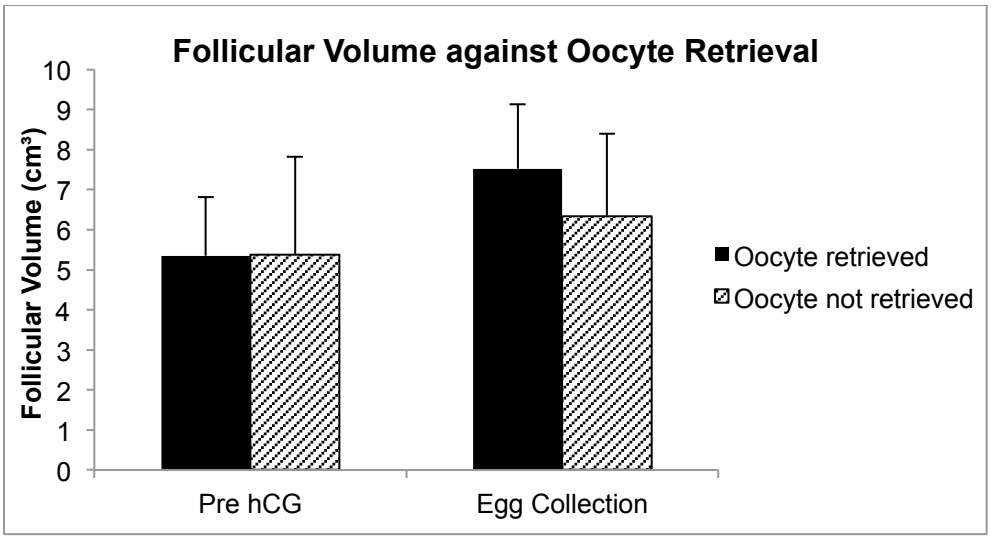

Figure 7.6. Follicular volume against oocyte retrieval

Error bars indicate SEM.

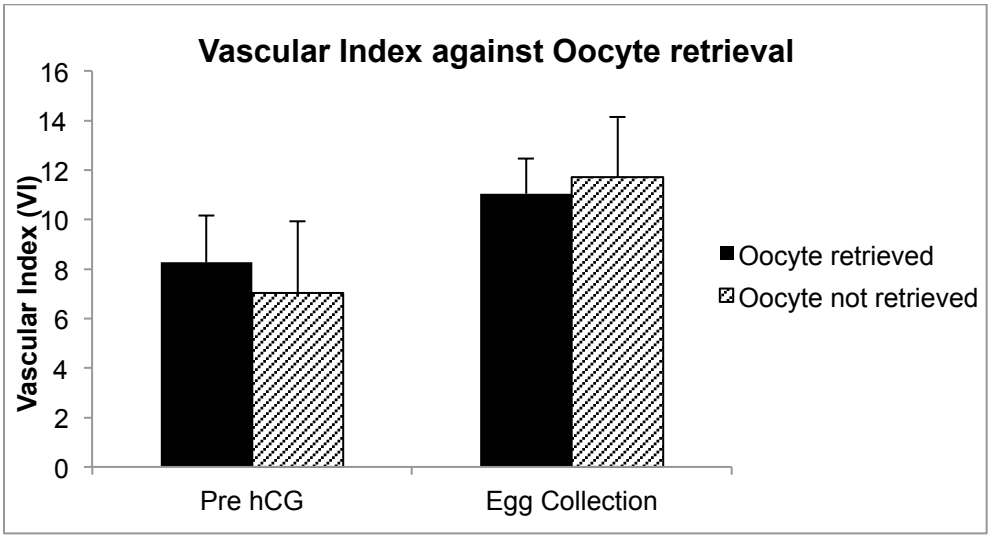

Figure 7.7. Vascular index against oocyte retrieval

Error bars indicate SEM.

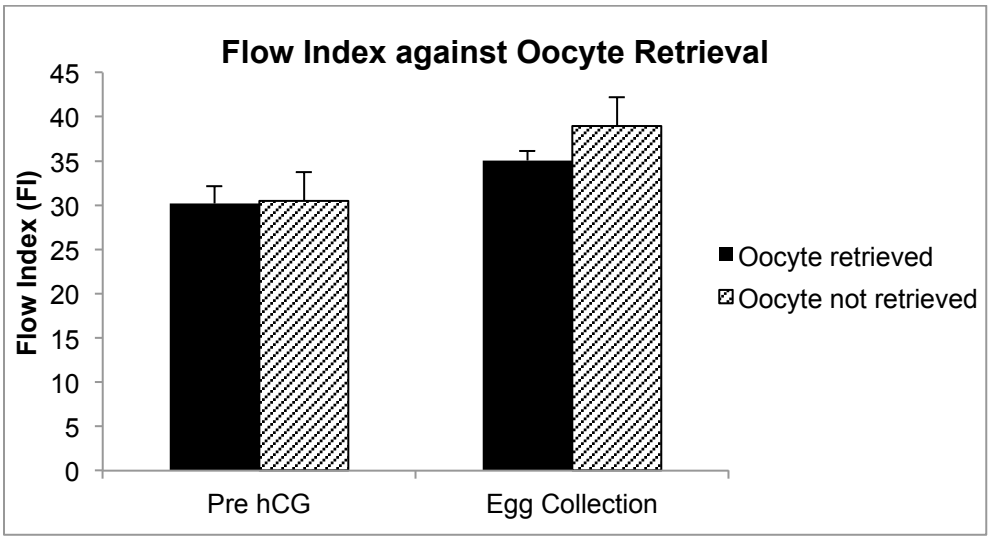

Figure 7.8. Flow index against oocyte retrieval

Error bars indicate SEM.

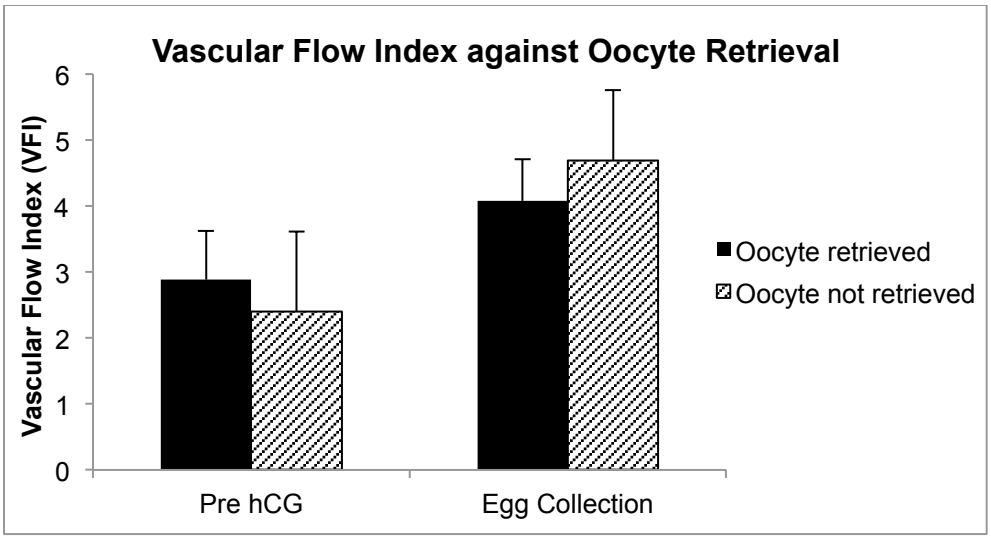

Figure 7.9. Vascular flow index against oocyte retrieval

Error bars indicate SEM.

7.5.4. Oocyte Fertilisation

The relationships between follicular size and the ultrasound vascular indices with oocyte fertilisation were examined. Measures of follicular size were not significantly related to oocyte fertilisation, although oocytes that were not fertilised did tend to come from follicles with a larger volume, as measured at both prehCG and egg collection (figure 7.11).

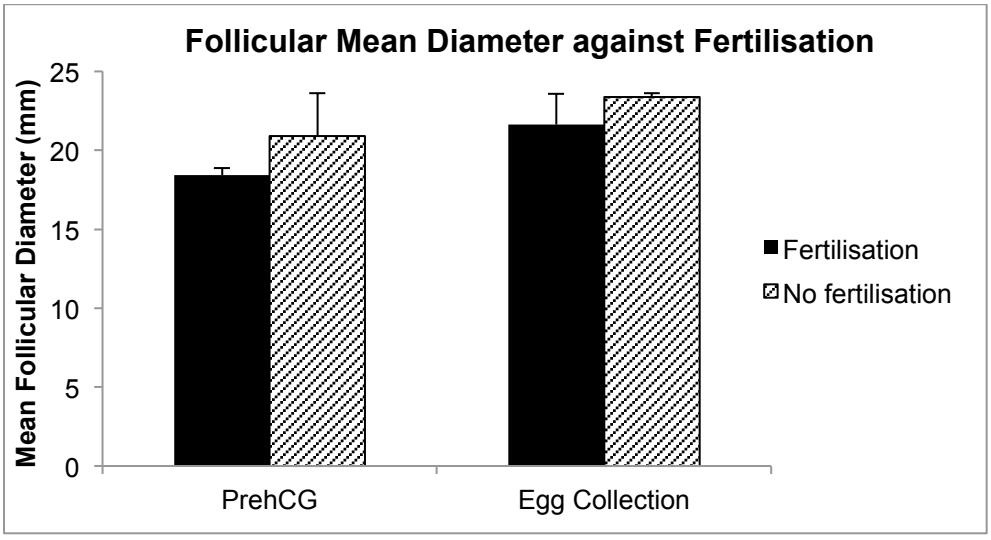

Figure 7.10. Mean follicular diameter against ooyte fertilisation

Error bars indicate SEM.

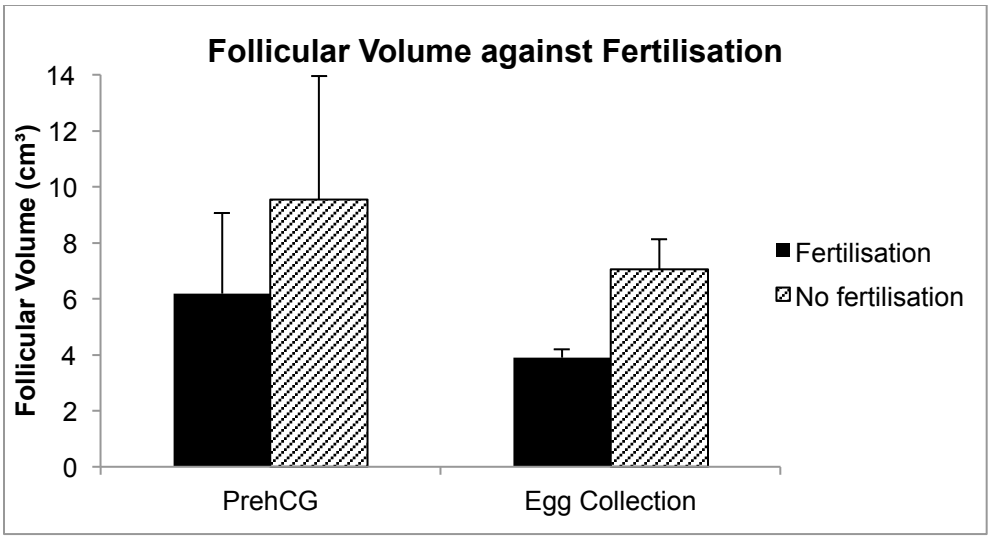

Figure 7.11. Follicular volume against oocyte fertilisation

Error bars indicate SEM.

Fertilisation rates were compared in relation to the vascular indices measured at prehCG and egg collection, as seen in figures 7.12 to 7.14 below. Vascular index was slightly lower at prehCG scan for follicles that did not contain an oocyte that fertilised, although this was not significant (P<0.06) (figure 7.12). Flow index and vascular flow index were significantly lower at prehCG scan for follicles that contained an oocyte did not fertilise (figures 7.13 and 7.14) (P<0.01 and P<0.05 respectively).

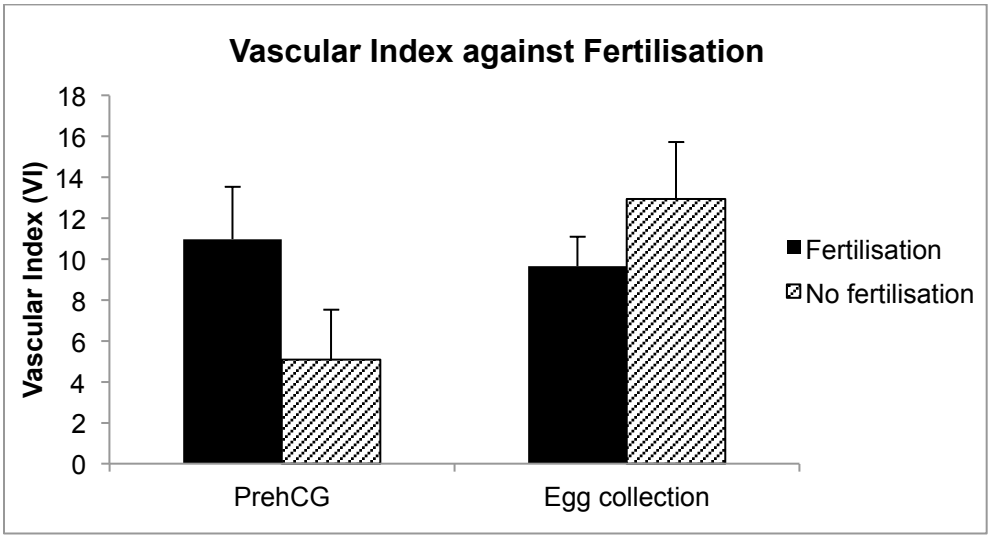

Figure 7.12. Vascular Index against oocyte fertilisation

Error bars indicate SEM.

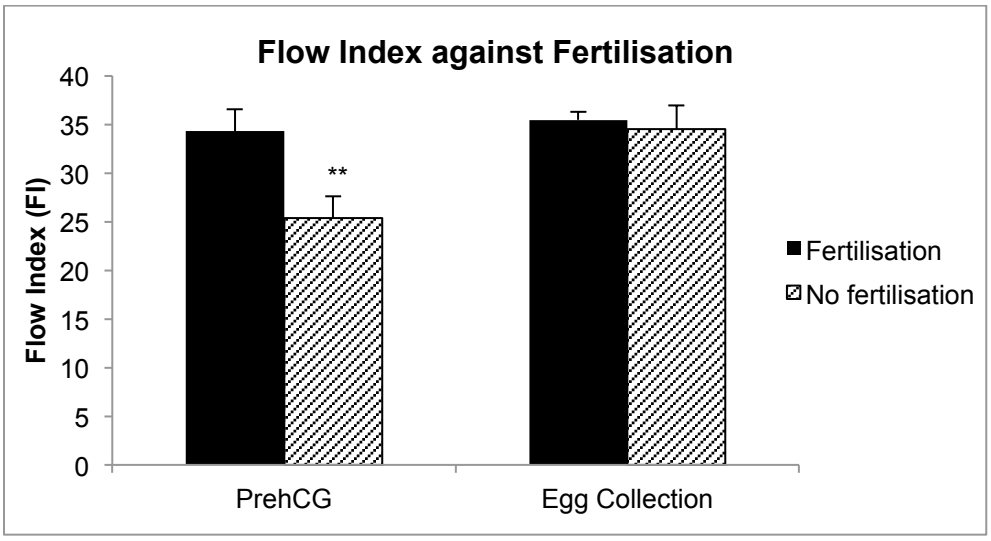

Figure 7.13. Flow Index against oocyte fertilisation

Error bars indicate SEM. ** P<0.01 compared to fertilised group.

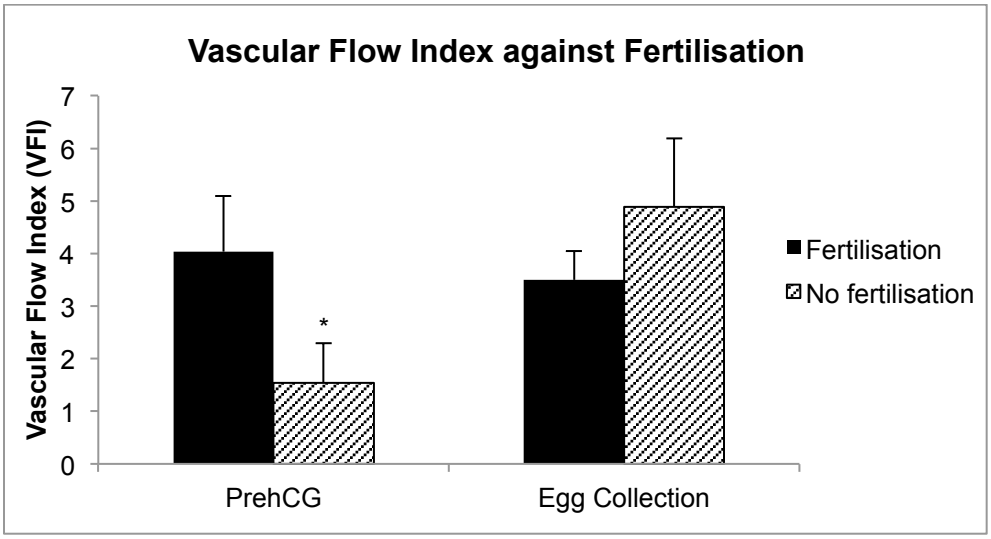

Figure 7.14. Vascular flow index against oocyte fertilisation

Error bars indicate SEM. * P<0.05 compared to fertilised group.

7.5.5. Embryo Transfer

The relationships between follicular size and ultrasound vascular indices with embryo transfer were examined. There was no significant relationship between follicular mean diameter and volume measured at prehCG and egg collection with embryo transfer (figures 7.15 to 7.19), however a total of just 3 of the embryos were transferred, and so statistical power was lacking.

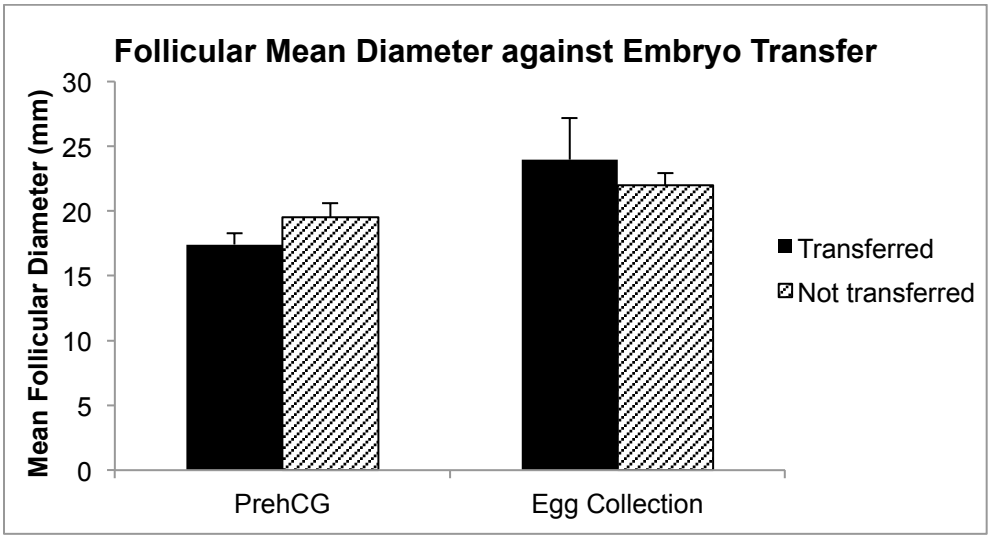

Figure 7.15. Mean follicular diameter against embryo transfer

Error bars indicate SEM.

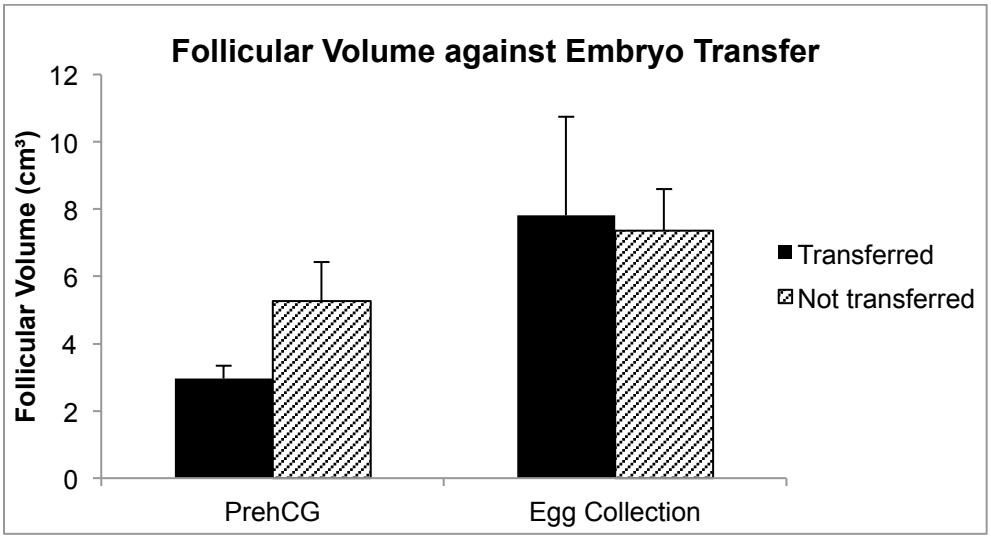

Figure 7.16. Follicular volume against embryo transfer

Error bars indicate SEM.

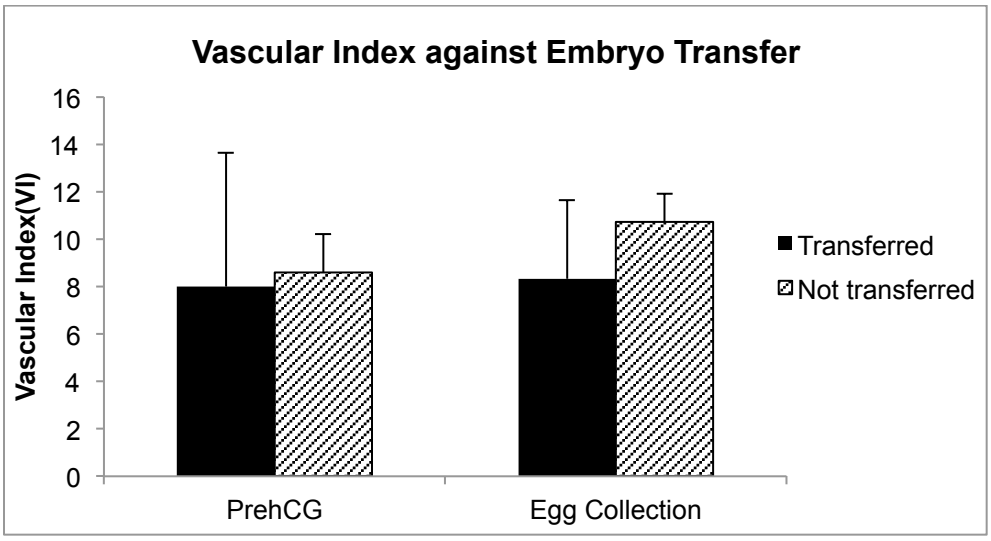

Figure 7.17. Vascular index against embryo transfer

Error bars indicate SEM.

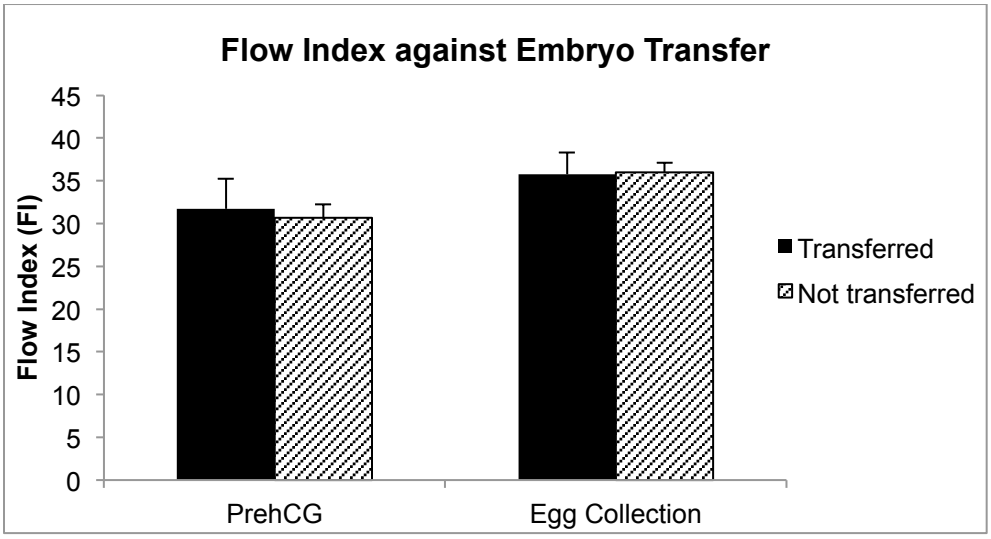

Figure 7.18. Flow index against embryo transfer

Error bars indicate SEM.

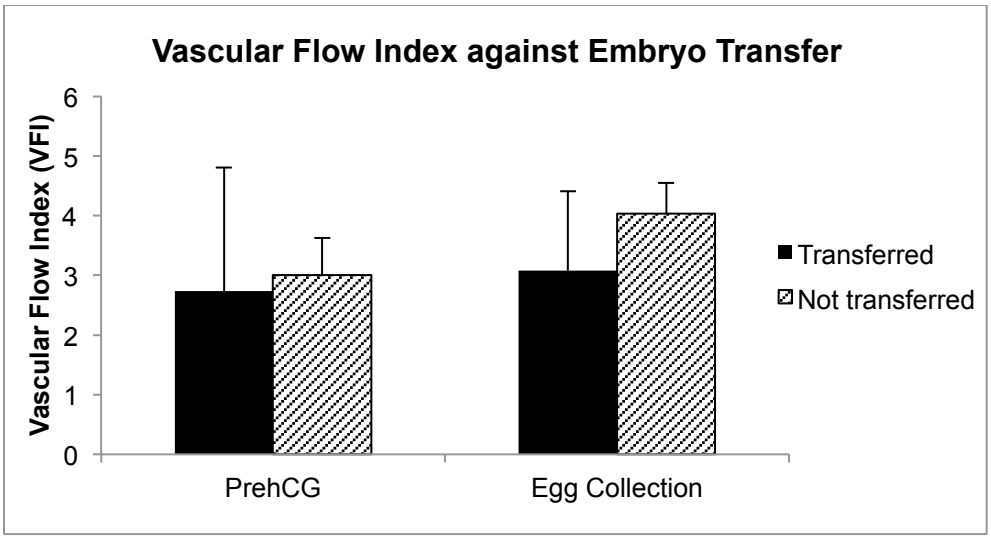

Figure 7.19. Vascular flow index against embryo transfer

Error bars indicate SEM.

7.5.6. Follicular Growth

The growth of the selected ovarian follicle was assessed by calculating the difference between the measurements at pre hCG scan with those at egg collection, thereby determining how to what degree the follicles increased in size following administration of hCG. Follicular growth was examined both in terms of mean diameter (figure 7.20) and volume (figure 7.21). Although it seemed that follicular growth from prehCG to egg collection was negatively correlated to oocyte retrieval and fertilisation, these relationships were not significant.

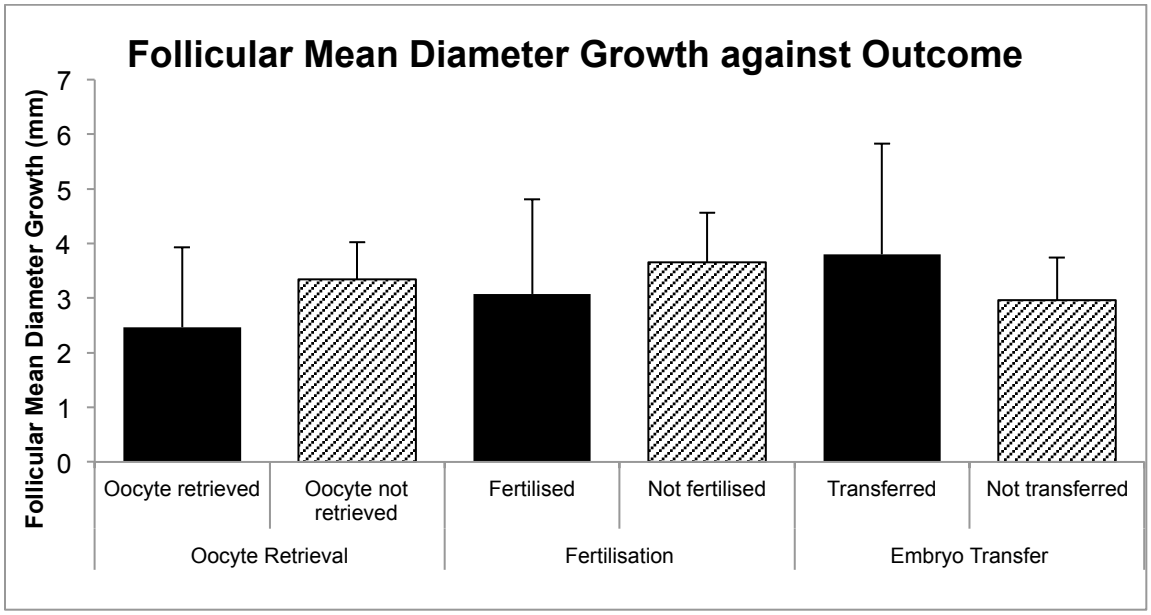

Figure 7.20. Follicular mean diameter growth against oocyte retrieval, fertilisation and embryo transfer

Error bars indicate SEM.

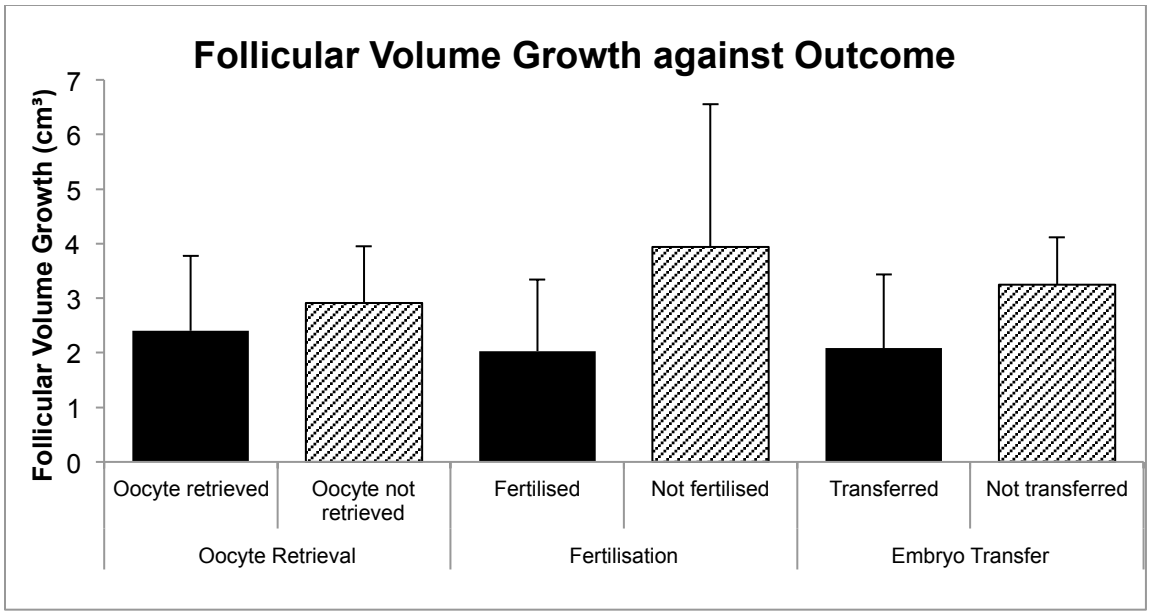

Figure 7.21. Follicular volume growth against oocyte retrieval, fertilisation and embryo transfer

Error bars indicate SEM.

The follicular volume was plotted against the mean follicular diameter for all follicles at prehCG and egg collection, to investigate the relationship between the two (figure 7.22). It was found that a third order polynomial relationship exists between the mean follicular diameter and the follicular volume.

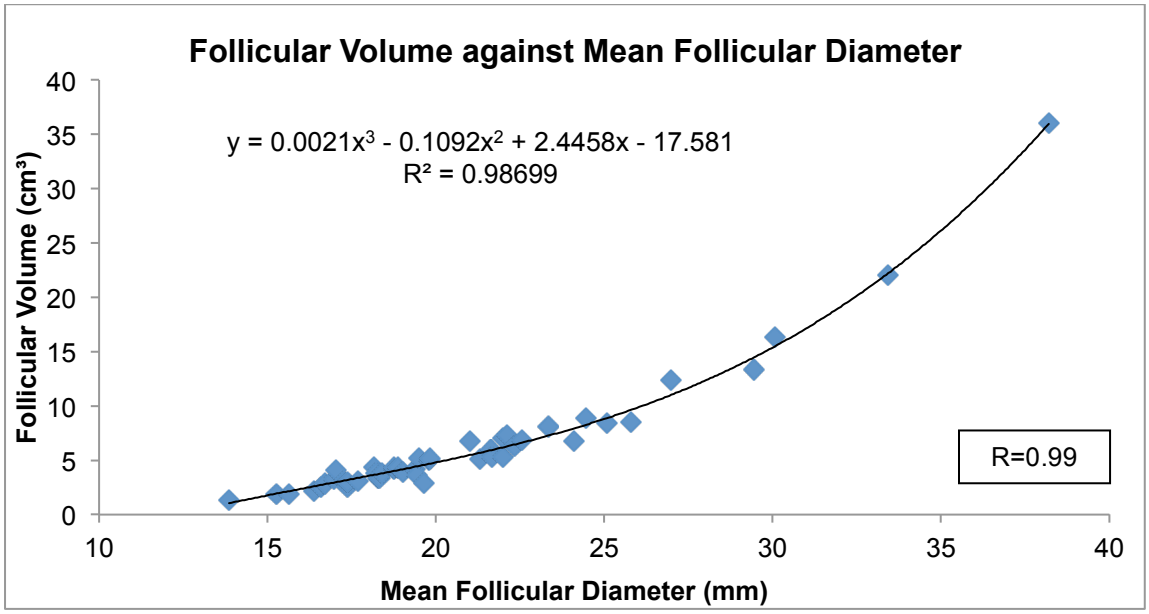

Figure 7.22. Mean follicular diameter against follicular volume

7.5.7. Patient Age

The patient age was compared to the developmental stage achieved by the selected follicle (table 7.2 and figure 7.23). None of these relationships were found to be significant.

	Oocyte retrieved	No oocyte	Fertilisation	No fertilisation	Embryo transferred	Embryo not transferred
Mean age	34.6 ± 0.97	33.0 ± 4.00	34.6 ± 1.29	35.4 ± 1.93	35.0 ± 2.08	34.4 ± 1.73
n	21	2	12	7	3	8

Table 7.2. Mean patient age of oocyte retrieval, fertilisation and embryo transfer (±SEM).

There was no significant correlation found between patient age and developmental stage. Developmental stage checkpoints are outlined in table 7.3 below.

Stage	Developmental Stage
0	No oocyte collected
1	MII oocyte
2	Fertilisation
3	4 cell @ day 2
4	<6 cells @ day 3
5	6 cells @ day 3
6	>6 cells @ day 3
7	Early blastocyst
8	Expanding blastocyst
9	Complete blastocyst

Table 7.3. Summary of numbered developmental stages according to the final follicle outcome. Grey boxes indicate final endpoints as transferrable embryos and blastocyst.

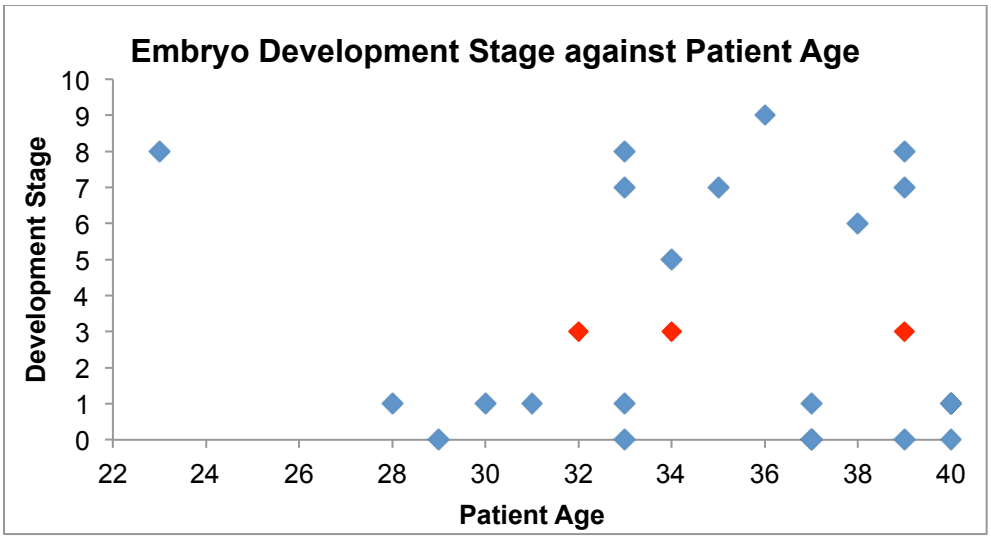

Figure 7.23. Patient age against oocyte development stage

Red markers indicate embryos that were transferred.

7.5.7.1. Follicular size and vascular indices

The patient age was compared to the mean follicular diameter and volume measured on ultrasound. A line of best fit revealed a very slight positive trend in both cases, however these were not significant and the slope of the trendlines is almost flat, therefore these graphs are not shown. This would be an expected result as there should not be a correlation between age and follicular size due to the fact that hCG is administered only when a minimum of 3 follicles reach a target size of 18mm, regardless of patient age.

Follicular mean diameter and volume growth from prehCG to egg collection was compared to patient age. Although the lines of best fit suggested a positive relationship to follicular mean diameter growth, this was not statistically significant (data not shown).

Ultrasound measured vascular indices were compared to patient age, at both prehCG and egg collection scans (figure 7.29). Linear regression analysis revealed a significant negative relationship between vascular index at prehCG and patient age (figure 7.29a; $P<0.05$). The same relationship was observed for vascular flow index at prehCG scan (figure 7.29c; $P<0.05$) but not for flow index (figure 7.29b).

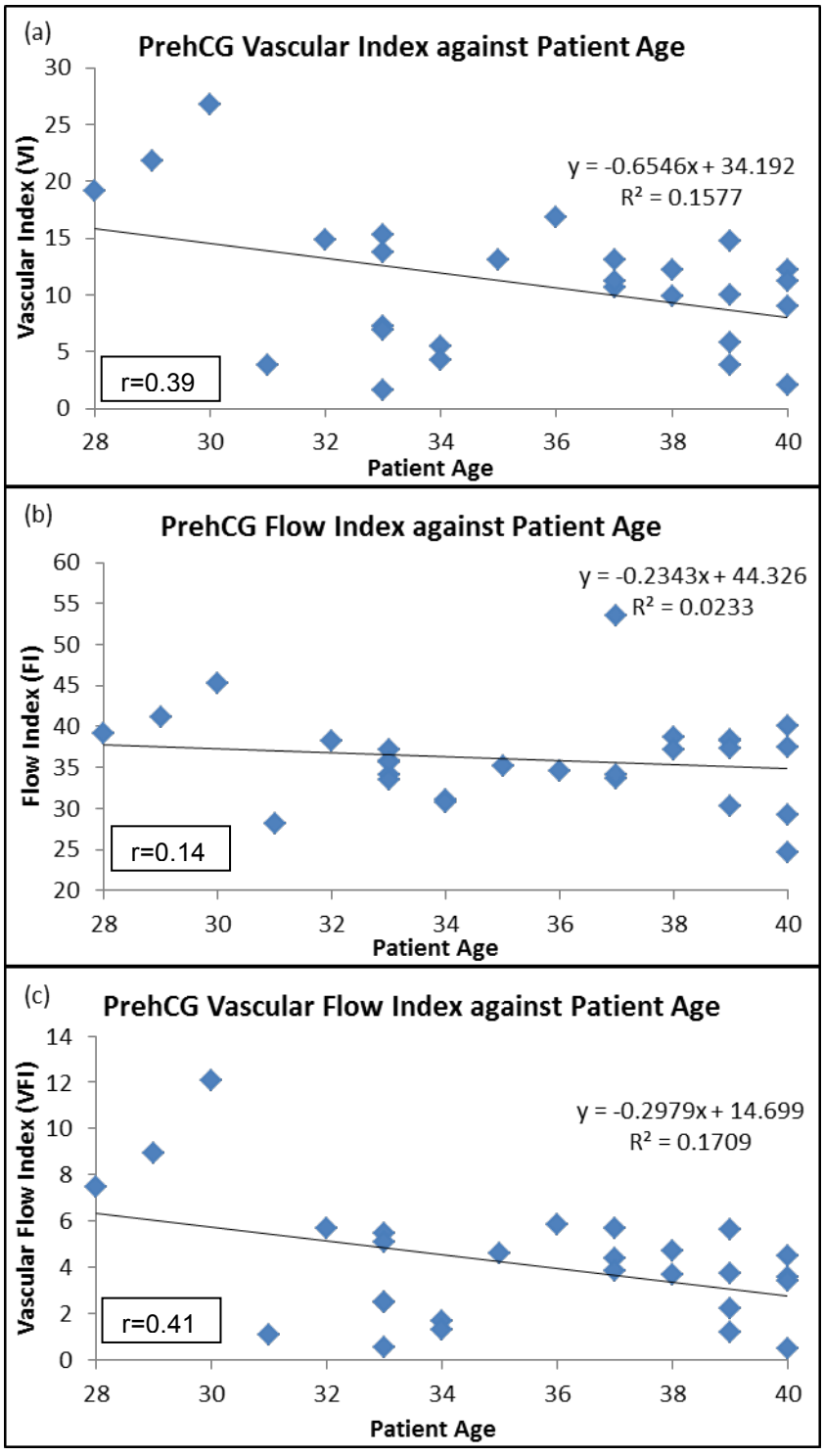

Figure 7.24. PrehCG vascular indices against patient age

The ultrasound vascular indices were also compared to patient age at egg collection and although negative trends were observed between VI and VFI and patient age were observed, these were not significant. As a result the graphs are not shown.

7.5.7.2. Follicular Fluid Constituents

Neither oestradiol, progesterone, AMH or BMP15 concentrations in the follicular fluid were significantly associated with patient age when grouped into ages 30 years and under (n=4), 31 to 35 (n=8), and greater than 35 years of age (n=9) (figure 7.25). GDF9 was unable to be measured in follicular fluid as concentrations were below detectable levels.

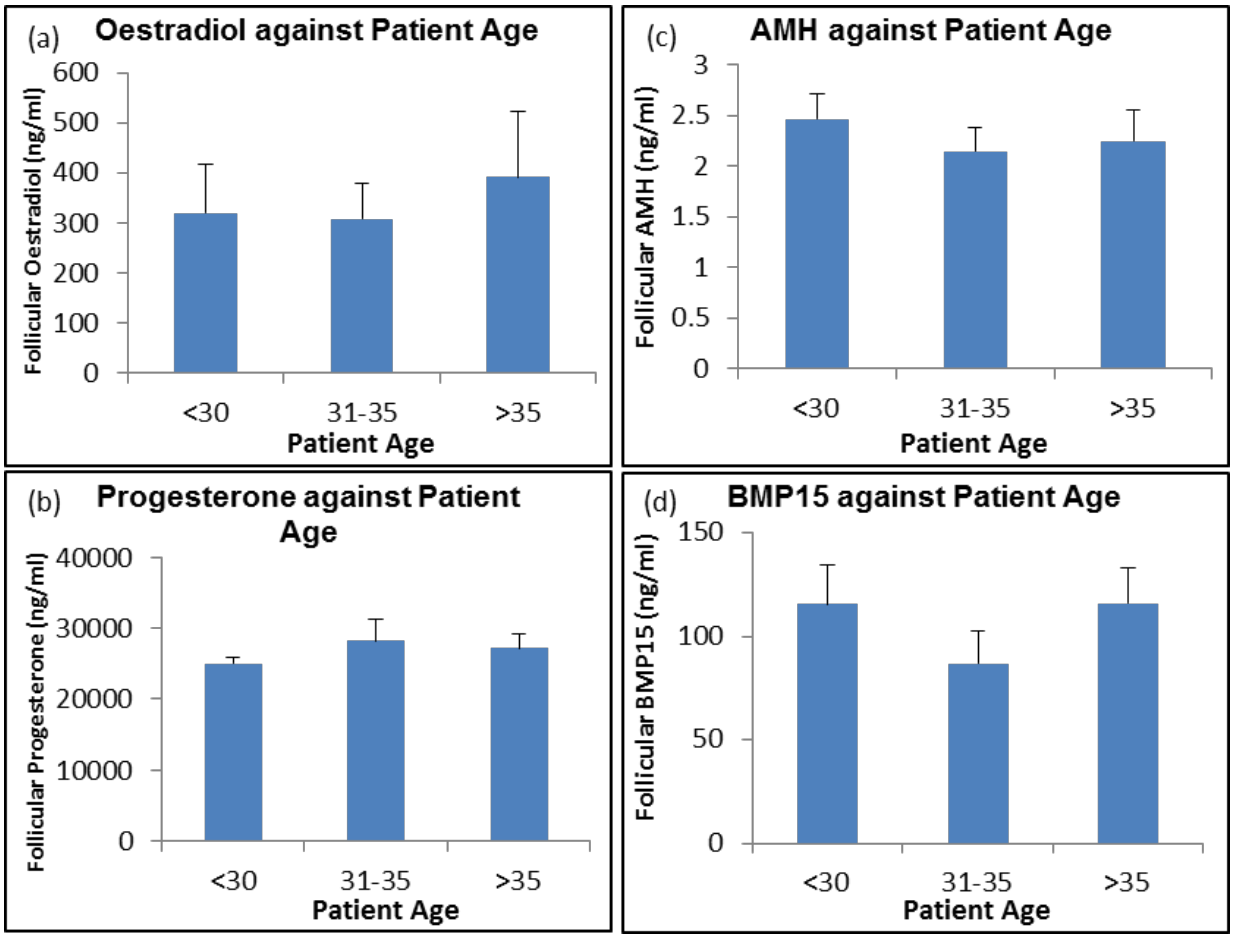

Figure 7.25. Follicular fluid (a) oestradiol, (b) progesterone, (c) AMH and (d) BMP15 concentrations against grouped patient age

Error bars indicate SEM.

7.5.8. Follicular Aspiration

The number of aspirations (and flushes) required to retrieve an oocyte was compared with the developmental stage achieved by the resulting embryo, as outlined in table 7.3, in order to ascertain whether flushing of the follicle is detrimental to the oocyte within. There was no significant correlation between number of follicular aspirations and the development of the embryo (figure 7.26).

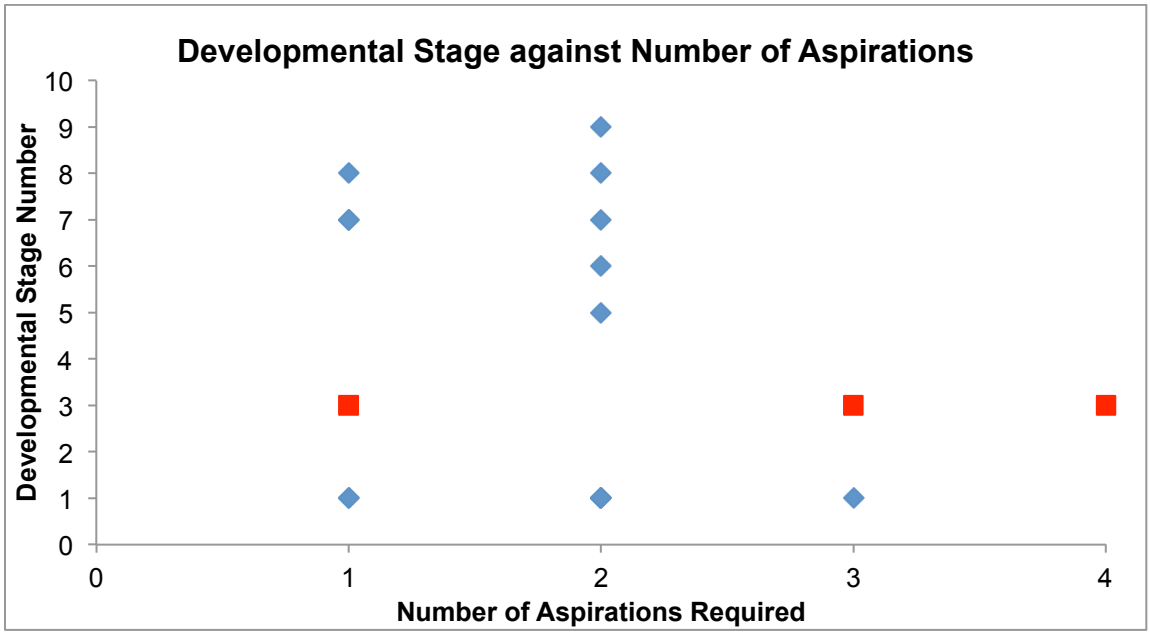

Figure 7.26. Developmental stage outcome against number of aspirations required to obtain an oocyte

Red squares indicate transferred embryos.

7.5.9. Follicular Fluid Steroid Hormones

Following measurement of oestradiol and progesterone in the follicular fluid by ELISA, the concentrations were compared to oocyte fertilisation and embryo transfer, follicular size and growth, and finally patient age. Comparison to oocyte retrieval could not be made because only one of the follicular fluid samples came from a patient from which an oocyte could not been collected.

Figure 7.27 below shows the oestradiol and progesterone concentration in the follicular fluid against oocyte fertilisation. No significant relationship was observed between oestradiol and fertilisation, although oestradiol concentration was higher in the follicular fluid of oocytes that fertilised successfully.

Progesterone concentration in the follicular fluid was significantly higher in the follicles which yielded an oocyte that fertilised successfully (P<0.05).

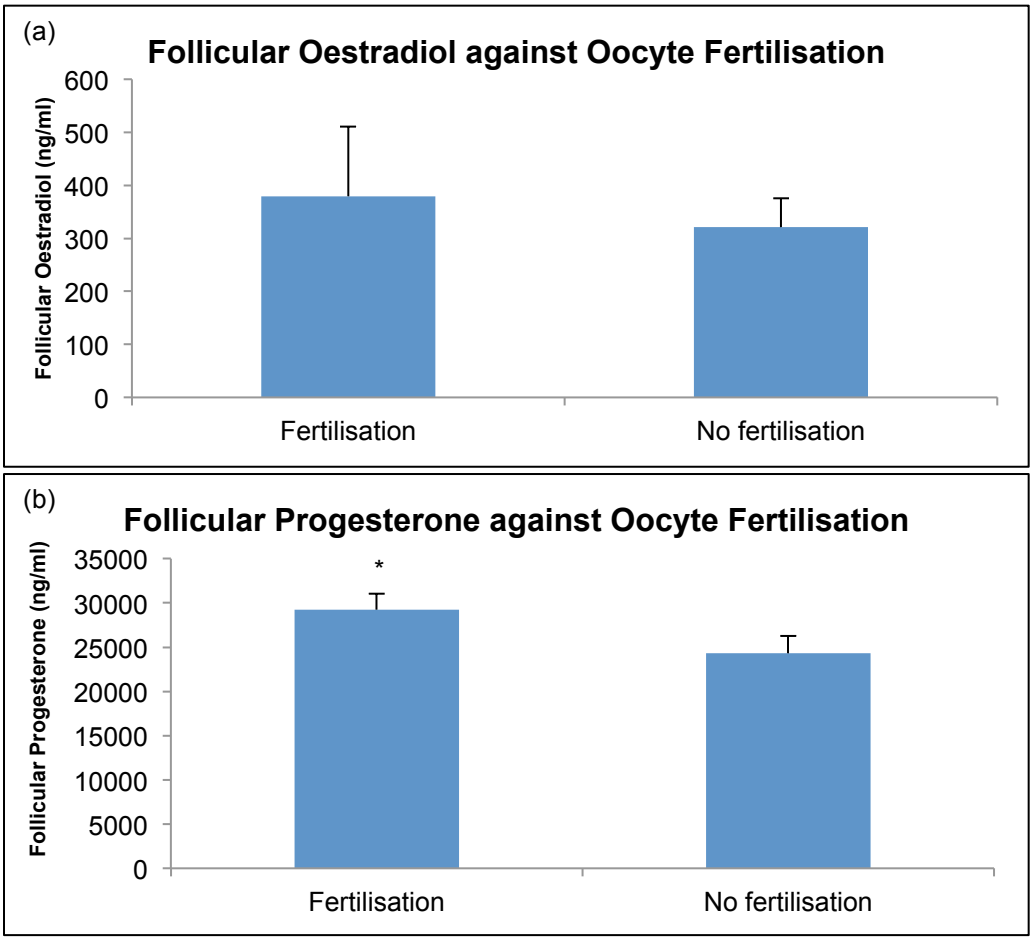

Figure 7.27. Follicular fluid (a) oestradiol and (b) progesterone against oocyte fertilisation

*P<0.05. Error bars indicate SEM.

Figure 7.28 shows the oestradiol and progesterone concentration in the follicular fluid against embryo transfer. Although the relationships observed were not significant probably due to small numbers, follicular oestradiol concentration was higher in the group of embryos that were transferred.

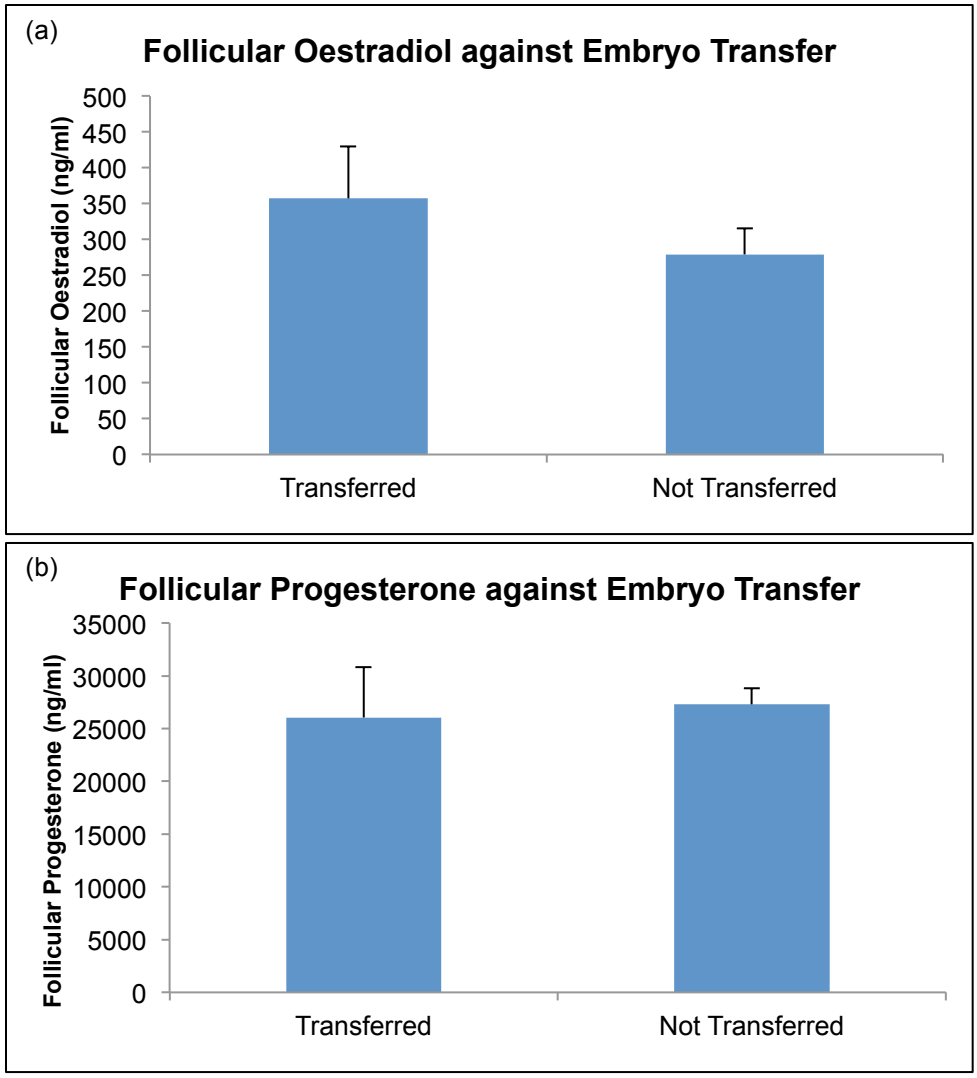

Figure 7.28. Follicular fluid (a) oestradiol and (b) progesterone against embryo transfer

Error bars indicate SEM.

Ratio of oestradiol to progesterone was compared to consequent oocyte fertilisation and embryo transfer, neither of which was found to be significant (figure 7.29). As concentrations of progesterone are approximately 1000 fold higher than those of oestradiol in the follicular fluid, the ratio of oestradiol in ng/ml to progesterone in µg/ml was used as described by Xia and Younglai (2000).

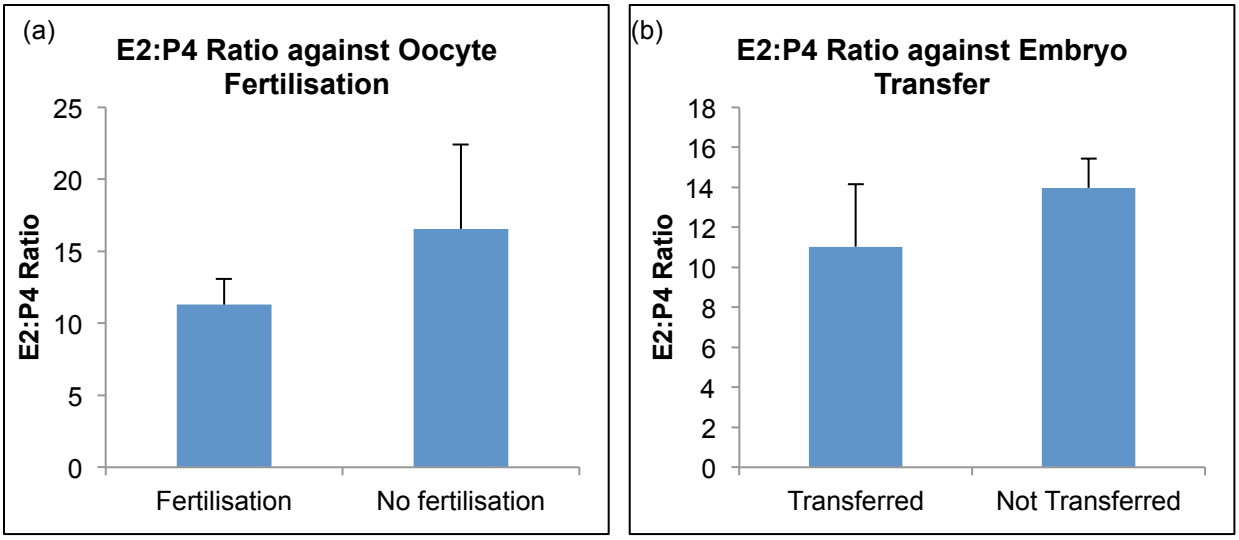

Figure 7.29. E2:P4 ratio against (a) oocyte fertilisation and (b) embryo transfer

Error bars indicate SEM.

The relationship between follicular fluid oestradiol and progesterone concentrations and follicular size parameters measured both at prehCG scan and egg collection was examined. Follicular oestradiol and follicular mean diameter at prehCG ultrasound scan were positively correlated according to a line of best fit second order polynomial regression (figure 7.30a). Follicular volume was also used as a proxy for follicular size, and the relationship to follicular fluid oestradiol was also examined (figure 7.30b). Linear regression analysis revealed that oestradiol was significantly positively correlated to follicular volume measured at prehCG ($P<0.01$).

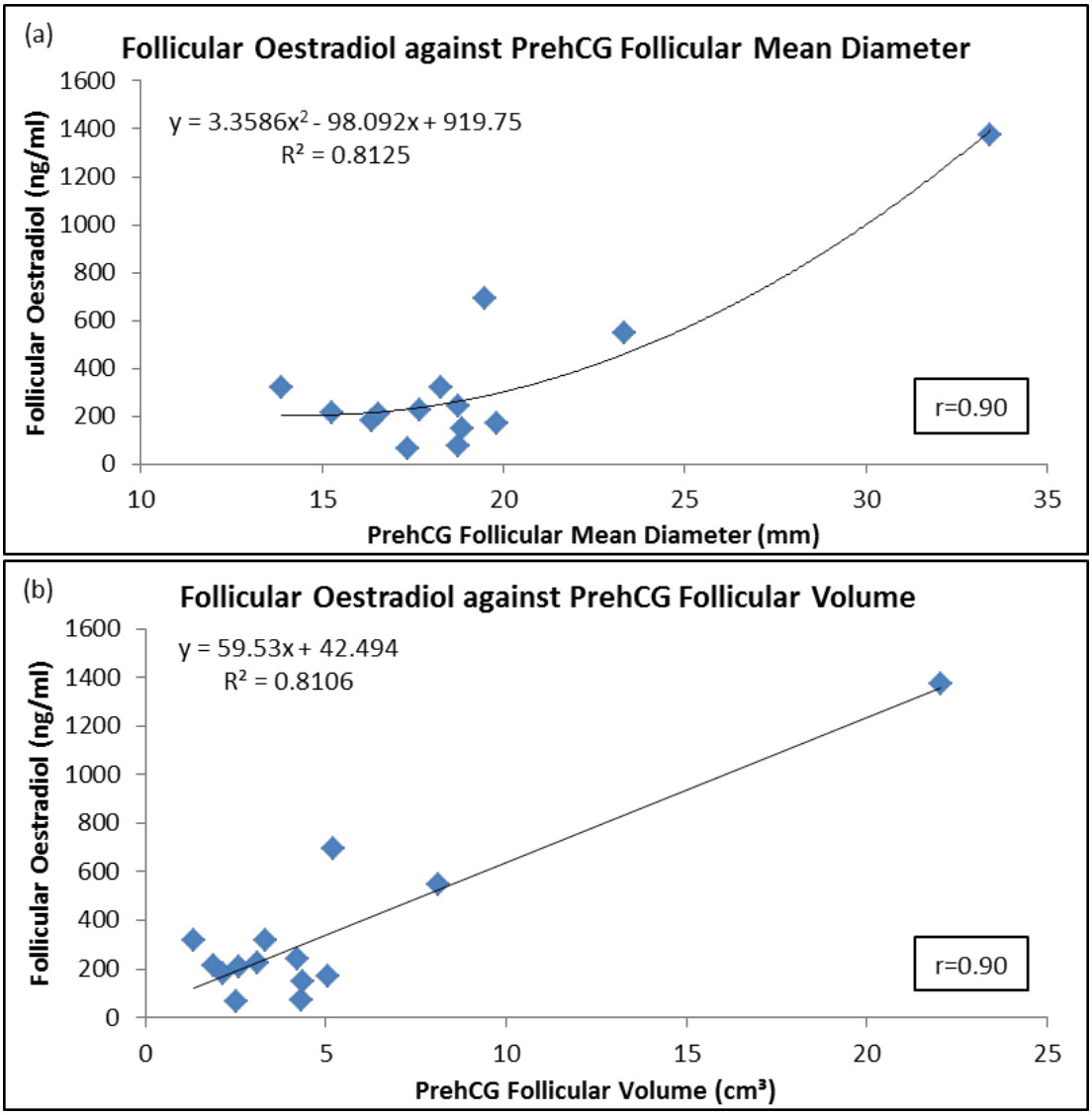

Figure 7.30. Follicular fluid oestradiol against prehCG follicular (a) mean diameter and (b) volume

The follicular mean diameter and volume was also measured at egg collection, and so the oestradiol concentration in the follicular fluid was also examined against these parameters. Again, oestradiol was found to positively correlate to follicular mean diameter and volume following the same trends observed at prehCG (data not shown).

Follicular fluid oestradiol concentration was compared to the growth of the follicles between prehCG and egg collection scans. Figure 7.31 below shows that there is no significant relationship to mean diameter growth, but a significant positive relationship to volume growth as analysed by linear regression ($P<0.05$).

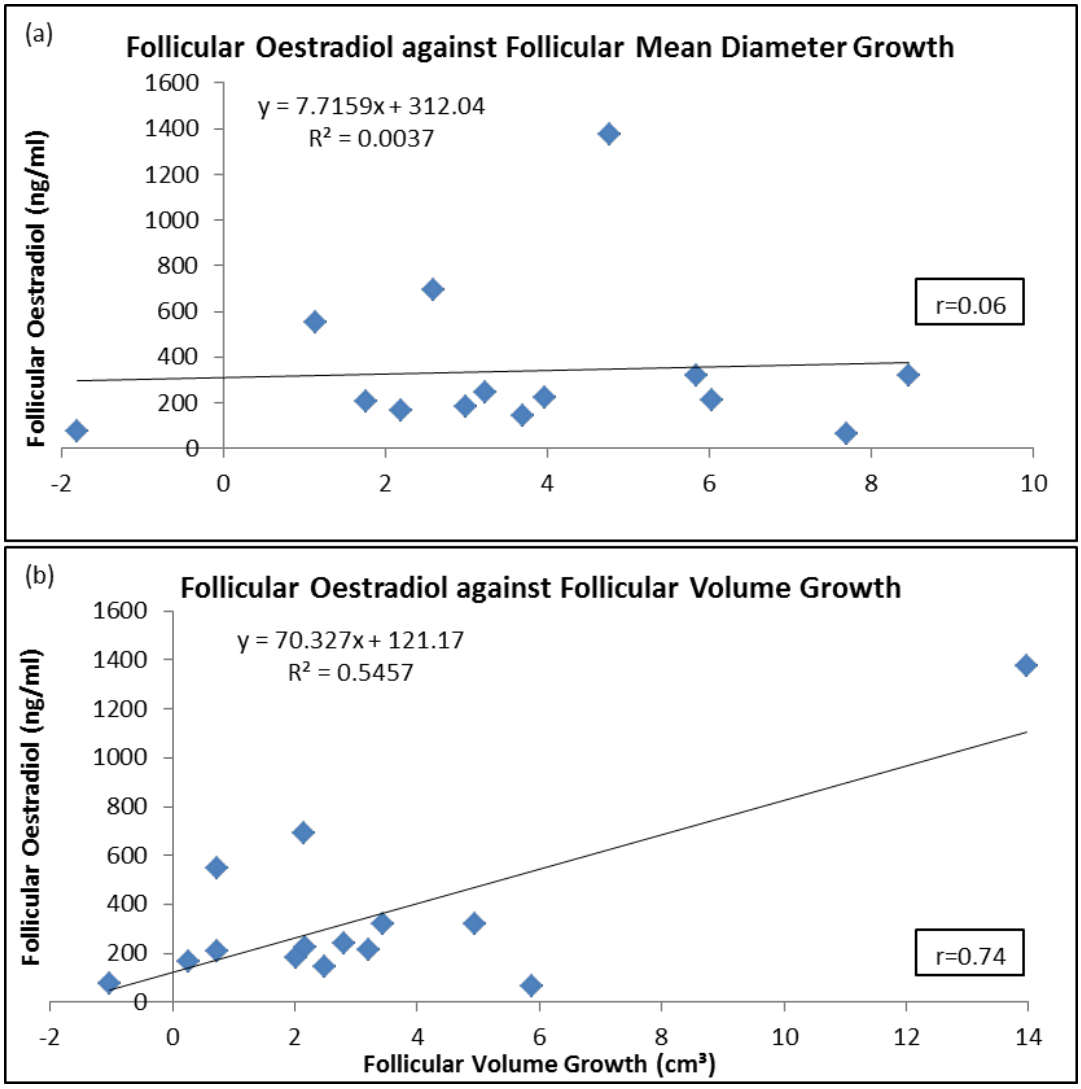

Figure 7.31. Follicular fluid oestradiol against (a) follicular mean diameter growth and (b) volume growth

Follicular fluid oestradiol had no correlation to vascular indices measured at prehCG and egg collection and so the data is not shown. Correlation coefficients were less than 0.31 in all cases.

Progesterone concentration in the follicular fluid was compared to follicular size and growth. There was found to be no significant correlation between progesterone and follicular size measured at prehCG and egg collection (correlation coefficients less than 0.14 in all cases), and no significant correlation to follicular growth (data not shown; correlation coefficients less than 0.21). Progesterone concentration also

had no correlation to vascular indices measured at prehCG and egg collection. Correlation coefficients to VI, FI and VFI were less than 0.45 in all cases.

7.5.10. Follicular Fluid TGFβ members

The TGFβ superfamily members AMH, BMP15 and GDF9 were measured in the follicular fluids collected at egg collection by ELISA in order to compare their concentration against oocyte and embryo development, as well as follicular size and growth between prehCG scan and egg collection.

7.5.10.1. AMH in Follicular Fluid

Intrafollicular AMH concentration was not significantly different in follicles that had successfully fertilised, nor in the follicles from which a transferred embryo originated (figure 7.32).

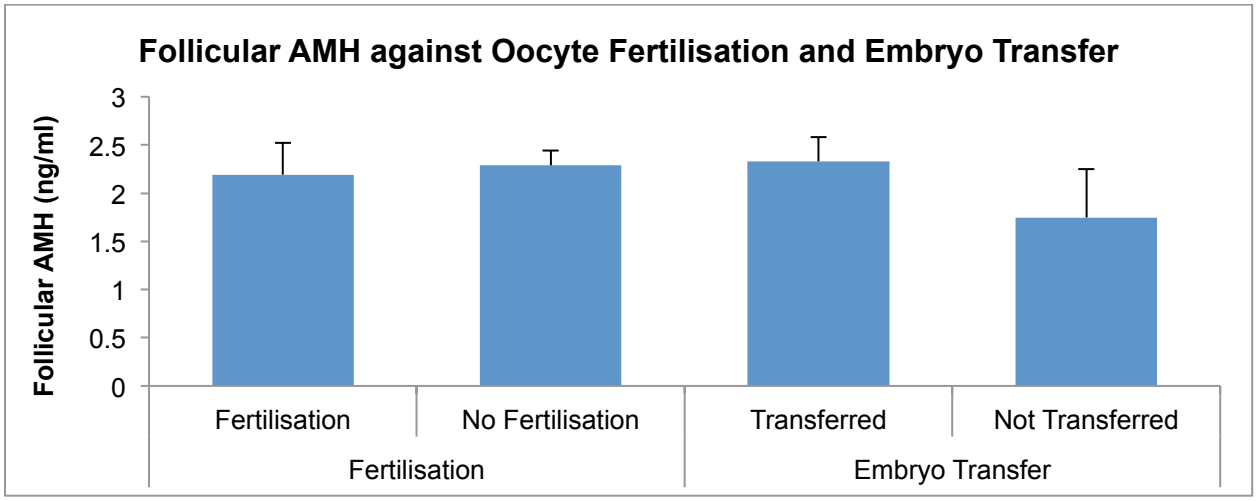

Figure 7.32. Follicular AMH against oocyte fertilisation and embryo transfer

Error bars indicate SEM.

AMH in the follicular fluid was compared to follicular mean diameter and volume, measured at prehCG scan and at egg collection (figure 7.33). Linear regression analysis showed that intrafollicular AMH concentration had a significantly positive linear correlation to mean diameter and volume at prehCG ($P<0.01$ in both cases; volume data not shown).

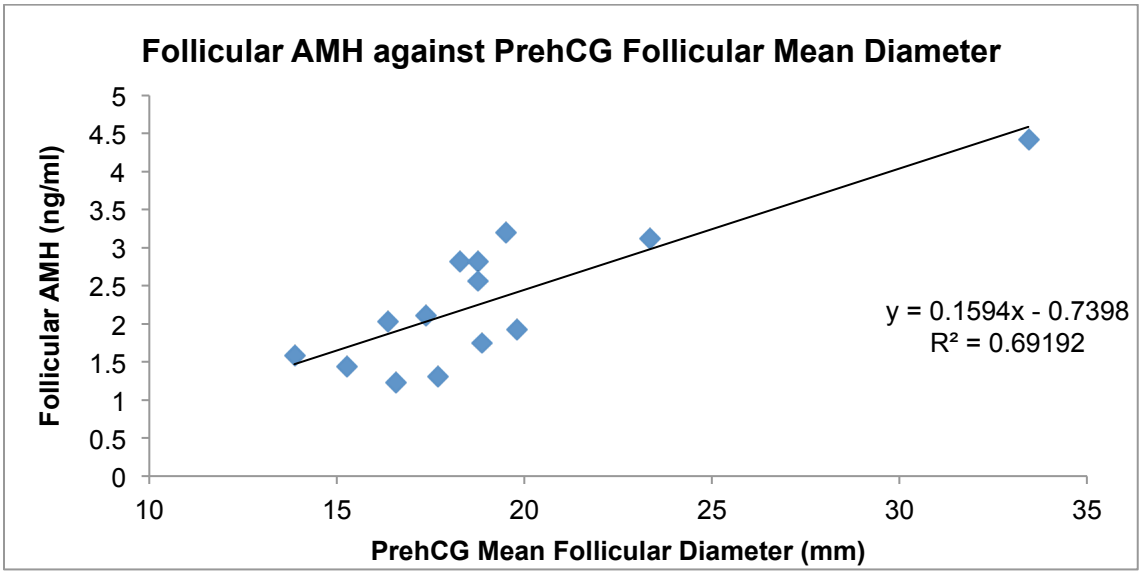

Figure 7.33. Follicular AMH against prehCG follicular mean diameter

Follicular fluid AMH concentration was compared to prehCG follicle size groups of <17mm, 17-18mm, 18-19mm and >19mm (figure 7.34a). The larger follicle diameter groups were found to have significantly higher AMH levels than those of a smaller size. When comparing to follicular volume, intrafollicular AMH concentration was significantly different only between the lowest and highest prehCG follicle volume groups (<3cm³ and >5cm³; figure 7.34b) (P<0.01).

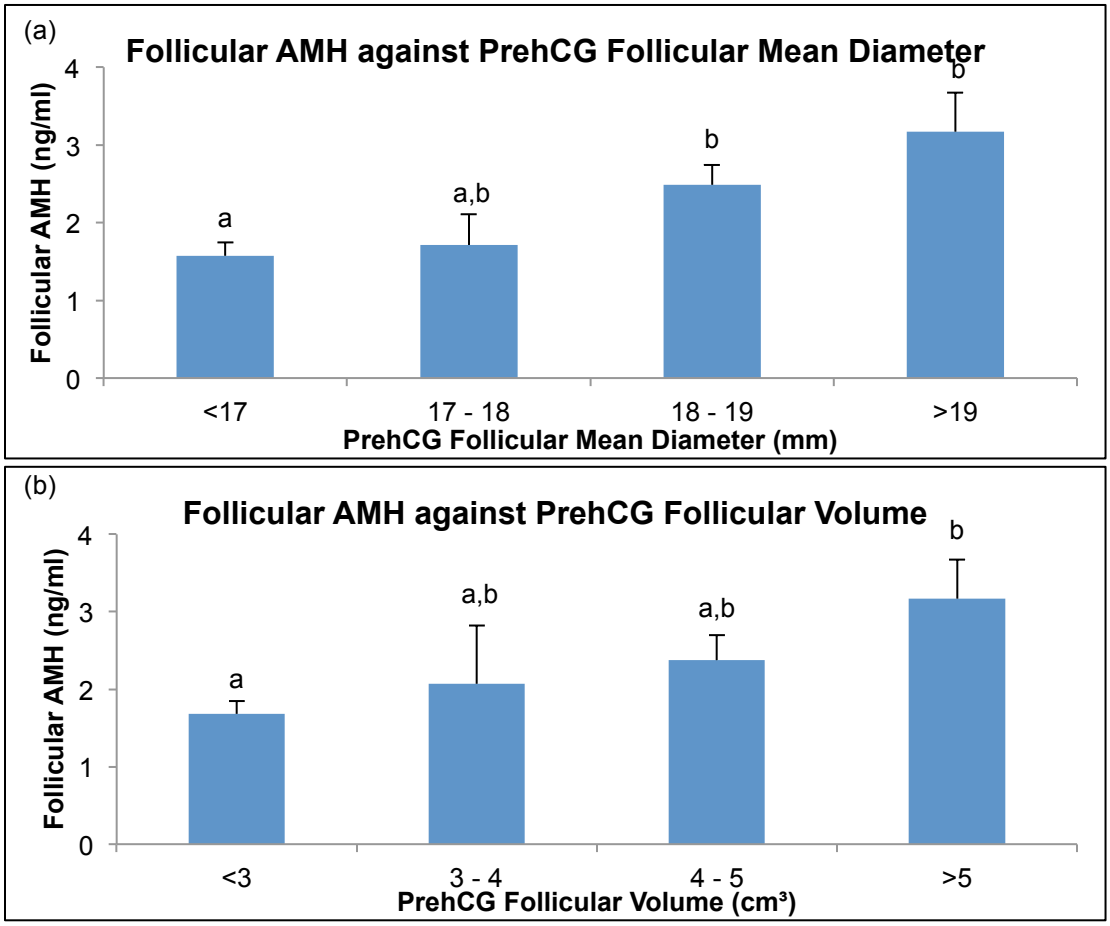

Figure 7.34. Follicular AMH against grouped prehCG (a) follicular mean diameter and (b) volume

Different letters indicate statistically significant differences between grouped follicular mean diameters (a) P<0.05; (b) P<0.01. Error bars indicate SEM.

At egg collection, the differences in AMH concentration between the follicular diameter size groups were not significantly different (figure 7.35a), however AMH concentration was significantly lower in follicles with a volume of 4-6cm^3 (figure 7.35b).

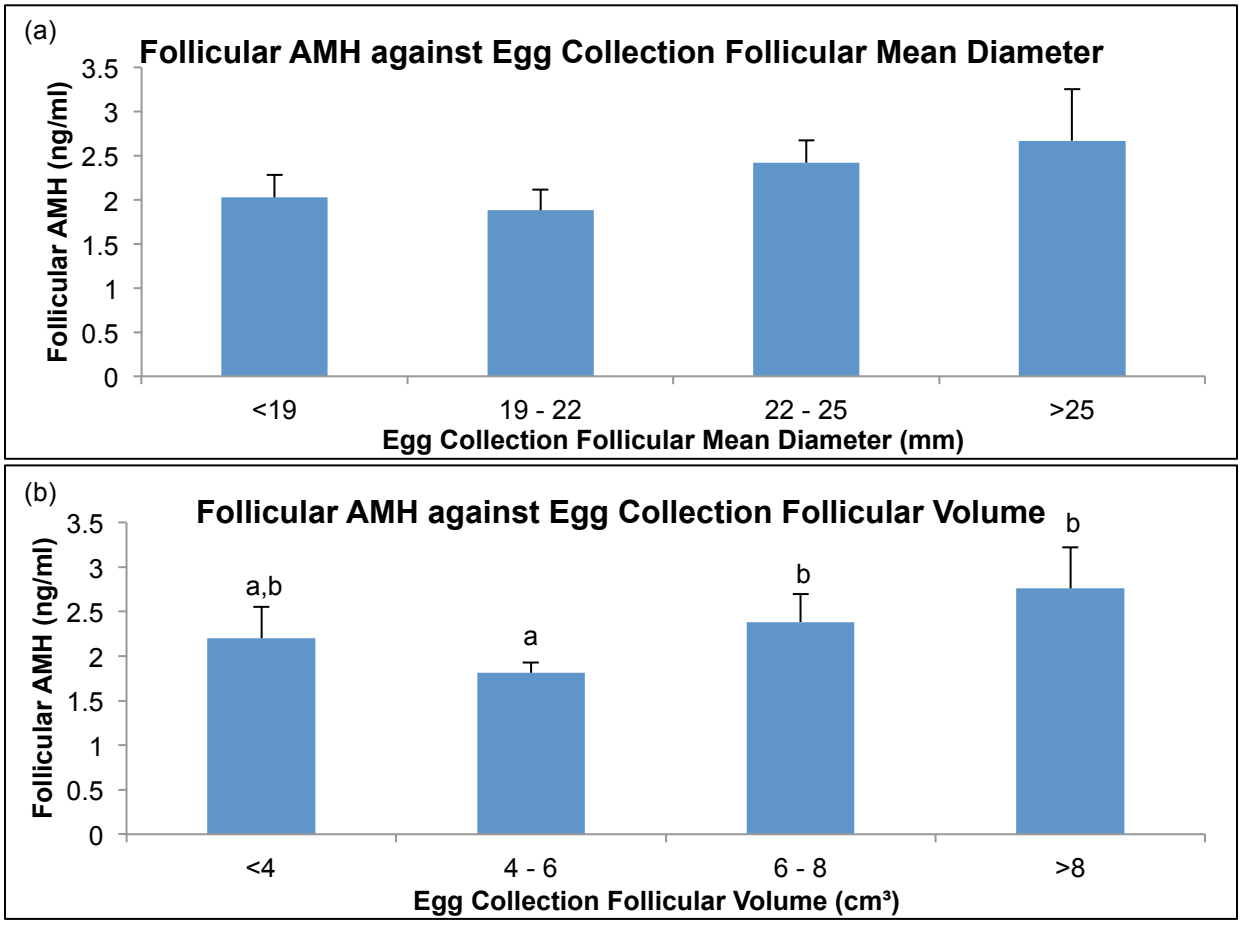

Figure 7.35. Follicular AMH against grouped egg collection (a) follicular mean diameter and (b) volume

Error bars indicate SEM.

There was no significant relationship observed between AMH concentration in the follicular fluid and follicular growth from prehCG to egg collection (data not shown). Correlation coefficients with mean diameter growth and volume were 0.20 and 0.51 respectively.

In addition, there were no significant correlations to vascular indices measured at prehCG or egg collection. Correlation coefficients to VI, FI and VFI were less than 0.3 in all cases (data not shown).

7.5.10.2. BMP15 in Follicular Fluid

BMP15 in the follicular fluid was found to have a significant positive relationship with the follicular mean diameter measured at prehCG scan following linear regression ($P<0.05$). Follicles with a mean diameter of less than 17mm had significantly lower intrafollicular BMP15 concentration than those larger than 18mm (figure 7.36a). Following linear regression, follicular BMP15 did not have a significant correlation to follicular volume measured at prehCG scan, however as seen in figure 7.36b, follicles with a voume of less than 3cm³ had significantly lower BMP15 concentrations than those in the larger follicle volume groups ($P<0.05$).

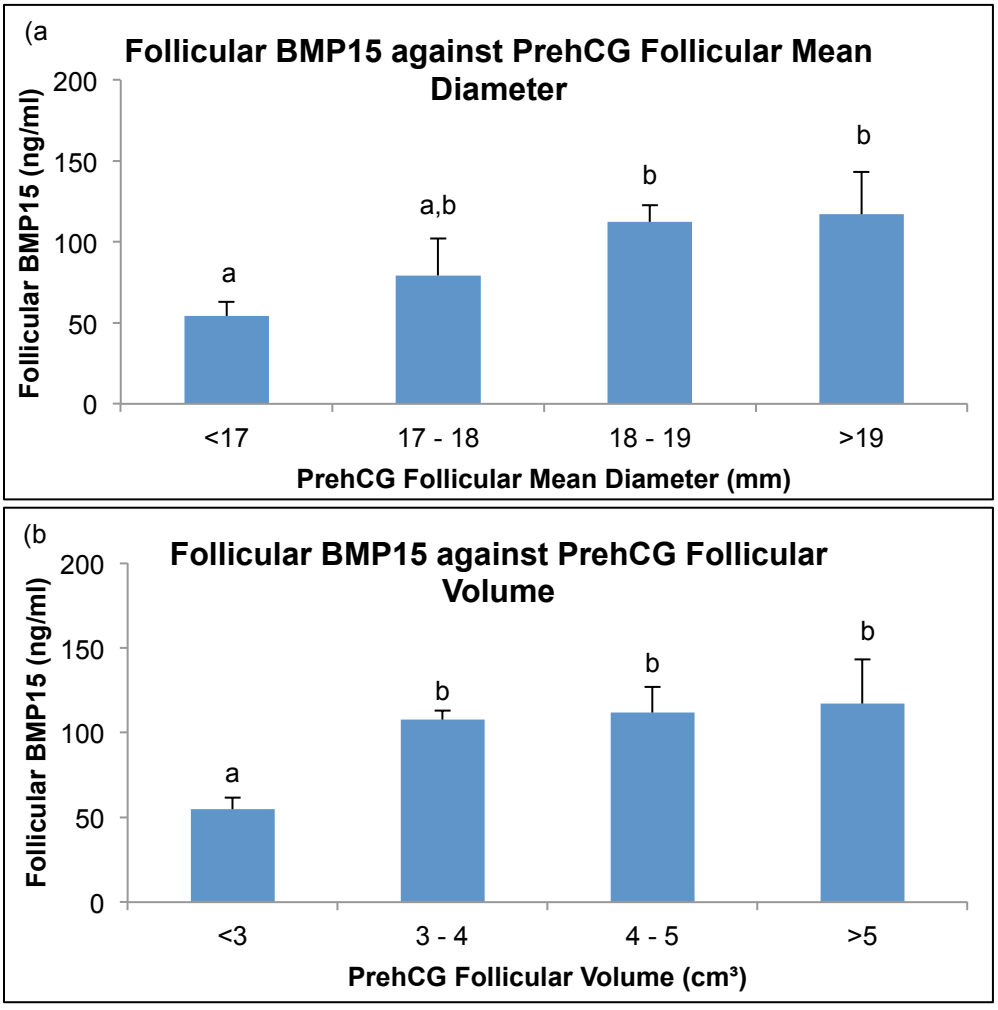

Figure 7.36. Follicular BMP15 against grouped prehCG (a) follicular mean diameter and (b) volume

Different letters indicate statistically significant difference between follicular mean diameter groups ($P<0.05$). Error bars indicate SEM.

BMP15 in the follicular fluid was also compared to follicular size measured at egg collection. No significant correlation to follicular mean diameter was observed, and there was no significant difference between the mean diameter groups (figure 7.37a). The same was found when intrafollicular BMP15 concentration was examined against to follicular volume, although follicles with a volume of 4-6cm³ did have slightly decreased BMP15 concentrations compared to the other groups (P<0.05) (figures 7.37b).

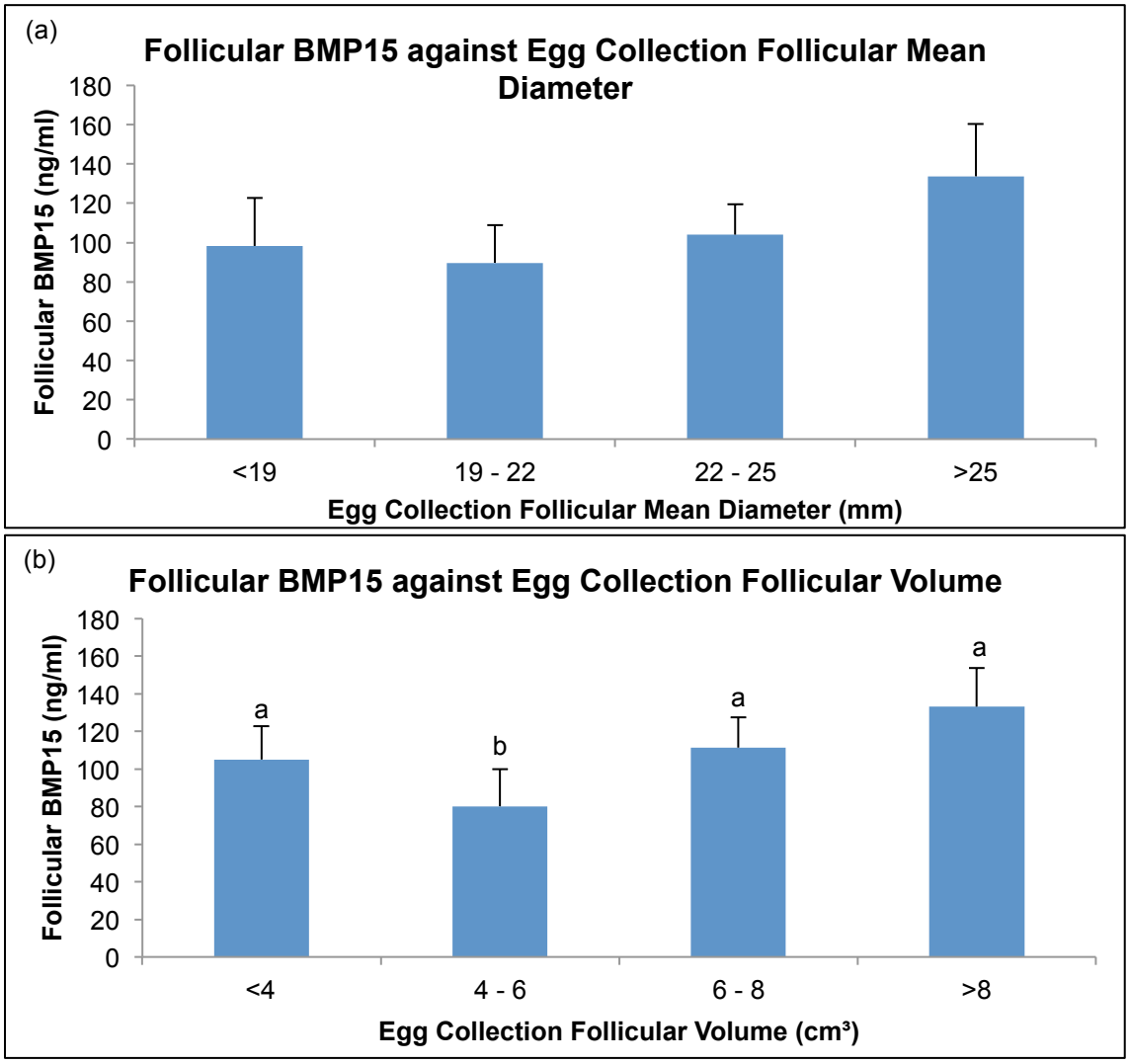

Figure 7.37. Follicular BMP15 against grouped egg collection (a) follicular mean diameter and (b) volume

Different letters indicate statistically significant difference between follicular volume groups (P<0.05). Error bars indicate SEM.

Follicular fluid BMP15 concentration did not correlate to vascular indices measured at prehCG or egg collection and so the data is not shown. Correlation coefficients were less than 0.19 in all cases.

7.5.10.3. GDF9 in Follicular Fluid

GDF9 was measured in the follicular fluid by ELISA. The concentrations present in the follicular fluid were found to be undetectable by this method.

7.5.10.4. Intrafollicular hormone and growth factor interactions

The steroid hormones and TGFβ superfamily members measured in the follicular fluid were compared to each other to examine any relationships between their concentrations. Interestingly, AMH and BMP15 concentrations were found to be significantly positively correlated following linear regression analysis ($P<0.05$) (figure 7.38). No other interactions between intrafollicular hormones and factors were observed.

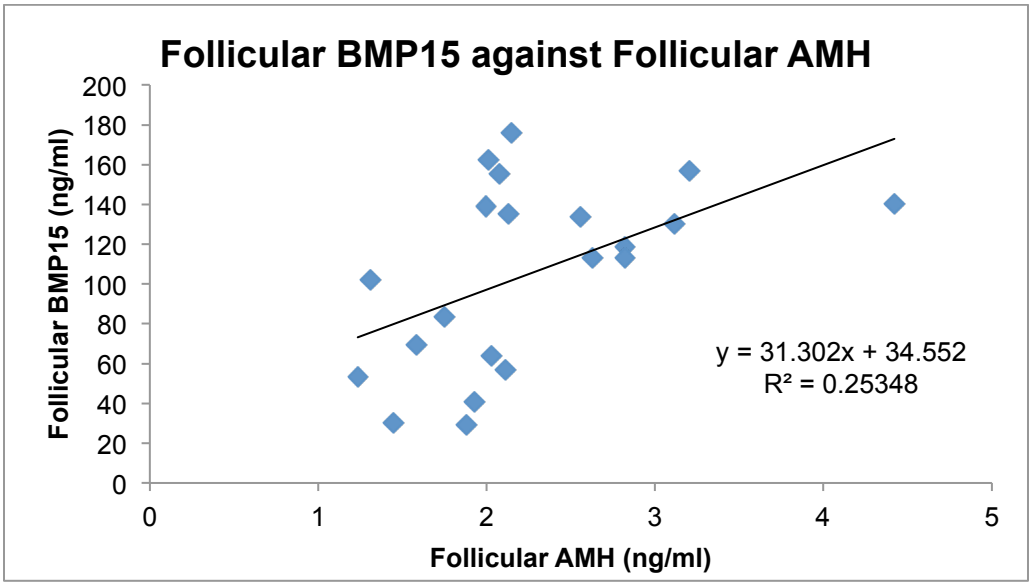

Figure 7.38. Follicular BMP15 against follicular AMH

7.5.11. Ovarian Stimulation Drugs

There were found to be no significant correlations between ovarian stimulation drug and follicular vascular indices, follicular size or follicular fluid constituents.

7.6. Discussion

Clearly the primary major limitation of the clinical study was the small number of patients. Although a lower number of patients was recruited into the trial than initially hoped, the feasibility and the extensive potential of the project was clearly demonstrated, and it is likely that recruitment will recommence, and the trial continue in the future once adequate staffing in the unit has resumed.

Many of the results observed were indicative trends, but were not statistically significant due to small sample sizes. This was particularly detrimental to the analysis when examining the embryo developmental relationships, as there were only one or two individuals that fell into each developmental stage. As a result the data was analysed in terms of the largest possible groupings; oocyte retrieval, fertilisation and embryo transfer (although again the sample size was just 3 in the transfer group).

In addition, although granulosa and cumulus cells were collected from each patient, due to the nature of commencing the trial in a relatively busy IVF unit, unfortunately only one sample per patient could be obtained. As a result the mRNA array expression of angiogenic factors was unable to be performed due to the inherent variation of expression levels within individual patients. If this trial does proceed in the future, 2 samples per patient will be collected so that gene expression analysis can be performed by PCR.

7.6.1. Follicular Size

Overall, comparison of mean follicular diameter and volume to oocyte, retrieval, fertilisation and embryo transfer, measured at both prehCG and egg collection did not reveal a significant relationship. In addition, analysis of the follicular growth between these two scan timepoints did not yield any significant relationships. This is likely to be due to the nature of the study using infertility patient participants, as all of the follicles aspirated were relatively large as a result of the stimulation regime used in the IVF process.

Consequently, the follicle size range of 13 to 38mm diameter in this study would mean that all of the follicles studied were of pre-ovulatory size, and so there was unlikely to be a marked difference observed in terms of IVF outcome.

A small number of studies have investigated a possible relationship between the size of the follicle and the success of IVF, although few have been able to examine implantation and pregnancy rates as individual follicle aspiration did was not performed. For example, Wittmaack *et al.* (1994) observed higher fertilisation and cleavage rates with oocytes from follicles more than 1ml in volume, however no difference in embryo quality was found, and so concluded that once fertilised, oocytes had the same implantation potential regardless of their follicular size. In clinical IVF, follicles of 1ml are considered to be relatively small, with a mean diameter of less than 15mm (as seen in figure 7.22). For example, in the clinic that this study was conducted, ovulation would not be induced until a minimum of 3 follicles had reached 18mm in diameter.

Dubey *et al.* (1995) found that follicles greater than 16mm had oocytes with higher fertilisation rates, but again, this is smaller than the minimum size required for ovulation induction in a clinical setting. Bergh *et al.* (1998) also observed a higher fertilisation rate in oocytes from larger follicles under the same criteria, however both of these studies made these observations based on a conventional IVF program, not ICSI. Bergh *et al.* also went on to discover that in the ICSI group of patients, there was no significant difference in fertilisation rates in the large and small follicle groups.

Clearly, follicles of a very small size will possess much lower developmental potential (although ICSI can sometimes overcome this to some extent), however in the size cohort clinically relevant for ovulation induction and egg collection, there seems to be little evidence that a follicle 16mm in diameter would have a lower chance of fertilisation than a follicle of 18mm diameter. As a result, it is unlikely to serve as an adequate indicator of consequent embryo development, as found in this study.

7.6.2. Vascular Indices

Ultrasound measurement of vascular indices yielded some particularly interesting results in relation to oocyte fertilisation. At the prehCG scan, it was found that follicles containing an oocyte that failed to fertilise had significantly lower peri-follicular blood flow index ($P<0.01$) and vascular flow index ($P<0.05$)

than the group that fertilised successfully. Vascular index was also approaching significance (P<0.06) with the same relationship. These relationships were not found to be present at egg collection.

From these results it seems that follicles with a larger local blood flow yield oocytes with a higher fertilisation capacity. These follicles would be provided with increased oxygen to the somatic cells, as well as vital nutrients needed for somatic cell growth and differentiation, and more importantly oocyte development (Van Blerkom et al., 1997), as well as circulatory and local endocrine signals that directly influence development. In addition, increased flow would result in an increased capacity for removal of carbon dioxide and waste metabolites that could be detrimental to the oocyte, especially in terms of chromosomal abnormalities (Gaulden, 1992; Van Blerkom *et al.*, 1997). These factors could contribute to the development of the oocyte in a more favourable environment that would ultimately dictate the consequent outcomes of fertilisation, embryo development and implantation, as demonstrated by several studies over recent years (Chui et al., 1997; Van Blerkom, 1998; Borini *et al.*, 2001; Mercé, 2002; Shrestha et al., 2006).

There is a distinct lack reports of ultrasound peri-follicular vascular indices measured at prehCG scan in relation to IVF outcome in the literature, however Nargund *et al.* (1996) did implicate measurement of follicular vascular indices as a strong candidate for predicting oocyte fertilisation and embryo quality in a study examining blood velocity. Various other studies measuring blood flow at egg collection have also concluded that blood flow to the follicle is especially important to follicular development with respect to oocyte developmental competence (Chui *et al.*, 1997; Coulam *et al.*, 1999; Huey *et al.*, 1999). Vlaisavljević *et al.* (2010) recently demonstrated that administration of hCG has a direct effect on peri-follicular vascular dynamics as measured by software reconstruction of the vascular network from a power Doppler ultrasound scan. It was observed that before hCG, blood flow to the follicle is supplied through a dominant vessel, and that once hCG is administered, the vascular dynamics around the follicle change so that a more balanced flow is achieved, and so blood supply becomes more uniform around the follicular perimeter. As the volume of follicles increases following the administration of hCG, the response is an equivalent increase in perifollicular vascular activity to meet the demands of the peri-ovulatory follicle. Quantification of vascularity and flow may therefore not be the whole story in terms of follicular development, as the distribution of these vessels may also play an important part. As a result, it seems

that measurement of vascular indices at prehCG and egg collection could yield potentially divergent results and conclusions.

The main drawback of the vast majority of the studies in the literature is that none have tracked individual oocytes from collection to embryo transfer, and as a result the conclusions that can be drawn in relation to oocyte and embryo quality are somewhat limited. In addition, power Doppler measurement of peri-follicular blood flow has only been performed at the time of egg collection, and to date there have been no reportings of utilization of the technique at prehCG scan to predict oocyte quality and IVF outcome. From this standpoint, this makes the POEM study quite unique, and the potential to investigate a huge array of data is even more apparent.

7.6.3. Patient Age

Analysis of the data did not reveal a significant relationship between patient age and oocyte retrieval, fertilisation or embryo transfer, nor was there a significant correlation to the embryo developmental timepoints. This was primarily as a result of the lack of numbers in each group.

However, this study was able to demonstrate that the vascular indices measured by 3D power Doppler at prehCG decrease significantly with increasing patient age, and although these relationships were not significant at egg collection, there was again a negative trend associated with age. This finding supports the results of a number of other studies that have also concluded that as the patient age increases, the vascular supply to the ovarian stroma as well as individual follicles becomes impaired (Kupesic *et al.*, 2003; Ng *et al.*, 2004; Costello *et al.*, 2006).

Previous studies have indicated that oocyte quality decreases with age, which is normally as a result of increased prevalence of chromosomal abnormalities (Costello *et al.*, 2006). One of the possible causes of these defects could be associated with decreased perifollicular blood flow, resulting in reduced available oxygen to the somatic cells surrounding the oocyte, that are then unable to provide pyruvate needed for oocyte metabolism (Van Blerkom *et al.*, 1997). It is likely, however, that vascularity of the individual follicle is directly affected by the oocyte itself by means of a signalling cascade ultimately resulting in the secretion of locally acting angiogenic factors (Fraser, 2006). Alternatively it is also possible that oocyte

quality could be purely dependant on follicular vascularity, driven by factors external to the follicle. Consequently the negative relationship between peri-follicular vascularity and advancing age could either be as a result of decreased oocyte quality or because of impaired angiogenic function within the ovary, which is unclear at this time.

7.6.4. Follicular Fluid Steroid Hormones

Steroid hormone measurement in the follicular fluids revealed correlations between oestradiol and oocyte fertilisation and embryo transfer, as well as follicular size and growth. Oestradiol concentration was higher in follicles which yielded an oocyte that successfully fertilised, as well as those from which an embryo was transferred, and although this was not statistically significant, trends were observed. Progesterone in the follicular fluid was also higher in follicles yielding an oocyte that consequently fertilized, and this was found to be significant ($P<0.05$). Therefore it seems that the microenvironment in which the oocyte resides could be highly influential to its later development, especially the ability to fertilise. The granulosa and theca cells, that produce oestradiol and progesterone respectively, must have a local effect on the oocyte contained within the follicle.

Relatively few studies have examined the concentration of steroid hormones in relation to oocyte development, and those that have been performed have frequently contradicted each other. Some have shown oestradiol and progesterone to correlate to oocyte fertilisation and embryo cleavage (Wramsby *et al.*, 1981; Botero-Ruiz *et al.*, 1984; Kreiner *et al.*, 1987; Kobayashi *et al.*, 1991; Mendoza *et al.*, 1999; Ilkhanizadeh *et al.*, 2010; Lamb *et al.*, 2010), whereas others have found no relationship (Franchimont *et al.*, 1989; Rosenbusch *et al.*, 1992; Tavmergen *et al.*, 1992; Fujiwara *et al.*, 2000; Asimakopoulos *et al.*, 2008). It does, however, seem logical that healthy granulosa cells from healthy ovarian follicles, would have increased steroidogenic capacity and therefore higher steroid hormone concentrations would be found in the follicular fluid. In addition, oestradiol and progesterone are thought to play a part in the angiogenic development of the follicle (Morales *et al.*, 1995; Shimizu and Miyamoto, 2007), and so follicles with higher concentrations in the follicular fluid may have increased vasculature and hence superior development, although no significant relationships between steroid hormones and ultrasound vascular indices were found in this study.

The difficulty with standardizing follicular fluid constituents as predictors of embryo development, is that there is a plethora of factors that will ultimately influence the results of any study. For example, gonadotrophin stimulation has been shown to affect follicular fluid composition, due to the FSH effect on somatic cell steroidogenesis, which in turn has a direct effect on the oocyte within (Mendoza *et al.*, 2002). As a result, the treatment regime that the patient receives will affect follicular fluid composition, and therefore when attempting to identify follicular fluid predictors of embryo development, it is vital that the study subjects receive identical stimulation protocols. In this study the patient ovarian stimulation regime was not dictated or indeed affected by the trial itself, and consequently patients received human menopausal gonadotrophin, recombinant FSH, or recombinant FSH with recombinant LH. Although statistical analysis revealed no relationships between stimulation drug administered and follicular fluid constituents, vascular indices and follicle size, the fact that the patients did not all receive identical stimulation treatment regimes could be considered to be one limitation to the study, though this would be very difficult to achieve given that drug type and dosages differ between individuals.

In addition to the stimulation drugs administered to the patients, the infertility treatment method itself will have an effect of the outcome of the study; unless ICSI is used to fertilise the oocytes, the maturity of the oocyte is uncertain as this can only be assessed indirectly by examining the morphology of the cumulus oophorus and corona radiata (Mendoza *et al.*, 1999). Consequently, immature oocytes would undoubtedly have been included in a number of the previously mentioned follicular fluid studies, which could have biased the results; another potential reason for discrepancy in the literature.

Another side to the argument is that the follicular fluid is simply a waste product reservoir of the oocyte and somatic cells that may not reflect the health status of the oocyte at all, and that measuring its constituents would indicate nothing more than the viability oocyte and somatic cells. However, even if this is the case, it is unlikely that there is not a single indicator of oocyte quality within the follicular fluid in which the oocyte develops.

7.6.5. Follicular Fluid TGF-β Factors

7.6.5.1. AMH in Follicular Fluid

This study did not find a significant correlation between AMH concentration measured in the follicular fluid and oocyte fertilization and embryo transfer. Previous studies published to date provide conflicting evidence regarding this relationship. In an IVF patient study, Takahashi *et al.* (2008) observed that oocytes from follicles with higher follicular AMH concentrations had higher fertilisation rates and Fanchin *et al.* (2007) even found high AMH to directly reflect increased embryo implantation rates. However, other studies found no correlation to any outcome parameters (Jancar *et al.*, 2008), and even that AMH may be negatively correlated to oocyte quality (Cupisti *et al.*, 2007). It has been demonstrated previously that atretic follicles do not express or secrete AMH in rats, sheep or humans (Baarends *et al.*, 1995; Bezard *et al.*, 1987; Weenen *et al.*, 2004), indicating that at the very least that if no AMH is present in the follicular fluid, the follicle is likely to be atretic and hence the oocyte within of compromised health. These conflicting studies warrant further research into the relationships between AMH and oocyte quality and developmental competence.

In this study, follicular fluid AMH concentration was observed to positively correlate to follicle size, although this relationship was only significant between the smallest and largest follicles. This was an unexpected result and requires greater numbers to confirm, as there were relatively low numbers in each size group. Currently data is very limited regarding the relationship between follicular size and intrafollicular AMH with regard to large peri-ovulatory follicles in IVF treatment cycles. In normal unstimulated human cycles, AMH is expressed by granulosa cells in a specific window of follicular development from the early stages, up until the late antral stage, when expression becomes low or absent altogether (Baarends *et al.*, 1995; Fanchin *et al.*, 2007; Seifer and MacLaughlin, 2007). However, the effects of ovarian stimulation regimes on AMH secretion have not yet been explored, and it possible that gonadtrophin stimulation and hCG may have an effect on the follicular developmental characteristics at these late stages of development, and so further research is required in this area.

AMH did not significantly correlate to steroid hormone levels in the follicular fluid in this study. This result is supported by the work recently published by Grøndahl *et al.* (2011), who measured AMH expression in granulosa and cumulus cells collected from patients in ART treatment cycles. AMH and aromatase expression were not correlated in these large antral follicles, which suggests that at this final stage of development, AMH and oestradiol production are independent of each other. It is thought that in small antral follicles at least, AMH suppresses steroidogenesis by inhibiting aromatase expression, however Grøndahl *et al.* (2011) found that in larger antral follicles in stimulated cycles this result was not observed, possibly because in these follicles there is decreased AMH expression, which in turn may have a decreased effect on aromatase. Therefore it seems that in larger follicles, as studied in this trial, aromatase expression, and hence oestradiol production, occurs independently of AMH expression and secretion.

BMP15 and AMH concentrations in the follicular fluid displayed a significant positive correlation, which could be a function of follicular size rather than a direct relationship, as both of these factors increased in concentration along with follicular mean diameter and volume. However, it seems more likely that oocyte-secreted factors could affect the surrounding cumulus cells by preventing cellular differentiation and increase AMH production, as shown by AMH immunohistochemistry performed on ovarian sections (Campbell, unpublished results), the staining of which is localized only to the cumulus cells (Weenan *et al.*, 2004). Also Grøndahl *et al.* (2011) observed that cumulus cell AMH expression was maintained though the later stages of development, while granulosa cell expression was depressed. This provides further evidence that suggests a local effect of the oocyte on AMH production by somatic cells, although the relationship between follicular AMH and oocyte development is clearly quite complex and requires further research.

7.6.5.2. BMP15 and GDF9 in Follicular Fluid

BMP15 measured in the follicular fluid was positively correlated to follicular size, both in terms of mean diameter and volume, which linear regression revealed to be significant. This effect was most prominent with the prehCG scan measurements. Smaller follicles had significantly lower BMP15 concentrations than

the larger size groups. Although this relationship was observed overall, if the largest follicle is removed from the analysis, this was no longer found to be significant compared to the egg collection scan measurements, indicating that overall BMP15 did not strongly correlate to follicular size at this timepoint. The reason for this could be due to the administration of hCG causing follicle maturation, and so at this point the role of the oocyte in preventing premature luteinization through production of oocyte-secreted factors is no longer in effect.

There have been no reported studies measuring BMP15 or GDF9 concentrations in the follicular fluid of ovarian follicles to date, and so it is not known at what concentrations these two factors are normally present in the follicular environment. Two previous studies have used Western blotting to measure the oocyte-secreted factors in follicular fluid, however this technique does not allow fully quantitative measurement of concentrations in the fluid (Wu *et al.*, 2007; Gode *et al.*, 2011), and can only be used as a relative measure between samples. Wu *et al.* (2007) found that BMP15 protein levels were higher in the follicular fluids from follicles containing oocytes that fertilised successfully, as well as embryos that cleaved and developed successfully. Follicular oestradiol was found to positively correlate to BMP15, and progesterone negatively so, further indicating a role for BMP15 as an anti-luteolytic factor produced by the oocyte (Shimasaki *et al.*, 2004). Conversely, Gode *et al.* (2011) recently observed no relationship between BMP15 protein in the follicular fluid and IVF outcome, and so the potential for this factor as a predictor of embryo development needs further research. However this latter study did find GDF9 to positively correlate to oocyte maturation and embryo quality, and so this could serve as a more appropriate factor to measure.

Our study was unable to detect GDF9 levels in the follicular fluid, as concentrations were below the threshold 0.156ng/ml of the ELISA, which is probably as a result of hCG administration decreasing GDF9 levels to undetectable levels. The study was unable to determine at what concentrations GDF9 is present in human follicular fluid, however if levels are too low to be detected by ELISA, it suggests that it is unlikely GDF9 concentrations would be predictive of embryo development anyway, as there would be such small differences between samples. Much like BMP15, it is likely that hCG-induced follicular maturation causes the oocyte to cease production of these factors, the role of which is to prevent

premature luteinization, and so intrafollicular concentration of GDF9 at egg collection is at a negligible level at this point.

In conclusion, our study was unable to demonstrate a relationship between the TGFβ superfamily members AMH, BMP15 and GDF9 concentrations and oocyte/embryo developmental parameters, and therefore it seems that measurement of concentrations in the follicular fluid could not be used in a clinical setting to predict oocyte developmental competence. However, the study has shown that AMH and BMP15 may interact in the pre-ovulatory follicle

7.7. Conclusion

In conclusion, this clinical trial has identified several targets for future research into potential predictors of oocyte and embryo development that have not yet been explored in detail. There is currently insufficient data to provide definitive links, but there is some indication that follicular vascularity and follicular steroid hormones may be related to oocyte developmental competence. While peri-follicular blood flow appears to have strong links to the development of the oocyte within, this requires further research. Measurement of oocyte secreted factors in the follicular fluid at egg collection seems to be fairly limited in terms of the relationship to oocyte developmental capacity due to the administration of hCG decreasing these factors in the follicular fluid significantly.

Chapter 8 – General Discussion

It has been reported that around 10% of couples fail to conceive within a year of attempting to become pregnant naturally (Fauser *et al.*, 2005). In the developed world the tendency to postpone having children has increased the incidence of ovarian ageing, which considerably affects fertility (Lambalk *et al.*, 2004). As a result of this change in lifestyle to delay having children, the field of infertility treatment in reproductive medicine has now become commonplace not just in the developed world, but also across the globe. In fact, it is thought that between 1 and 3% of all children born in the Western world are from IVF (Fauser and Edwards, 2005).

There is now awareness of the contribution of infertility treatment to multiple gestations and births, which are associated with increased perinatal morbidity and mortality, in addition to considerable financial implications to the National Health Service in the UK, as a result of increased need for surgical and medical procedures for both mother and child. The costs of infertility treatment are far outweighed by the direct perinatal costs of multiple births following IVF treatment (Collins, 2002), and as a result the shift toward single embryo transfer has now taken place. However, as a consequence of IVF clinics implanting a single embryo, where before 2 were transferred, success rates in these clinics dropped considerably in many cases, and although research in the field was taking place before this change, there has been a copious increase in order to ensure that success rates are brought back up to where they were previously, and beyond.

One of the most challenging aspects of IVF, and arguably one of the areas that has attracted the most attention in terms of research, is selection of the embryo with the best possible chance of developing and implanting to lead to a pregnancy. In order to do this, research has focused on what makes a 'good or bad' oocyte and embryo, as current clinical practice is to examine the oocyte and embryo under the microscope, and formulate an assessment of its quality. However, this morphological examination is subjective, and weakly correlated with pregnancy (Guerif *et al.*, 2007), and as a result there is a desperate need to find that 'golden marker' of embryo quality that will enable embryologists and clinicians to make the crucial decision of which embryo to transfer to the patient.

Some argue that improving embryo selection methods may be becoming a dead-end in terms of reaching the final goal of optimising IVF success, and that improving cryopreservation may serve as a more promising avenue of research (Mastenbroek *et al.*, 2011). However, this is a fairly single minded way of looking at the problem, and although improving crypreservation may well provide a helpful way of storing oocytes and embryos, if the quality of the embryo is not known the value of this is very limited.

Of course, laboratory research, which underpins the ability to assess the developmental capacity of an oocyte or embryo in the clinic, is absolutely crucial. This work described in this thesis has demonstrated this, from targeting oocyte-secreted factors affecting granulosa cells in culture, to how blood moves around the mammalian ovary, scaled up to a clinical trial examining these factors and ovarian blood flow *in vivo*, in the species which the research is ultimately aiming to benefit.

8.1. Summary of research findings

There are still gaps in the jigsaw of knowledge that forms the complex molecular mechanism of oocyte development; in particular, the oocyte and its microenvironment.

The research described in this thesis has focused on the interactions between the oocyte and its surrounding somatic cells, specifically concerning the role of oocyte-secreted factors in regulating follicular development in terms of both avascular granulosa cell function and follicular vascularity and hypoxia, both *in vitro* and *in vivo*.

In the experiments described in Chapter 4, granulosa cell cultures provided further evidence for a role of a combination of BMP15 and GDF9, as well as BMP6, in the prevention of luteinization by increasing oestradiol and suppressing progesterone production. Furthermore, co-culture results indicated a further role for the COC in promoting somatic cell proliferation, suggesting the COC can directly affect the fate of its own development by producing factors that have a positive effect on its surrounding cells, and in turn, the individual microenvironment in which it resides. The *in vivo* infusion study supported the *in vitro* evidence suggesting that oestradiol synthesis is regulated by these factors in combination, as the passive

immunization against the combination of BMP15 and GDF9 had a negative effect on steroidogenesis in some of the animals, however more experimentation is required to confirm this effect.

Further cell cultures described in Chapter 4 explored the effects of hypoxia in the ovarian follicle induced by increased media depth, high seeding density and treatment with chemical agents, which in all cases were found to have a considerable detrimental effect on granulosa cell steroidogenesis. During hypoxic conditions the follicle reacts by producing angiogenic factors in order to increase follicular vasculature and hence overcome these conditions so that normal development can resume under optimal conditions.

Although the development of clinical ultrasound technology has come a long way since its advent over 50 years ago, and has facilitated the prediction of ovarian response to stimulation in the IVF clinic, as yet there have been no concrete links between ultrasound parameters and oocyte quality. Even though highly sensitive power Doppler is able to detect diminutive blood flow in capillaries well below the surface of the skin, there has been no concensus reached to ascertain the use of the technology in a clinical setting to relate to follicular blood flow and oocyte quality.

Measurement of follicular vascularity and blood flow could be considered to be an indirect indication of the hypoxic state of the follicle, although this has not yet been demonstrated in the clinical trial described in Chapter 7. It seems relatively clear that the follicle requires a significant degree of blood flow in order to provide it with the endocrine and molecular signals needed for adequate development, however the mechanisms by which the vascular network is formed and regulated *in vivo* are not yet clear.

Overall this thesis has elucidated some of the interactions involved in follicular development with regard to the relationship between the oocyte and somatic cells, however considerable research will be required in the future to investigate the complex system that exists in the ovary, as summarized in figure 8.1. Of course, these factors not only interact with each other, but are also affected, and affect, additional external endocrine and molecular stimuli, such as gonadotrophins, which are not included in the diagram for simplicity.

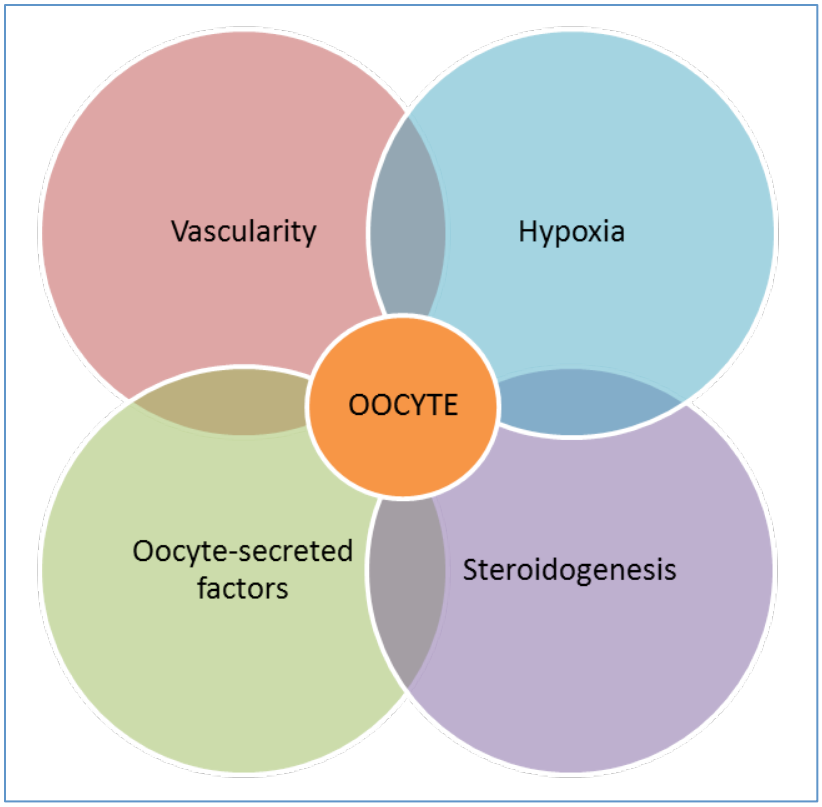

Figure 8.1. Oocyte interactions with intrafollicular and extrafollicular factors

8.2. Limitations of the research

In both the *in vitro* and *in vivo* experiments, one of the major limitations was that the oocyte secreted factors of ovine origin proved extremely hard to obtain. This meant that mouse GDF9 and human BMP15 had to be used in the *in vitro* cultures, which differ structurally to those of the sheep. McNatty *et al.* (2005a) found that mouse and ovine GDF9 had different effects on both bovine and ovine granulosa cell cultures *in vitro*, indicating that the oocyte-secreted factor species of origin has an affects its mechanism of action in culture. This group also observed these divergent effects of factors originating from different species when culturing rat granulosa cells (McNatty *et al.*, 2005b). Consequently, it is not possible to make a concrete statement from our *in* vitro work that the cooperative activity of hBMP15 and mGDF9 observed in these ovine granulosa cell cultures is physiologically representative, and so further experimentation is required.

The primary limitation in the clinical trial was partly the amount of time required to obtain ethics approval, as the trial was able to run for just a few months as opposed to a full year as planned. In addition, the restricted number of aspirations performed per patient limited the number of replicates considerably, as it was originally planned to aspirate two follicles per patient. However, due to the experimental nature of the techniques employed, such as single embryo culture, it was decided that in order to avoid a potentially severely detrimental effect on IVF success rates in the unit it was necessary to commence the trial aspirating one follicle per patient. Because of this, the planned molecular analyses were not possible until two aspirations per patient could be performed.

Another limitation of the clinical trial methodology is that in IVF, the oocytes, which are collected at MII, have considerably reduced oocyte-secreted factor production at this stage, and so the value of measurement of these factors in the follicular fluid at aspiration may be somewhat limited. Ideally these oocytes would be collected at GV stage, however for obvious reasons in an IVF setting this would not be possible.

The primary focus of the research described in this thesis has been on embryo quality and development, but what has not been explored is the maternal side of establishment of pregnancy.

Favourable uterine conditions, more specifically good endometrial receptivity, are needed for the embryo to implant, and is a whole different field of research that is ongoing not just in our IVF clinic, but in research facilities around the world. Even the highest possible quality embryo will not be able to implant in a uterus that does not offer favourable conditions for development, and this is thought to be one of the causes of the relatively common problem of recurrent miscarriage.

Study of endometrial receptivity is relatively limited in the IVF setting, but the work that has been carried out has concentrated on the non-invasive ultrasound assessment of the uterus. The measurement of blood flow, using the technology described in this thesis, has in particular been linked to uterine receptivity, as well as numerous other factors including thickness, shape and general condition of the uterine lining (Dechaud *et al.*, 2008).

8.3. Future work

Doubtlessly research into how the oocyte interacts with its follicular environment, as well as how its environment affects its development, will continue to be a key area of study in the future. There is presently a huge number of questions to be answered in this field, the answers to which will lead to significantly better understanding of not just oocyte development and ovarian physiology as a whole, but also potentially huge developments in clinical assisted reproduction.

The work described in this thesis has provided some of these answers, but there are of course new avenues of research to explore and ideas to investigate, leading on from this.

8.3.1. *In vitro* culture

The culture method used in the granulosa cell cultures has been designed to replicate as closely as possible the physiological environment of the follicular compartment, and has performed exceptionally well in almost every experiment performed. This further confirms the potential and value of this culture system, developed by Campbell *et al.*, capacity to explore the molecular aspects of follicular somatic cell growth and differentiation has only been touched upon in this research. To further investigate the effect of oocyte-secreted factors on the granulosa cells, it would seem logical as a next step to examine SMAD phosphorylation by GDF9, BMP15, and the two in combination. This was, in fact, attempted but due to time constraints it was not possible to complete this particular experiment. Reader *et al.* (2011) recently reported the results of *in vitro* rat granulosa cell cultures in which the GDF9/BMP15 combination signalling pathway was investigated. This study showed differing signalling cascades of GDF9 and BMP15 from different species, and overall the results suggested that the molecular complexes formed by these two factors are likely to act not just through the SMAD 2/3 pathway, but also via non-SMAD signaling pathways. A similar result was also observed by Mottershead *et al.* (2011), although both of these studies concluded that more research was needed to further investigate these findings.

Oocyte culture was not performed to any great degree in the *in vitro* work, and there is potentially a wealth of information to be gained from co-culturing the somatic cells with oocytes and COCs. Following

on from the granulosa cell cultures, it would be interesting to see what factors affect the synthesis and secretion of oocyte-secreted factors, particularly in relation to endocrine signaling.

Furthermore, the effect of hypoxia on the oocyte itself could be explored *in vitro* using a similar methodology to the granulosa cell cultures, perhaps by examining the effect on oocyte-secreted factor production serving as an indicator of oocyte health. This would be extremely valuable as there has not yet been a direct link shown between oxygen availability and oocyte-secreted factor production.

8.3.2. *In vivo* experiments

The infusion experiment was almost as much about researching and optimizing the surgical procedure of ovarian cannulation as it was investigating the effect of blocking the effects of GDF9 and BMP15, as this technique is still fairly novel. However, overall the work was a success, and the results from the infusion study itself are encouraging.

Due to the small number of animals involved in the experiment, and not all of them responding to the infusion, the next step experiment would be focused on repeating the procedure with more animals, to obtain further results to support the hypothesis that the GDF9/BMP15 combination antiserum does indeed inhibit steroidogenesis. Leading on from this, the effect on the reproductive cycle could be explored, as a number of published studies have indicated a role for these factors in the regulation of ovulation, however none have investigated the effects of the two factors in combination.

Conversely, it would be very interesting to examine the effect of infusing recombinant oocyte-secreted factors into the ovarian vasculature, although availability of these factors at a significantly lower cost would be a prerequisite for this to take place! It is possible that in the distant future administering oocyte-secreted factors could have a role in ovarian stimulation, but there is a long way to go before any such work could become commonplace.

8.3.3. Clinical research

Following the promising results of the clinical trial, the study is set to continue in the future, following a similar format to the original, in order to further investigate the observed relationships between follicular

fluid factors and oocyte developmental competence. In addition to the endocrine and growth factor profiling of the follicular fluid, the methodology is expected to change to allow the collection of two sets of follicular aspirates, and therefore enable the quantitive analysis of gene expression by the oocyte somatic cells, which this preliminary trial was not able to do.

The potential of the trial to reveal new information about oocyte developmental competence and ultimately optimization of IVF, is considerable. Single embryo culture, tracking the oocyte from the moment it is aspirated from the follicle, through fertilisation, culture, and transfer is a feat in itself, but the POEM study has shown that it is possible. In fact, this study was the first to track a human pregnancy from pre-hCG right through to live birth. In addition, and perhaps more importantly, the clinical trial has shown that single culture is not only feasible, but enables the study of any somatic cell marker, follicular fluid constituent, ultrasound parameter, and a number of other measures, to be compared to oocyte quality and embryo development, which will undoubtedly prove invaluable for future research in the unit.

8.4. Conclusion

In summary, this thesis has explored some of the complex interactions that exist within the mammalian ovary, from a cellular to a whole organism level, which ultimately lead to the creation of life. Although some of the results of the research have elucidated some of these interactions, in these have lead to yet more questions; however this is arguably the finding of any credible research.

This research has provided further evidence that the oocyte plays a key part in the development of the follicle, by optimizing its microenvironment to ensure the most ideal conditions for its own requirements. It seems that the potent oocyte-secreted factors secreted for this purpose have a considerable effect on the somatic cells that surround the oocyte itself, and potentially the vascular network that surrounds the follicle, through this interaction.

As more is discovered every day in this fast-paced area of work, it is exciting to look to the future with anticipation of what research will reveal about ovarian physiology, as well as how this will translate into a developments in the IVF clinic.

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Appendix

1. Cell Culture Media

Dissection medium

500ml Medium 199 (Sigma M7528) or DMEM (Sigma D5671)

2.5ml Penicillin/Streptomycin (Sigma P0906)

5ml Fungizone (Sigma)

10ml HEPES (if not already in media) (Sigma H0887, 1M)

Granulosa medium

500ml McCoys 5A with sodium bicarbonate (Sigma M8403)

0.5g BSA (Sigma A9056)

5ml Pen/Strep

7.5ml L-Glutamine (Sigma G2150, 200mM)

10ml HEPES

250µl Transferrin (10mg/ml)

100µl Selenium (20µg/ml)

143µl Testosterone or androstenedione (1mg/ml)

50µl IGF-I LR3 (100µg/ml)

50µl Insulin (100µg/ml)

50µl FSH (10µg/ml)

2. Assay Buffers

Radioimmunoassay PGel buffer (0.05M)

Per litre distilled water:

2.3g Sodium di-hydrogen orthophosphate

6.3g Disodium hydrogen orthophosphate

9.0g Sodium chloride

1.0g Sodium azide

1.0g Gelatin

ELISA Coating buffer (0.05M carbonate buffer)

2.1g sodium bicarbonate

2.65g sodium carbonate

500ml distilled water

ELISA Assay buffer (PBS/0.1%BSA)

10 PBS tablets in 1 litre distilled water

0.5g BSA

ELISA Wash buffer (PBS/0.05% Tween)

1 PBS tablet in 1 litre distilled water

0.5mls Tween 20

Blocking solution (PBS/3% BSA)

5 PBS tablets in 500mls distilled water

15g BSA

3. Tissue Processing

Bouins Fixative

Picric acid	75ml
Formaldehyde	25ml
Glacial acetic acid	5ml

Tissue processing method

75% ethanol	1 hour
90% ethanol	1 hour
95% ethanol	1 hour
3 x 100% ethanol	1 hour each
3 x Xylene	45 minutes each
3 x Parafin wax	45 minutes each

Haematoxylin & eosin staining method

2 x Xylene	3 minutes
2 x Ethanol	3 minutes
70% ethanol	3 minutes
Distilled water	3 minutes
Harris haematoxylin	10 minutes
Running tap water	5 minutes
1% HCl in 70% Ethanol	2 dips
Scotts tap water	2 dips
Running tap water	5 minutes
1% eosin	2 minutes
Running tap water	90 seconds
2 x ethanol	2 minutes

Xylene	2 minutes
Xylene	3 minutes

Allowed to air dry then mounted in DPX

Aromatase immunohistochemistry BOND-MAX™ automated protocol

Peroxide block

Wash

Marker (primary antibody) 1 hour

Wash

Post primary IgG

Wash

Polymer (secondary antibody –poly HRP)

Wash

DAB

Water

Hematoxylin counterstain

Water

Wash

Water