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The role of Fas and Fas ligand in apoptosis during regression of the corpus luteum

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**THE ROLE OF FAS AND FAS LIGAND
IN APOPTOSIS DURING
REGRESSION OF THE CORPUS
LUTEUM**

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ABSTRACT

Apoptosis, a form of physiological cell death, has been found to occur during regression of the corpus luteum (Juengel *et al*, 1993; Dharmarajan *et al*, 1994). The pathways involved in this process, however, have yet to be specified. One possible mediator of corpus luteum regression is the Fas (or APO-1 or CD95) receptor, a transmembrane protein which induces apoptosis in the cell when ligated. In order to further confirm this hypothesis, the present study establishes and quantitates the presence and regulation of Fas receptor and Fas ligand (FasL) in the rat corpus luteum during pregnancy and post-partum. The animals used were sexually mature (10-12 week old) female Wistar rats. The presence of Fas and FasL in the rat corpus luteum at various stages of pregnancy and post-partum was investigated by immunohistochemistry using an anti-Fas monoclonal antibody and an anti-FasL polyclonal antibody. FasL was localised in corpora lutea throughout pregnancy, whilst Fas was localised at day 1 of pregnancy and at the time of luteolysis. Information on the steady state mRNA levels of FasL was obtained using relative quantitative reverse transcription PCR (RT-PCR) analysis of RNA isolated from rat ovaries at various stages of pregnancy and post-partum. Expression of FasL mRNA increased significantly at day 22 of pregnancy, just prior to parturition, and decreased by day 3 post-partum. The ability of an anti-rat Fas monoclonal antibody to block spontaneous apoptosis in corpora lutea placed in an *in vitro* culture model with serum-free medium was examined by analysis of extracted

DNA using 3'-end labelling. Treatment with an anti-rat Fas monoclonal antibody demonstrated a reduction in the occurrence of spontaneous apoptosis. Roles for Fas receptor and Fas ligand in corpus luteum function were proposed, and the suitability of the *in vitro* corpus luteum culture model for future studies investigating molecular mechanisms of Fas-mediated apoptosis in the corpus luteum was discussed.

I certify that this thesis does not, to the best of my knowledge and belief:

- (i) incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;*
- (ii) contain any material previously published or written by another person except where due reference is made in the text; or*
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1. INTRODUCTION

Apoptosis, a form of physiological cell death, allows tissue remodelling and cellular replacement to occur quickly, without damaging surrounding tissue. It can be distinguished from necrosis, which is cell death resulting from physical injury or trauma, by morphological characteristics such as cellular condensation, membrane blebbing, cleavage of DNA into oligomers of 185 base pair fragments, and packaging of cellular contents into membrane-bound apoptotic bodies. These apoptotic bodies are phagocytosed by surrounding cells or macrophages. Examples of apoptotic cell death occur during development (Hurle, 1988; Clarke, 1990), in the elimination of autoreactive T and B lymphocytes (MacDonald and Lees, 1990) and during the degradation of rabbit uterine epithelium following ovariectomy (Rotello *et al*, 1989).

One of the physiological processes in which apoptosis has been implicated is regression of the corpus luteum, an organ within the ovary which differentiates from the ruptured follicle following ovulation. The main function of the corpus luteum is secretion of progesterone, to maintain the lining of the uterus for implantation and for the support of a fertilised ovum. Regression of the corpus luteum occurs at the end of each ovarian cycle or when it is no longer required for the maintenance of pregnancy (Johnson and Everitt, 1995, pg. 71-72). Little is known about the mechanisms involved in this process, however apoptotic cell death has been found to occur during luteal regression

in cattle (Juengel *et al*, 1993), pseudopregnant and pregnant rabbits (Dharmarajan *et al*, 1994; Dharmarajan *et al*, 1997), pregnant rats (Ke *et al*, 1997; Sridaran *et al*, 1997) and marmoset monkeys (Fraser *et al*, 1995).

As a range of stimuli may trigger apoptosis and many molecular pathways have been identified which facilitate apoptotic cell death, it is necessary to identify the mediator(s) of apoptosis in regressing luteal cells. One possibility is the Fas (or APO-1 or CD95) receptor, a transmembrane protein which induces apoptosis in the cell following ligation. Although the exact mechanisms by which the Fas / Fas ligand system induce apoptosis have yet to be identified, it is known to be utilised extensively by the cells of the immune system (Watanabe-Fukunaga *et al*, 1992; Ju *et al*, 1995; Dhein *et al*, 1995; Oshimi *et al*, 1996).

Within the reproductive system, the expression of mRNA for the Fas receptor has been detected in the mouse ovary (Watanabe-Fukunaga *et al*, 1992), and Fas appears to be involved in the regression of vaginal epithelium following ovariectomy and during the estrus cycle in the mouse (Suzuki *et al*, 1996). Fas receptor mRNA is also expressed by human granulosa and luteal cells, and Fas monoclonal antibodies can induce apoptosis in these cells when they are pretreated with interferon gamma in culture (Quirk *et al*, 1995). In addition, abundant expression of the Fas receptor in the regressing corpus luteum of the normal adult human ovary has been reported (Kondo *et al*, 1996).

These findings indicate that Fas-mediated apoptosis may have a role in the mechanisms of regression of the corpus luteum. The present study examined the regulation of Fas receptor and Fas ligand in the rat corpus luteum during pregnancy and post-partum to examine this hypothesis. At the same time, the suitability of the *in vitro* corpus luteum culture model for future studies into the mechanisms of Fas-mediated apoptosis in the corpus luteum was assessed.

2. LITERATURE REVIEW

2.1. Introduction

Regression of the corpus luteum involves the death of considerable numbers of cells and significant tissue remodelling within a short period of time. The mechanisms contributing to luteolysis (functional and structural regression of the corpus luteum) are not well understood and many factors appear to interact to produce the changes seen in this process. Although apoptosis of luteal cells occurs at the time of regression, the pathways involved have yet to be identified. Recent studies indicate that the Fas receptor, a transmembrane protein capable of inducing apoptosis in the cell when ligated, is expressed by luteal cells at the time of regression, thus providing a rationale for further studies into the role of the Fas / Fas ligand system in corpus luteum function.

2.2. The Corpus Luteum

2.2.1. Background

The corpus luteum, an organ within the ovary which differentiates from granulosa and lutein cells of the ruptured follicle following ovulation, is essential for successful reproduction in mammals. Its main function is secretion of the steroid hormone progesterone, which maintains the uterine lining in a state

ready for implantation and development of a fertilised ovum. Towards the end of each ovarian cycle or when no longer required for the maintenance of pregnancy, the corpus luteum undergoes functional regression, when the amount of progesterone secretion is dramatically reduced. This is followed by structural regression, with degeneration of the luteal cells. Figure 1 illustrates the levels of progesterone secretion throughout the 23 days of rat pregnancy. Note that the levels are highest at around day 16 of pregnancy and they decline dramatically just prior to parturition.

Whilst functional regression is necessary to allow the next ovarian cycle to commence, structural regression is also essential, as the ovary must rid itself of obsolete tissue in order to maintain its uniform size in relation to other organs.

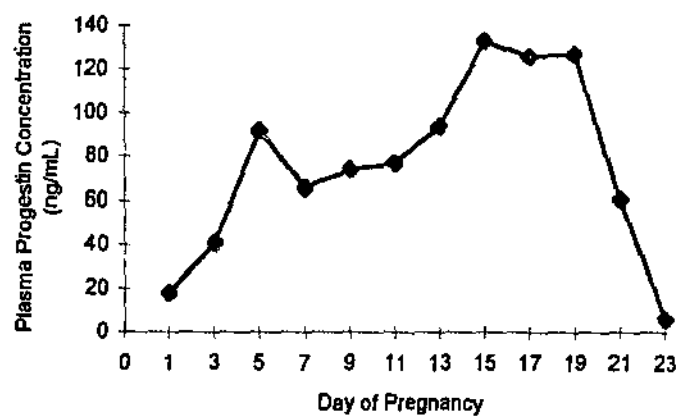


FIGURE 1: Serum Levels of Progesterone Throughout Rat Pregnancy
(SOURCE: Uchida *et al*, 1970)

2.2.2. The Rat Estrus Cycle

The following section outlines the nature of the estrus cycle and histology of the rat corpus luteum, as natural regression of the pregnant rat corpus luteum was the model used for this study.

The estrus cycle of the rat generally lasts for four days and comprises four stages. Proestrus is characterised by the rapid growth of follicles and regression of corpora lutea from the previous cycle. The follicles then grow to their maximum size during estrus. Ovulation occurs early in the next stage, metestrus. In the latter part of this stage new corpora lutea are formed from differentiated granulosa and lutein cells of the ovulated follicles. During diestrus, the newly formed corpora lutea and follicles for the next cycle grow (Hebel and Stromberg, 1986, pg. 231).

Corpora lutea from previous cycles regress slowly in the rat, so that three or more generations can be seen in the ovary at any time during a specific estrus cycle (Hilliard, 1973). After several cycles the corpora lutea involute, form scar tissue which is known as corpora albicans, and eventually disappear. Figure 2 depicts a whole ovary from a rat at day 22 of pregnancy and the histology of the rat ovary. Individual corpora lutea which have been dissected from a rat ovary and the histology of the corpus luteum are shown in Figure 3.

The gestation time in rats is 23 days, with the corpus luteum reaching maximum function at day 16 of pregnancy, as illustrated in Figure 1. Functional regression of the corpus luteum, evidenced by a decrease in progesterone secretion, begins two to three days before parturition. The number of luteal cells in the rat corpus luteum stays constant (Meyer and Bruce, 1979; Dharmarajan *et al*, 1989), indicating that cell death does not occur at this time. Structural regression of luteal cells becomes apparent at day 1 post-partum and the weight of the corpus luteum of pregnancy continues to decline over numerous estrus cycles following parturition (Taya and Greenwald, 1982).

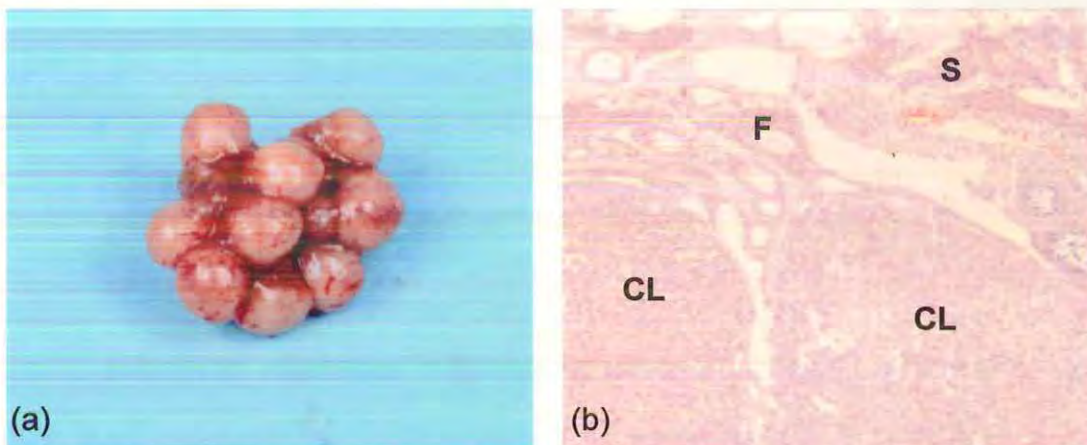


FIGURE 2: The Rat Ovary

- (a) Whole Ovary From a Rat at Day 22 of Pregnancy.
- (b) Section of Rat Ovary Stained with Haematoxylin and Eosin, 10x. CL - Corpus Luteum, F - Follicle, S - Stroma.

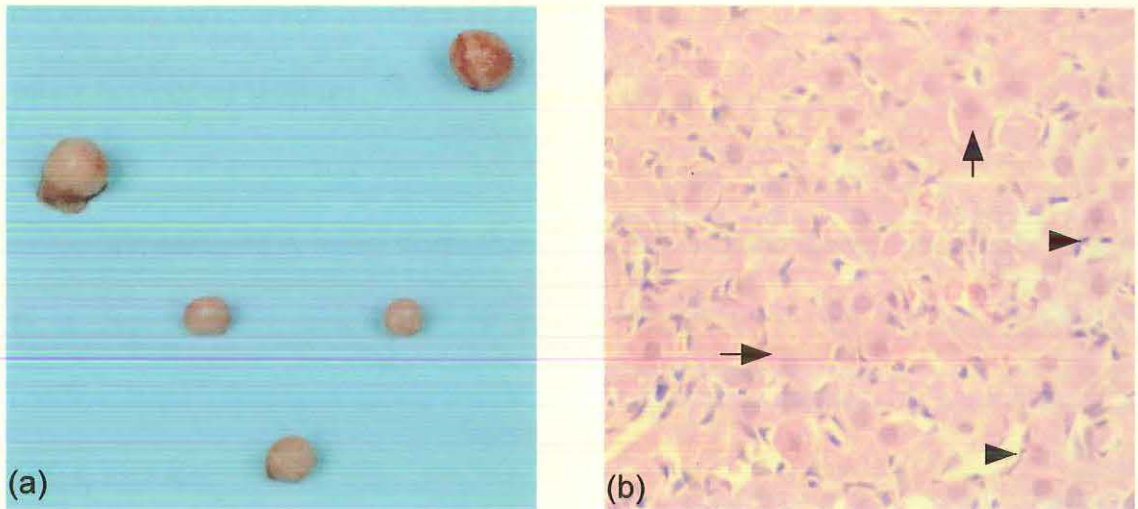


FIGURE 3. The Rat Corpus Luteum
 (a) Individual Corpora Lutea Dissected from a Rat Ovary at Day 22 of Pregnancy.
 (b) Section of Rat Corpus Luteum Stained with Haematoxylin and Eosin, 40x. Arrows - Luteal Cells, Arrowheads - Endothelial Cells.

2.2.3. Proposed Mechanisms of Corpus Luteum Regression

Hormonal influences in luteolysis differ from species to species and are not well characterised. The reduction in progesterone secretion prior to luteolysis has been attributed to secretion of prostaglandin $F_{2\alpha}$ in the sheep, cow, guinea-pig and horse (Johnson and Everitt, 1995, pg. 72), however a similar signal in other mammals has not been identified. Thus, luteolysis in some species may be initiated by withdrawal or insufficient supply of luteotrophic substances, whilst in others luteolysis follows the release of a luteolytic substance. The matter is further confused by evidence that some luteotrophic hormones may have luteolytic properties, either through an age

dependent differential reaction by the luteal cells, or when in combination with other hormones (Hilliard, 1973).

Whatever the initial hormonal influence, regression of the corpus luteum is a cyclic event which occurs in all mammals. Cellular and molecular mechanisms proposed to be involved in luteal cell regression (Juengel *et al*, 1994) include:

- (1) reduction of blood flow,
- (2) formation of oxygen radicals, and
- (3) infiltration of immune cells.

Reduction of blood flow to the corpus luteum at the time of regression could be expected to have a role in luteolysis, by diminishing the amounts of luteotrophic substances available to luteal cells. Rates of blood flow in relation to functional regression of the corpus luteum have been investigated by many researchers using various animals and models of regression, with variable results reported (Bruce *et al*, 1984). In the rat, blood flow to the corpus luteum did not appear to be reduced in natural regression at the end of pseudopregnancy (Pang and Behrman, 1979) or pregnancy (Bruce *et al*, 1984). Nor was it reduced by prostaglandin-induced regression (Pang and Behrman, 1981). Furthermore, examination by light microscopy showed no evidence of vascular degeneration during natural regression of the pregnant rat corpus luteum (Bruce *et al*, 1984). Thus blood flow does not appear to be a significant

factor in corpus luteum regression in the rat (Bruce *et al*, 1984) although it may have a role in luteolysis in other species.

Oxygen radicals and reactive oxygen species are highly charged and/or reactive products of physiological chemical processes produced in cells during aerobic metabolism. They are capable of causing damage to tissue due to their highly reactive nature. Consequently, cells have developed defence mechanisms, such as antioxidant enzymes. Research indicates, however, that oxygen radicals and reactive oxygen species assist in a variety of reproductive functions, including capacitation (a process involving plasma membrane changes to spermatozoa so that they are able to fertilise an ovum), the binding of sperm to the zona pellucida of the ovum, and ovulation (Riley and Behrman, 1991). Small amounts of hydrogen peroxide, a reactive oxygen species, have been shown to exert luteolytic effects on rat ovarian cells (Behrman and Preston, 1989). Also, production of hydrogen peroxide rapidly increases in rat ovaries during the natural luteal regression that occurs in late pseudopregnancy, and in regression induced by treatment with prostaglandin $F_{2\alpha}$ (Riley and Behrman, 1991), further indicating a role in luteolysis.

As granulosa cells do not differentiate into luteal cells until the onset of sexual maturation, luteal tissue is not present during the time that the immune system is adapting to "self". This unique characteristic of the corpus luteum has led to the proposal that immune mechanisms may be involved in corpus luteum

regression (Bukovsky *et al*, 1995), and numerous studies indicate that the immune system may have a role in luteolysis. Bukovsky *et al* found in 1977 (cited in Bagavandoss *et al*, 1990) that existing corpora lutea persisted in adult rats treated with antithymocyte serum. Both macrophages and activated T lymphocytes were present in the rabbit corpus luteum throughout the luteal phase, with the number of macrophages increasing significantly during the latter stages of regression, both in pseudopregnancy and following parturition (Bagavandoss *et al*, 1990). Further, Class II major histocompatibility complex (MHC) antigens, which activate T lymphocytes when presented by cells in conjunction with antigens that trigger immune responses, have been identified in:

- human granulosa cells with autoimmune dysfunction (Hill *et al*, 1990),
- cultured bovine luteal cells treated with interferon gamma (Fairchild and Pate, 1989),
- bovine large luteal cells, increasing throughout the estrus cycle to reach a maximum before luteolysis (Fairchild Benyo *et al*, 1991),
- the rat corpus luteum during pregnancy and pseudopregnancy, increasing at the time of luteolysis (Bränström *et al*, 1994), and
- human granulosa lutein cells, substantially increasing during the late luteal phase (Bukovský *et al*, 1995).

Fairchild Benyo *et al* (1991) hypothesised that the expression of Class II MHC antigens on luteal cells potentiates immune responses involved with luteolysis. Whilst T lymphocyte infiltration of the corpus luteum did not appear to alter

significantly throughout the luteal phase in rats (Bränström *et al*, 1994), these cells were present in the regressing corpus luteum of the rabbit (Bagavandoss *et al*, 1988), and human (Bukovský *et al*, 1995). Thus, from research to date, immune cells do appear to have a role in corpus luteum function which could be related to a reduction in progesterone secretion and/or luteolysis.

It is important to note that each of the above proposed mechanisms involved in luteal regression are not mutually exclusive. Indeed, immune mechanisms could possibly lead to vascular degeneration and increased concentrations of oxygen radicals. The apoptosis which occurs during luteolysis may be triggered by one, or a combination, of these factors or by conditions yet to be identified.

2.3. Apoptosis

2.3.1. Background

Since the advent of the cell theory in the nineteenth century, scientists have known that all cells must eventually die. Before the early 1970's, however, most research into cell death focused either on necrosis, cell death resulting from physical insults, such as trauma, infectious agents and exposure to toxins, or on the mechanisms of ageing. The significance of physiological cell death was not fully realised until after the process was characterised morphologically by Kerr *et al* (1972). This regulated process of cell death was

named apoptosis from the Greek word for falling (as does a leaf from a tree), referring to detachment of the dying cells from the extracellular matrix.

Apoptosis is necessary for normal function in organisms as it allows tissue remodelling and cellular replacement to occur quickly with little or no damage to surrounding cells. It is also a pathway which may be taken by cells which have been significantly, but not fatally, damaged by physical insult. Examples of apoptotic cell death occur during development (Hurle, 1988; Clarke, 1990), in the elimination of autoreactive T and B lymphocytes (MacDonald and Lees, 1990; Sambhara and Miller, 1991), during the degradation of endometrial cells (Rotello *et al*, 1989), and in cytotoxic T lymphocyte-mediated killing of target cells (Cohen *et al*, 1985).

Cell death by apoptosis follows from a range of stimuli or from the withdrawal of growth factors. Depending on the cell type and stimuli, apoptotic cell death may occur by a variety of molecular pathways, however, the observed morphological characteristics are generally uniform. Induction involves the activation of specific proteins which function in various aspects of this process. Occurring in single cells or small clusters of cells, apoptosis is characterised by cellular condensation, membrane blebbing, cleavage of DNA into oligomers with multiples of 185 base pair fragments (the DNA is cleaved at internucleosomal sites), and packaging of cytoplasmic and nuclear contents into membrane-bound vessels known as apoptotic bodies. Surrounding cells or

macrophages phagocytose these apoptotic bodies, preventing the release of harmful cellular contents in the extracellular matrix.

Identification and confirmation of apoptotic cell death can be provided by a variety of methods. Those most commonly used include the observation of morphological characteristics, 3'-end labelling, *in situ* labelling of double strand DNA breaks with terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labelling (TUNEL), and the comet assay. The morphology of dying cells can be viewed by light or electron microscopy. As stated previously, features which distinguish apoptotic cells from necrotic cells include the presence of cellular condensation, chromatin condensation, membrane blebbing and the production of apoptotic bodies. By comparison, cells dying by necrosis are evidenced by cellular swelling, lysis of the cells and the presence of inflammation in the surrounding tissue. Viewing the morphology of cells is an important and useful tool to confirm apoptotic cell death in the tissue of interest, as cell types and locations can be identified. This technique, however, is not a practical choice for use in quantitative studies, as morphological characteristics of apoptosis may only be seen in a small number of cells at any one time and cell counting methods are time-consuming.

Both 3'-end labelling and TUNEL involve labelling of DNA which has been fragmented. DNA extracted from tissue is used for 3'-end labelling, where the 3'-ends of cleaved DNA are labelled with a radioactive nucleotide ($\alpha^{32}\text{P}$ -

ddATP). The labelled DNA is fractionated on an agarose gel from which an autoradiograph is developed. DNA from cells undergoing apoptosis show ladders with multiples of around 185 base pairs in size, indicating that the DNA has been cleaved at internucleosomal sites, while DNA from necrotic cells is characterised by a smear, indicating the random breakdown of genetic material which occurs in this type of cell death (Tilly and Hsueh, 1993). The 3'-end labelling technique is a useful tool for quantitative analysis of the extent of apoptosis, as the low molecular weight DNA bands from each lane in an agarose gel can be excised and counts per minute obtained from a scintillation counter. This method, however, cannot confirm which cell types in tissue samples are dying by apoptosis. On the other hand, TUNEL does allow the identification of which cells are dying, but does not distinguish between apoptosis and necrosis (Hsueh *et al*, 1994). The comet assay involves electrophoresis of cell preparations to draw charged DNA from the cell nucleus (Fairbairn *et al*, 1995). Fragmented DNA forms a characteristic "comet-like" tail as it migrates further than intact DNA, and so apoptotic cells can be rapidly identified (Fairbairn *et al*, 1995). Two methods of detection are often used in tandem, in order to balance the relative advantages and disadvantages of each.

2.3.2. Apoptosis and the Reproductive System

The reproductive system provides many examples of physiological processes which require extensive tissue remodelling and cell death during normal function. Thus, it is not surprising that the occurrence of apoptosis has been confirmed in various reproductive cell types undergoing regression and attrition. Numerous studies of the relationships between hormone-dependent cells and apoptosis have been conducted.

In the male, apoptosis was found to occur in the ventral lobe of the rat prostate gland following the post-castration cessation of testosterone secretion (Kyprianou and Isaacs, 1988). The rat prostate has since been developed as an *in vivo* model for studying molecular mechanisms of apoptosis, as a considerable number of cells undergo apoptosis and the length of time taken for the ventral prostate cells to die is predictable (Colombel and Buttyan, 1995).

Apoptosis has also been implicated in the deletion of germ cells during normal spermatogenesis. Up to 75% of the potential number of mature sperm which can be produced from germ cells in the adult testis are lost (Billig *et al*, 1996). The morphological characteristics observed in spermatogonial cell death are the same as those described in cells undergoing apoptosis (Allan *et al*, 1992). Depletion of testosterone and gonadotropin by administration of an antagonist to gonadotropin-releasing hormone was associated with a significant

increase in apoptosis of germ cells, as detected by 3'-end labelling and TUNEL (Hikim *et al*, 1995; Brinkworth *et al*, 1995). A role for apoptosis in prenatal male gonads has also been suggested, with the identification of apoptotic morphology in embryonic germ cells of the male mouse (Coucovanis *et al*, 1993). Follicle stimulating hormone (FSH) has been shown to influence the survival of germ cells in both immature and adult rats, with apoptosis (again evaluated by 3'-end labelling and TUNEL) increasing when FSH was specifically immunoneutralised (Shetty *et al*, 1996). Further, in samples of semen collected from a human fertility centre, a correlation was found between the percentage of sperm cells which showed characteristics associated with low fertility and the percentage of sperm cells which stained positive for DNA strand breaks using TUNEL (Gorczyca *et al*, 1993). The authors concluded that apoptosis may be involved in the physiological deletion of germ cells, possibly to eliminate defective cells (Gorczyca *et al*, 1993), however, other detection methods for apoptosis would need to be used to confirm this suggestion.

Apoptosis has also been found to occur in the female reproductive system and, as in the male, hormonal control does appear to influence physiological cell death in some situations. Ovariectomy, for example, results in apoptosis of uterine epithelial cells in rabbits (Rotello *et al*, 1989).

One of the most striking examples of tissue loss over time occurs in the ovaries of mammals. Before the onset of ovulation, 99.9% of the follicles

present in human ovaries at birth degenerate by a process known as atresia (Hsueh *et al*, 1994). In 1991, Hughes and Gorospe showed by identification of morphological characteristics and use of 3'-end labelling that rat granulosa cells of follicles induced to degenerate appear to undergo apoptosis. Similar findings were published by Tilly *et al* in the same year with naturally atretic chicken and porcine follicles. Hormones were found to control apoptosis in granulosa cells, with oestrogens preventing apoptosis and testosterone negating the effects of oestrogens (Billig *et al*, 1993). Whilst Billig *et al* (1993) found that progesterone had no effect on granulosa cell apoptosis, Peluso and Pappalardo (1994) reported that progesterone reduced the incidence of apoptosis in cultured rat granulosa cells and, further, that adhesion between cells enhanced the anti-apoptotic effects of progesterone.

2.3.3. Apoptosis in the Corpus Luteum

The first molecular evidence that luteal cells may undergo apoptosis was presented by Zeleznik *et al* (1989). Similarities in descriptions of the histological changes occurring in the corpus luteum during the luteal phase of pregnancy (Corner, 1956), and the cellular changes associated with apoptosis (Kerr *et al*, 1972), prompted Zeleznik *et al* (1989) to investigate the occurrence of apoptotic degeneration in luteal cells. As stated previously, one of the characteristics of apoptosis is the cleavage of DNA into oligomers of 185 base pairs. Cohen and Duke (1984) identified an endonuclease capable of

fragmenting DNA in this manner, so Zeleznik *et al* (1989) searched for, and detected, $\text{Ca}^{++}/\text{Mg}^{++}$ -dependent endonuclease activity in differentiated rat granulosa cells, but not in undifferentiated granulosa cells. Interestingly, a calcium ionophore had been shown to mimic the luteolytic effects of prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) in isolated luteal cells (Baum and Roseberg, 1977), perhaps because it activated an endonuclease responsible for DNA cleavage in apoptosis.

That luteal cells were actually capable of dying by apoptosis was initially confirmed by Juengel *et al* (1993). In both spontaneous and $\text{PGF}_{2\alpha}$ -induced luteal regression in cattle, apoptosis, detected by fractionating DNA on an ethidium bromide-stained agarose gel or 3'-end labelling, occurred after progesterone serum levels had begun to decrease. Hence, Juengel *et al* (1993) proposed that apoptosis was instrumental during structural regression of the corpus luteum but not during functional regression. This study also found that capillaries within the corpus luteum disappeared before fragmented DNA could be detected, indicating that endothelial cells were not undergoing apoptosis at the time of structural regression of luteal cells. It is possible that the decrease in progesterone secretion by luteal cells, or the disappearance of endothelial cells in the corpus luteum, may be early triggers leading to the apoptotic cell death seen in structural regression in cattle (Juengel *et al*, 1993).

In contrast, a correlation between the occurrence of apoptosis and functional luteolysis in the pseudopregnant and pregnant rabbit ovary has been found (Dharmarajan *et al*, 1994; Dharmarajan *et al*, 1997). An analysis of DNA integrity by TUNEL confirmed that those cells undergoing DNA fragmentation were luteal cells (Dharmarajan *et al*, 1994). The *in vitro* corpus luteum culture model was developed following the discovery that spontaneous apoptosis occurred in rabbit corpora lutea cultured in serum-free medium. This model was used to show that treatment with human chorionic gonadotropin, the hormone which sustains luteal function during the first trimester of pregnancy in humans, could suppress apoptosis in cultured rabbit corpora lutea (Dharmarajan *et al*, 1994).

Recently, *in vivo* treatment of rat corpora lutea with a gonadotropin-releasing hormone agonist during early pregnancy was found to induce apoptosis in luteal cells (Sridaran *et al*, 1997). The same study found that spontaneous apoptosis of luteal cells could be induced by culturing rat corpora lutea in serum-free medium (Sridaran *et al*, 1997). An analysis of DNA integrity by TUNEL indicated that some apoptosis is occurring at day 22 of rat pregnancy, increasing by day 1 post-partum (Ke *et al*, 1997).

Corpora lutea of marmoset monkeys undergoing induced regression following administration of either a $\text{PGF}_{2\alpha}$ analogue or gonadotropin-releasing hormone antagonist, showed morphological features in some degenerating

luteal cells which were characteristic of apoptosis, whilst other luteal cells appeared to be dying by necrosis (Fraser *et al*, 1995). Unfortunately, further experiments to confirm that apoptotic cell death was occurring, and to identify the extent of apoptosis, were not undertaken.

In summary, apoptosis has been found to occur during luteolysis in a range of mammalian species. The mechanisms and pathways involved in apoptotic cell death in the corpus luteum, however, have yet to be identified.

2.4. The Fas / Fas Ligand System

As stated in the previous section, the molecular pathway by which apoptosis occurs is dependent upon the cell type involved and the stimuli received. Thus, luteal cells undergoing apoptosis may use one of many pathways, or perhaps more than one pathway, during regression. One possible mediator involved in corpus luteum regression is the Fas (or APO-1 or CD95) receptor, a transmembrane protein which induces apoptosis in the cell when ligated.

2.4.1. Background

In 1989 two separate research groups developed mouse monoclonal antibodies, named Fas (Yonehara *et al*, 1989) and APO-1 (Trauth *et al*, 1989),

which were capable of inducing apoptosis in some human cell lines. The cytolytic activity was particularly apparent when the cells were also treated with interferon gamma (Yonehara *et al*, 1989). These antibodies had two possible modes of action: they either bound to an antigen which was a receptor with cytolytic activity and acted as a ligand by inducing apoptosis, or the antibodies bound to an antigen which was a receptor for a growth factor or a molecule essential for transduction of a growth signal, blocking the signal and causing cell death. Expression of human Fas cDNA in mouse cell lines made the cells susceptible to death induced by the Fas monoclonal antibody, indicating that the Fas receptor did transduce an apoptotic signal to the cell (Itoh *et al*, 1991). The Fas receptor was classified as a type I membrane protein (because the N-terminal of the protein was extracellular and the C-terminal lay within the cell), belonging to the tumour necrosis factor (TNF) receptor family. This grouping includes TNF receptor I (also capable of inducing cell death) and II, nerve growth factor receptor and lymphocyte antigens such as CD40 and OX40 (Itoh *et al*, 1991). Subsequently, the Fas and APO-1 antibodies were found to act upon the same receptor (Oehm *et al*, 1992).

Fas antigen mRNA expression was detected in the mouse thymus, heart, liver and ovary (Watanabe-Fukunaga *et al*, 1992), indicating that it was possibly involved in a variety of apoptotic processes. Deletion of a region at the C-terminus of Fas showed that this area was necessary for the transduction of cytolytic activity (Itoh and Nagata, 1993). Homology to the TNF receptor I was

significantly conserved in this region and the 68 constituent amino acids were termed the "death domain", highlighting their function in transducing an apoptotic signal to the cell (Itoh and Nagata, 1993).

Fas ligand, cloned in 1993, was found to be a type II transmembrane protein belonging to the TNF family (Suda *et al*, 1993). The structure and properties of Fas ligand were similar to those of TNF α (for example, Fas ligand appeared to function as a trimer and overexpression of the protein resulted in shedding of a soluble form which was capable of inducing apoptosis), but Fas ligand specifically activated the Fas receptor (Suda *et al*, 1993). Fas ligand mRNA expression in the rat was detected in spleen, testis, small intestine, kidney and lung (Suda *et al*, 1993).

As Fas and TNF had many similarities, Clément and Stamenkovich (1994) compared the two by transfecting fusion proteins consisting of the extracellular region of CD40 and the transmembrane and intracellular regions of Fas receptor, TNF I receptor or TNF II receptor into a human melanoma cell line which did not express these proteins. Cells were treated with the CD40 ligand and differences in the rates of cell death examined. While Fas receptor was capable of killing most cells within hours, the TNF receptors required two to three days of treatment with the CD40 ligand for cytotoxicity to occur (Clément and Stamenkovich, 1994), indicating either that different molecular mechanisms were involved in the induction of apoptosis or that the TNF receptors induced

apoptosis following a convoluted series of steps, whilst Fas-mediated apoptosis directly and immediately triggered the cell death pathway.

2.4.2. Fas and Fas Ligand in the Immune System

In the few short years since the Fas/Fas ligand system was discovered and characterised many important functions within the immune system have been shown to involve Fas-mediated apoptosis. The physiological significance of Fas expression in the thymus was first realised when it was found that mice carrying the lymphoproliferation (*lpr*) mutation had defects in the gene for Fas receptor (Watanabe-Fukunaga *et al*, 1992). Lymphoproliferation disorder in mice resembles human systemic lupus erythematosus and is characterised by lymphadenopathy, splenomegaly, production of many autoantibodies and the development of nephritis or arthritis (Watanabe-Fukunaga *et al*, 1992). Further evidence for this proposal was presented when generalised lymphoproliferative disease (*gld*) in mice, also characterised by lymphadenopathy and splenomegaly, was found to result from a point mutation in the gene coding for Fas ligand (Takahashi *et al*, 1994). The phenotypes indicate that Fas-mediated apoptosis is used in the thymus to delete negatively selected T cells which react to "self" antigens.

T-cell mediated cytotoxicity, previously thought to result only from formation of perforin pores in target cells, can occur by the Fas/Fas ligand

system. Fas-mediated apoptosis of target cells was demonstrated by cytotoxic T lymphocytes incapable of killing using perforin pores (Kägi *et al*, 1994). Target cells from *lpr* mutant mice, however, could not be killed by these T cells (Lowin *et al*, 1994). Thus, cytotoxic T lymphocytes are capable of expressing Fas ligand in order to induce apoptosis in target cells which express the Fas receptor. More recently, human natural killer cells have also been shown to be capable of inducing apoptosis in target cells by expression of Fas ligand (Oshimi *et al*, 1996).

As both *lpr* and *gld* mice showed defects in the deletion of peripheral activated T cells, a role for Fas in inducing apoptosis of activated T cells for immune suppression was proposed (Dhein *et al*, 1995; Ju *et al*, 1995). Activated T cells increased the expression of Fas receptor mRNA (Dhein *et al*, 1995) and markedly increased the expression of Fas ligand mRNA (Ju *et al*, 1995). Also, the death of activated T cells could be prevented by treating cells with an antibody capable of blocking Fas-mediated apoptosis (Dhein *et al*, 1995; Ju *et al*, 1995).

In summary, Fas-mediated apoptosis has been shown to have significant roles in immune system function, including deletion of self-reactive T lymphocytes in the thymus, T cell- and natural killer cell-mediated cytotoxicity and immune suppression of activated T cells.

2.4.3. Fas Ligand and Immune Privilege

The ability of some tissue sites to protect grafted tissue from rejection and of some tissue types to be grafted without being rejected is known as immune privilege. Attacks by the immune system in response to foreign antigens invariably result in damage to surrounding tissue and inflammation. In delicate or non-dividing tissue, as is found in the eye and brain, the effect of the cure may be worse than the cause. Also, in the case of pregnancy, foreign tissue must be accepted for successful reproduction. Protection from damaging immune reactions is achieved by a variety of physiological means, including blood-tissue barriers and immune suppression.

The expression of Fas ligand to facilitate the killing of activated T cells displaying the Fas receptor has been proposed as another mechanism of immune privilege (Bellgrau *et al*, 1995; Griffith *et al*, 1995). In confirmation, Sertoli cells of the testis express Fas ligand and are able to survive indefinitely when transplanted in the kidney capsule of allogeneic animals, whilst Sertoli cells from gld mice (which express non-functional ligand) are rejected (Bellgrau *et al*, 1995). Further, Griffith *et al* (1995) performed a series of experiments implicating Fas ligand-mediated immune privilege in the mouse eye. Both Fas ligand mRNA and protein were expressed in the eyes of normal mice. Inflammatory cells entering the anterior chamber of the eye in normal mice, but not in gld mice, died. No immune response was detected in the virally infected

eyes of normal mice, whereas gld mice exhibited inflammation and survival of immune cells in similarly infected eyes. Also, Fas-positive tumour cells died when exposed to isolated anterior segments of the eye in normal mice but not in gld mice, and Fas-negative tumour cells did not die (Griffith *et al*, 1995). Unfortunately, the mechanism(s) of cell death were not confirmed as being apoptosis, as the authors analysed DNA fragmentation by TUNEL alone.

Fas ligand has also been detected in human cytotrophoblasts of first trimester placental tissue sections and syncytiotrophoblasts of term placenta by immunohistochemical staining and Western blotting, indicating that its involvement in immune privilege may also extend to protection of the foetus (Runic *et al*, 1996).

2.4.4. Fas and Fas Ligand in the Reproductive System

Fas and Fas ligand mRNA and protein is expressed in a range of reproductive tissues (Watanabe-Fukunaga *et al*, 1992; Suda *et al*, 1993; Bellgrau *et al*, 1995; French *et al*, 1996). Examination of their role in these tissues, however, has only recently begun.

Steroid hormones such as testosterone, oestrogen and progesterone are essential for normal reproductive functioning, not least because of their role in the regulation of cell proliferation. Removal of the gonads (and, therefore,

cessation of testosterone secretion) in the normal male mouse resulted in expression of Fas receptor and apoptotic regression of the prostate and epididymis, whilst no regression was observed following gonadectomy of *lpr* mice (Suzuki, Matsuzawa and Iguchi, 1996). Similarly, Fas expression was increased and apoptotic cell death confirmed during ovariectomy-induced regression of normal mouse vaginal epithelial cells, but changes were not detected in *lpr* mice (Suzuki, Enari, Eguchi, Matsuzawa, Nagata, Tsujimoto and Iguchi, 1996).

Abundant expression of Fas receptor in regressing corpora lutea and atretic follicles of humans, as well as decreasing expression of Fas receptor in maturing oocytes has been detected using immunohistochemistry (Kondo *et al*, 1996). Confirmation of these findings was provided by Quirk *et al* (1995) who found that Fas mRNA was expressed by human granulosa / luteal cells. This study also showed that cultured granulosa / luteal cells pretreated with interferon gamma were capable of undergoing apoptosis induced by a Fas monoclonal antibody (Quirk *et al*, 1995).

2.5. Summary

The molecular mechanisms involved in regression of the corpus luteum, an essential process in the female reproductive system, are not well understood. Although apoptosis has been shown to occur during luteolysis

(Juengel *et al*, 1993; Dharmarajan *et al*, 1994; Fraser *et al*, 1995; Dharmarajan *et al*, 1997; Sridaran *et al*, 1997; Ke *et al*, 1997), the pathway(s) leading to cell death have yet to be identified. Fas mRNA is expressed by human granulosa and luteal cells, and Fas monoclonal antibodies are able to induce apoptosis in these cells when they are pretreated with interferon gamma in culture (Quirk *et al*, 1995). In addition, abundant expression of the Fas receptor in the regressing corpus luteum of the normal adult human ovary has been reported (Kondo *et al*, 1996). Further studies are required to examine the regulation of both Fas receptor and Fas ligand in the naturally regressing corpus luteum, and to clarify the role of the Fas/Fas ligand system in corpus luteum function.

3. ANIMALS

Throughout the research the experimental animals used were sexually mature (10-12 week old) female Wistar rats. They were housed at 21°C with 55% humidity in a 12 hours light / 12 hours dark cycle. The feed was autoclaved normal cubes and acidified water, both provided ad lib.

3.1. Mating and Gestation

Five female rats were caged with one male rat overnight to allow mating to occur. Day 1 was taken to be the day after successful mating. Rats were considered to be pregnant if sperm were present in a vaginal smear on Day 1. The gestation time for rats is 23 days (ie: the litter is born on day 23 of pregnancy). Table 1 shows the various pregnancy and post-partum stages used throughout this study.

Table 1
Stages of Pregnancy and Post Partum in the Rat used for this Study

STAGE	ABBREVIATION	DESCRIPTION
Day 1	D1	The day after successful mating
Day 8	D8	8 days after successful mating
Day 16	D16	16 days after successful mating
Day 22	D22	22 days after successful mating
Post-Partum Day 1	PP1	1 day after birth of litter
Post-Partum Day 3	PP3	3 days after birth of litter

3.2. Tissue Collection

Rats were heavily anaesthetised with a mixture of halothane and nitric oxide during the procedure. Ovaries and portions of spleen were excised as quickly as possible and treated as required for the particular protocol to be followed (either immunohistochemistry, RT-PCR or organ culture). After the removal of tissue, rats were killed by administration of a lethal dose of anaesthetic.

4. IMMUNOHISTOCHEMISTRY

4.1. Rationale

Immunohistochemistry is an established, routine histological technique used to identify antigens in tissue sections by means of antigen reaction with a labelled primary or secondary antibody (Robinson *et al*, 1990, pg. 413). Not only can the presence of Fas and Fas ligand be confirmed using this technique, but the proteins can also be localised to particular cells in tissue sections.

Antibodies, which belong to a group of proteins called immunoglobulins, are produced by B lymphocytes to bind selectively to foreign antigens. The region on the antigen to which the antibody binds is known as an epitope. There are an almost infinite number of antibodies which can be made, but individual B lymphocytes can only make one specific antibody. When launching an immune attack, a B lymphocyte is able to proliferate, forming clones which all secrete the same specific antibody.

Antibodies produced for immunohistochemistry may be either monoclonal or polyclonal. Monoclonal antibodies are produced by clones of one B lymphocyte, so they bind with one specific epitope on an antigen. Polyclonal antibodies are made by clones of different B lymphocytes which react to a specific antigen, so they bind to a variety of epitopes on the one

antigen (see Figure 4). As monoclonal antibodies bind to only one epitope, there is less chance of non-specific binding or cross-reactivity compared to polyclonal antibodies. However, if the epitope is concealed by tissue fixation no binding can occur, whilst polyclonal antibodies can bind to various epitopes which may not have been concealed on the antigen. Also, the time and cost involved in producing monoclonal antibodies is much higher than for polyclonal antibodies.

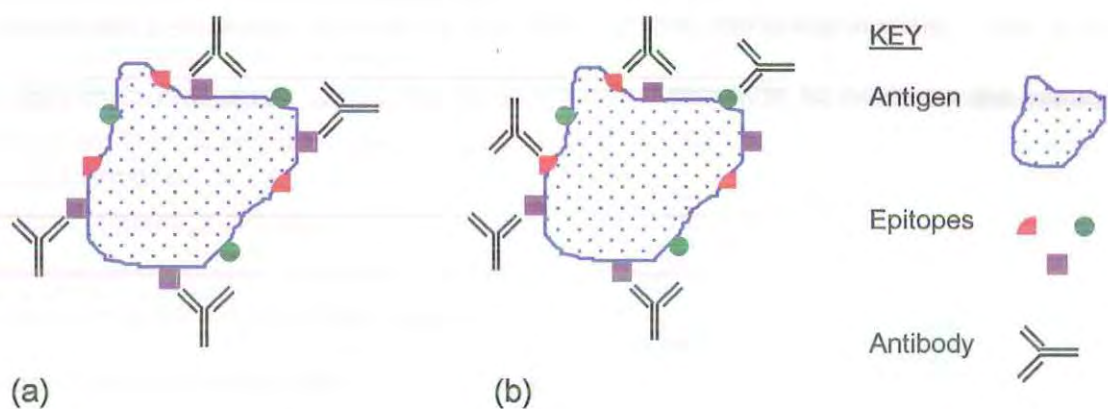


FIGURE 4: Monoclonal and Polyclonal Antibody Binding
 (a) Monoclonal antibodies bind to a specific epitope on an antigen.
 (b) Polyclonal antibodies bind to various epitopes on an antigen.

A variety of protocols have been developed for the detection of bound labelled antibodies. Immunoenzymatic staining using the enzyme horse radish peroxidase and the chromogenic substrate 3'3'-diaminobenzidine tetrahydrochloride (DAB) was chosen for both the Fas receptor and Fas ligand. The reaction between horse radish peroxidase and DAB in the presence of

hydrogen peroxide produces a brown end product which is stable and can be viewed by light microscopy.

The two-step indirect method of immunoenzymatic staining was used to detect Fas receptor in tissue sections (see Figure 5). After incubation with the unlabelled primary antibody (mouse anti-rat Fas monoclonal antibody), a horse radish peroxidase-labelled secondary antibody directed against the primary antibody was applied. As the primary antibody was produced in the mouse, the secondary antibody chosen bound with mouse immunoglobulins. The section was then incubated with DAB and hydrogen peroxide to produce the coloured end product.

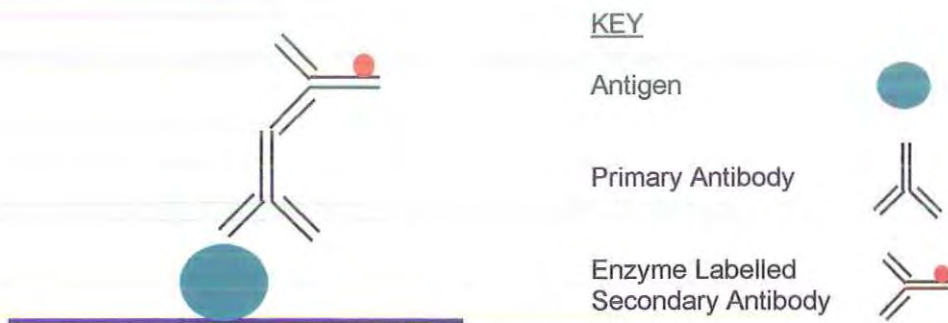


FIGURE 5: Two-Step Indirect Method of Immunoenzymatic Staining

A labelled avidin-biotin technique was used to detect the presence of Fas ligand (see Figure 6). This method utilises the high affinity between avidin or streptavidin and biotin. After the unlabelled primary antibody (goat anti-rat Fas ligand polyclonal antibody) was applied, sections were incubated with the

secondary antibody, which was biotinylated (covalently bound to biotin). As the primary antibody was produced in the goat the secondary antibody chosen bound with goat immunoglobulins. Streptavidin labelled with horse radish peroxidase was then applied to sections to bind with the biotin on the secondary antibody, and DAB and hydrogen peroxide were applied to produce the coloured end product.

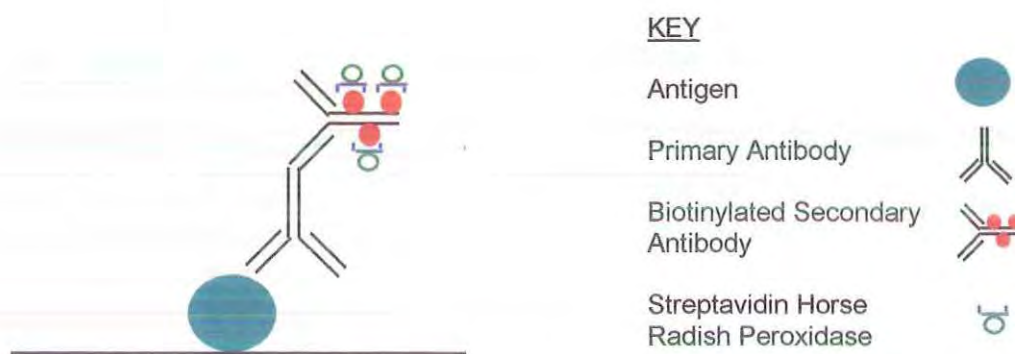


FIGURE 6: Labelled Avidin-Biotin Method of Immunoenzymatic Staining.

The Fas antigen has previously been localised in the human ovary using the avidin-biotin immunoperoxidase method (Kondo *et al*, 1996). It is necessary, however, to confirm the presence and localisation of both Fas receptor and Fas ligand at the time of corpus luteum regression, as both are required for induction of Fas-mediated apoptosis. Thus, the present study was designed to examine patterns in staining of corpora lutea at the same stage of pregnancy and post-partum, as well as at different stages. It was also planned that a comparison of results for Fas receptor and Fas ligand would be made.

4.2. Sample and Tissue Preparation

For the study, four rats from each stage of pregnancy and post-partum (i.e. days 1, 8, 16 and 22 of pregnancy and days 1 and 3 post-partum) were required, and one ovary from each rat was used. Portions of spleen were excised from rats for use as positive controls. Following excision, the ovaries and portions of spleen were fixed in 4% paraformaldehyde at 4°C for 24 hours. They were then kept in phosphate buffered saline (PBS) at 4°C until processing. Tissue was processed using a Shandon Citadel 1000 Tissue Processor with a 10 hour protocol, as outlined in Table 2.

Table 2
10 Hour Protocol for Tissue Processing

SOLUTION	INCUBATION TIME
50% Ethanol	1 hour
70% Ethanol	1 hour
90% Ethanol	1 hour
Absolute Ethanol	1 hour
Absolute Ethanol	1 hour
Absolute Ethanol	1 hour
Slidebright	1 hour
Slidebright	1 hour
Paraffin Wax	1 hour
Paraffin Wax	1 hour

After being placed within wax in a vacuum embedder for 30 minutes, the tissue was embedded in paraffin wax and stored at -20°C to allow the blocks to harden. Sections with a thickness of 5 microns were cut using a microtome. Four sections from each ovary, placed on separate slides, were required - one for detection of Fas receptor, one for detection of Fas ligand and two for use as

negative controls. A section of spleen was also placed on each of the slides for use as a positive control. The slides were incubated at 45°C overnight to dry.

4.3. Method

Paraffin wax was removed from the sections using toluene, and the slides were then rehydrated through alcohol solutions of decreasing concentrations. The sections were incubated in 3% hydrogen peroxide in methanol for 10 minutes and washed three times in fresh PBS.

When staining for Fas receptor, sections were first incubated in 1% sodium dodecyl sulphate (SDS) for 5 minutes and then washed in fresh PBS three times. Sections were incubated with primary antibody (mouse anti-rat Fas monoclonal IgG, 250µg/mL) diluted 1 in 100 with PBS plus 1% bovine serum albumin (BSA) at room temperature for 2 hours, then washed in fresh PBS three times. The secondary antibody (sheep anti-mouse immunoglobulin, 0.5mg/mL), which was diluted 1 in 100 with PBS plus 1% BSA plus 2% normal rat serum, was incubated on sections for 45 minutes. The slides were then washed in fresh PBS three times.

When staining for Fas ligand, sections were incubated with the primary antibody (goat anti-rat Fas ligand polyclonal IgG, 200µg/mL) diluted 1 in 50 with PBS plus 1% BSA at room temperature for 2 hours, then washed in fresh PBS

three times. Sections were incubated with the secondary antibody (biotinylated rabbit anti-goat IgG, 1.5mg/mL) diluted 1 in 50 with PBS plus 1% BSA plus 2% normal rat serum for 45 minutes, then washed in fresh PBS three times. Streptavidin horse radish peroxidase conjugate was diluted 1 in 50 with PBS plus 1% BSA and incubated on sections for 45 minutes. Slides were then washed in fresh PBS three times.

Negative controls for each section were treated in the same manner, but received diluent only in place of primary antibody. Sections of spleen were placed in each run as a positive control.

All sections were then incubated for 10 minutes with 3,3'diaminobenzidine tetrahydrochloride (DAB) solution and then washed in fresh PBS three times. Sections were counterstained by placing in haematoxylin for 6 seconds, then dehydrated through alcohol solutions of increasing concentrations and finally toluene. Slides were coverslipped using DPX mountant for viewing by light microscopy.

See Figure 7 for experimental design. Detailed protocols are contained in Appendix B.

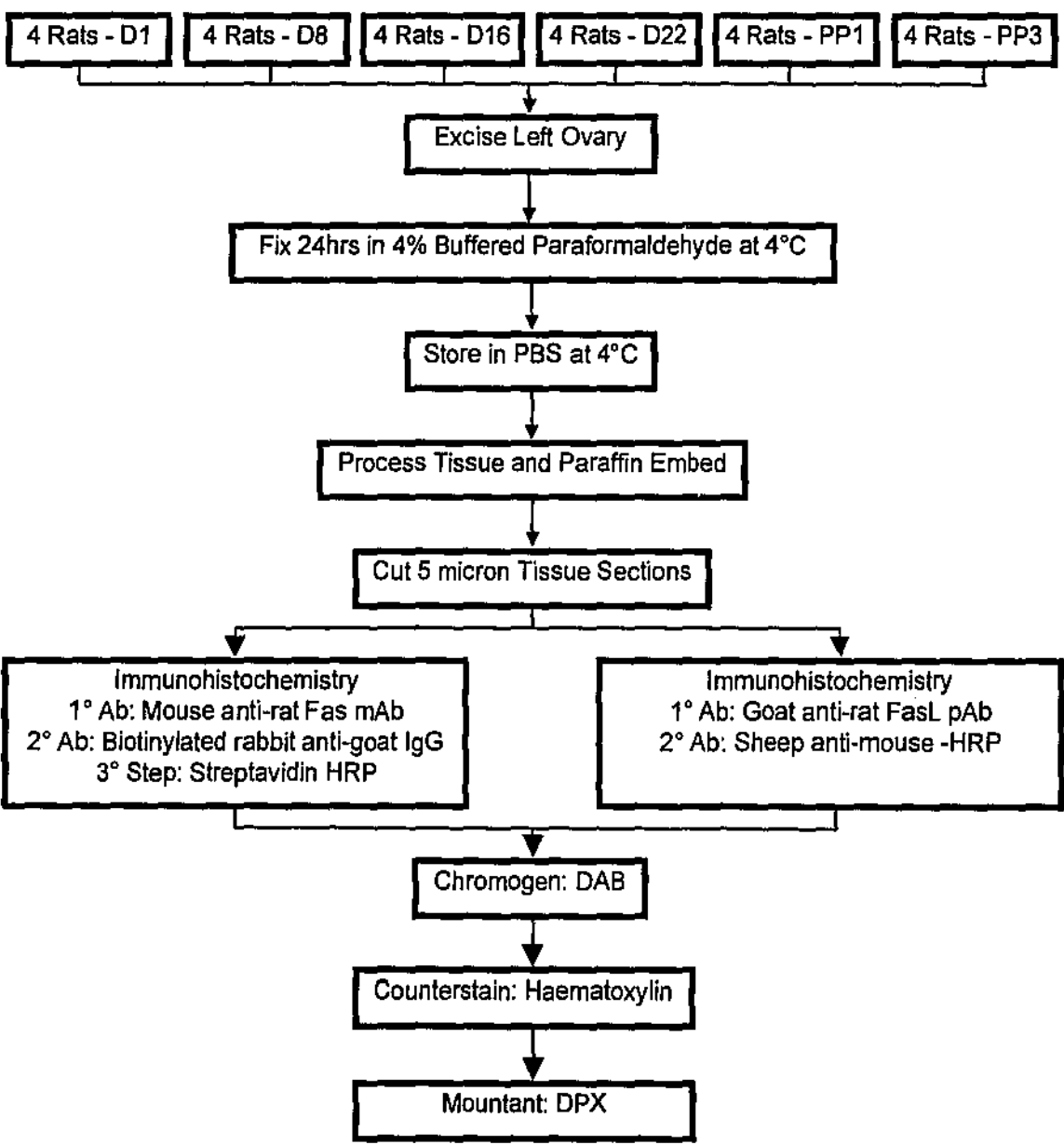


FIGURE 7: Experimental Design for Immunohistochemistry

4.4. Results

The presence of Fas receptor and Fas ligand within the rat corpus luteum during pregnancy and post-partum was established using immunohistochemistry.

4.4.1. Fas Receptor

Initially, the manufacturer's recommended protocol was followed when staining for Fas receptor. The primary antibody was diluted 1 in 500 and incubation times for primary and secondary antibodies were 30 minutes each. Staining was not observed in any of the sections tested under these conditions. Dilution of the primary antibody was reduced to 1 in 100 and incubation times were increased to 60 minutes for the primary antibody and 45 minutes for the secondary antibody. Very pale staining was seen with these conditions. The primary antibody was then diluted 1 in 50, with incubation times remaining unchanged. These parameters resulted in some nonspecific staining of negative control sections. The incubation time was increased to two hours and the primary antibody was diluted 1 in 100 to alleviate nonspecific staining. Prior to application of primary antibody the Fas antigen was further exposed with treatment of 1% SDS for 5 minutes to improve stain.

Pale immunohistochemical staining indicated that the Fas receptor was present in the rat corpus luteum at day 1 of pregnancy. No stain was seen in corpora lutea of rats at days 8 and 16 of pregnancy. Whilst pale staining was present at day 22 of pregnancy, the stain was most intense in the corpus luteum at day 1 post-partum. Staining was present, but less intense, at day 3 post-partum (see Figure 8). Within the corpus luteum, staining was concentrated in the cytoplasm of luteal cells. Endothelial cells displayed no positive staining for Fas receptor (see Figure 10). Negative control sections showed no nonspecific staining. Positive control sections (spleen) displayed scattered staining. The staining pattern and intensity in the spleen was consistent between immunohistochemical runs.

4.4.2. Fas Ligand

A 1 in 400 dilution of anti-rat Fas ligand polyclonal antibody with an incubation time of 60 minutes was initially used to localise Fas ligand, however no staining was seen with these conditions. Decreasing the dilution of primary antibody to 1 in 100 resulted in very pale staining which was unconvincing. The dilution of primary antibody was decreased further, to 1 in 50, but staining was not improved. Increasing the incubation time of the primary antibody (diluted 1 in 50) to two hours was found to improve the staining intensity dramatically, so this protocol was chosen for all sections.

Fas ligand was present in the rat corpus luteum at all stages of pregnancy and post-partum examined. At day 1 of pregnancy all but one corpus luteum in each rat ovary stained intensely (see Figure 12). The staining intensity decreased by day 8 and decreased further by day 16, with all corpora lutea staining positively at these stages. Expression of Fas ligand increased in corpora lutea at day 22 of pregnancy. Staining of Fas ligand was intense at day 1 post-partum and had decreased by day 3 post-partum (see Figure 11). Staining within the corpus luteum was seen in the cytoplasm of luteal cells. Endothelial cells did not stain positively for Fas ligand (see Figure 14). Nonspecific staining was not apparent in negative control sections. A characteristic scattered staining pattern was seen in the positive control sections of the spleen. The intensity of staining in the spleen was consistent between immunohistochemical runs.

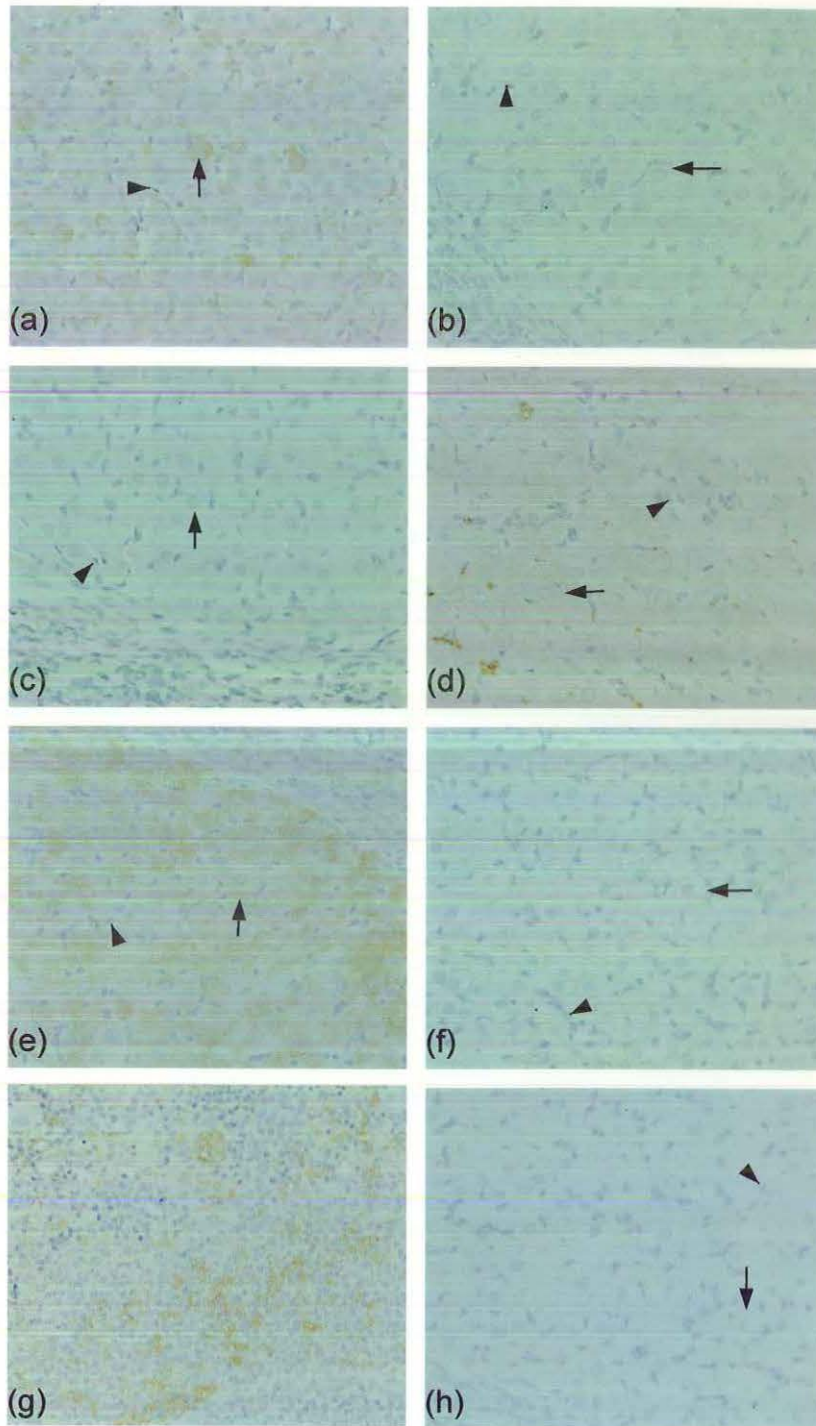


FIGURE 8. Rat ovary sections incubated with anti-Fas mAb and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 40x. Stain was present at day 1 of pregnancy, but not at days 8 and 16 of pregnancy. Pale staining was present at day 22 of pregnancy. Intensity of stain was highest at day 1 post-partum and was decreasing by day 3 post-partum. The positive control showed scattered staining, while the negative control showed no nonspecific staining. (a) Day 1 of Pregnancy. (b) Day 8 of Pregnancy. (c) Day 16 of Pregnancy. (d) Day 22 of Pregnancy. (e) Day 1 Post-Partum. (f) Day 3 Post-Partum. (g) Spleen, Positive Control. (h) Day 1 Post-Partum, Negative Control. Arrows - Luteal Cells. Arrowheads - Endothelial Cells.

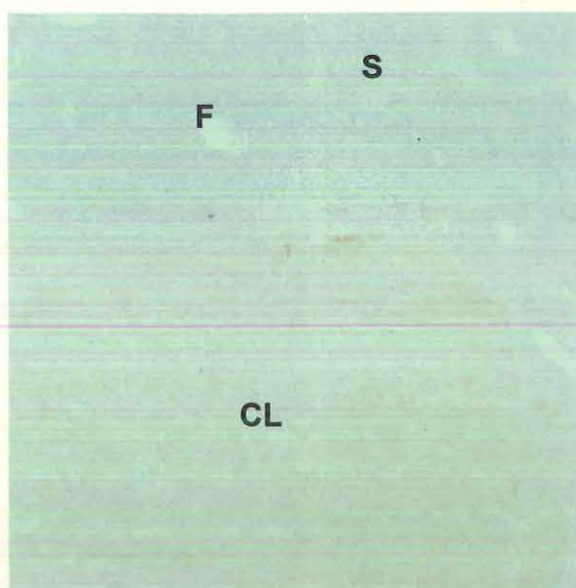


FIGURE 9. Stain for Fas in corpus luteum was distinct compared to the ovarian stroma and follicles. Rat ovary section incubated with anti-Fas mAb and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 10x. Day 1 Post-Partum. CL - Corpus Luteum. S - Stroma. F - Follicle.

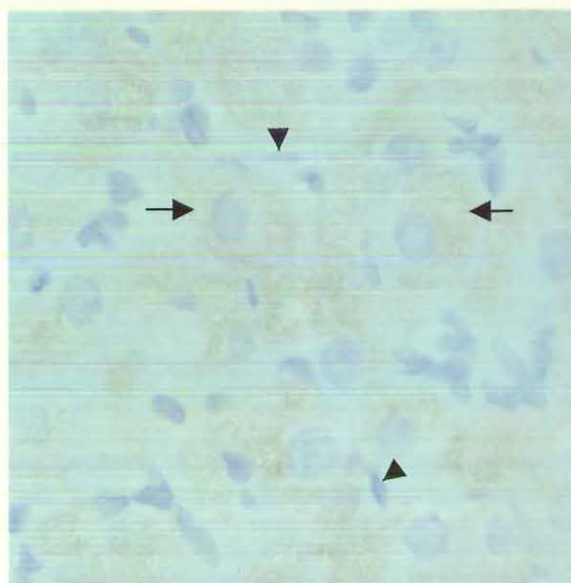


FIGURE 10. Stain for Fas within the corpus luteum was isolated to the cytoplasm of luteal cells. Endothelial cells did not stain positively for Fas receptor. Rat ovary section incubated with anti-Fas mAb and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 100x. Day 1 Post-Partum. Arrows – Luteal Cells. Arrowheads - Endothelial Cells.

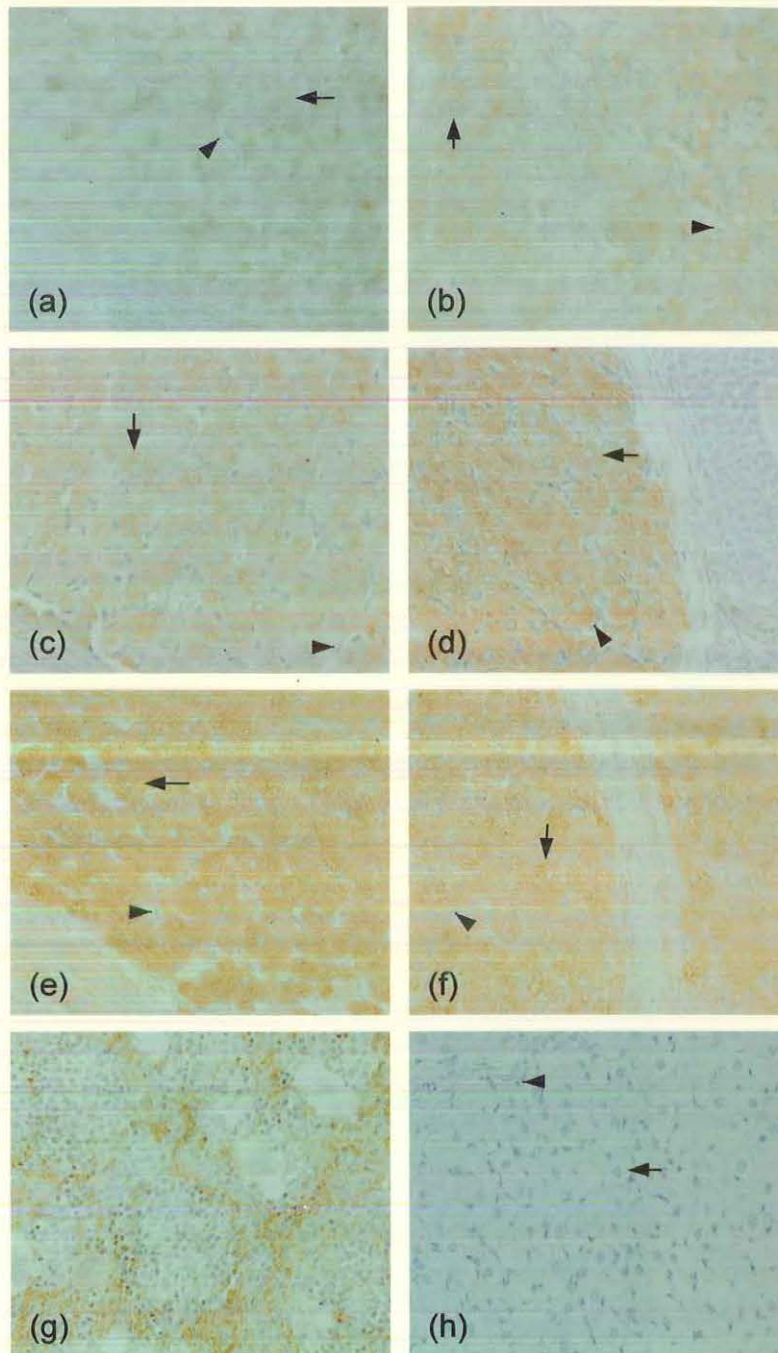


FIGURE 11. Rat ovary sections incubated with anti-Fas ligand polyclonal antibody and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 40x. Stain was present at all stages of pregnancy and post-partum. At day 1 of pregnancy all but one corpus luteum in each ovary stained intensely. The staining intensity decreased in day 8 and again in day 16 (all corpora lutea at these stages were stained), increasing at day 22 of pregnancy. Staining at day 1 post-partum was at its most intense. At day 3 post-partum the intensity of stain decreased slightly. The positive control showed scattered staining, while the negative control showed no nonspecific staining. (a) Day 1 of Pregnancy. (b) Day 8 of Pregnancy. (c) Day 16 of Pregnancy. (d) Day 22 of Pregnancy. (e) Day 1 Post-Partum. (f) Day 3 Post-Partum. (g) Spleen, Positive Control. (h) Day 1 Post-Partum, Negative Control. Arrows - Luteal Cells. Arrowheads - Endothelial Cells.

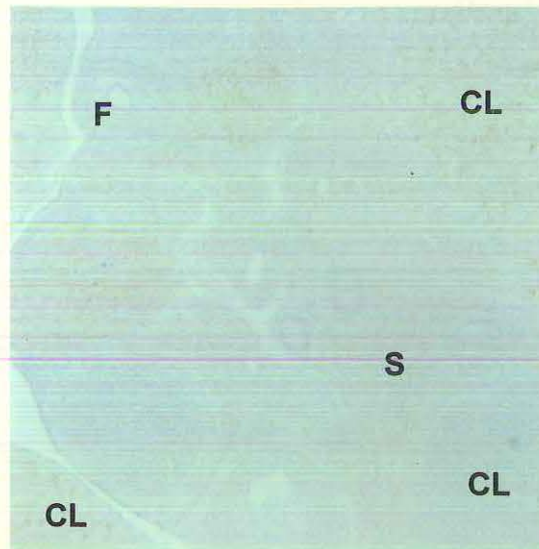


FIGURE 12. At day 1 of pregnancy all but one corpus luteum in each rat ovary stained positively for Fas ligand. Rat ovary section incubated with anti-Fas ligand polyclonal antibody and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 10x. Day 1 of Pregnancy. CL - Corpus Luteum. S - Stroma. F - Follicle.

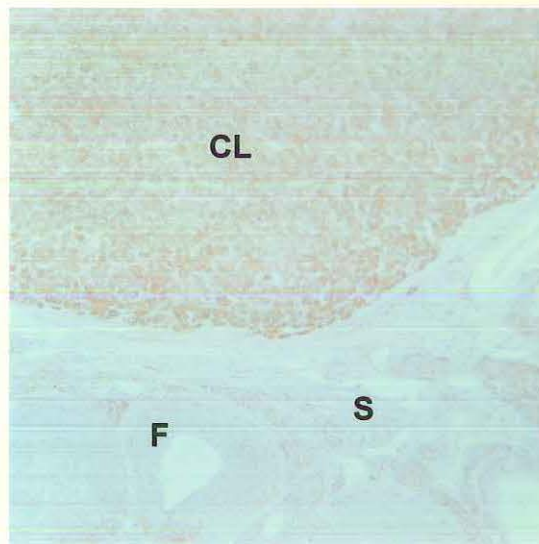


FIGURE 13. Whilst staining was observed in the ovarian stroma and some follicles, the staining intensity was highest in corpora lutea at day 1 post-partum. Rat ovary section incubated with anti-Fas ligand polyclonal antibody and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 10x. Day 1 Post-Partum. CL - Corpus Luteum. S - Stroma. F - Follicle.

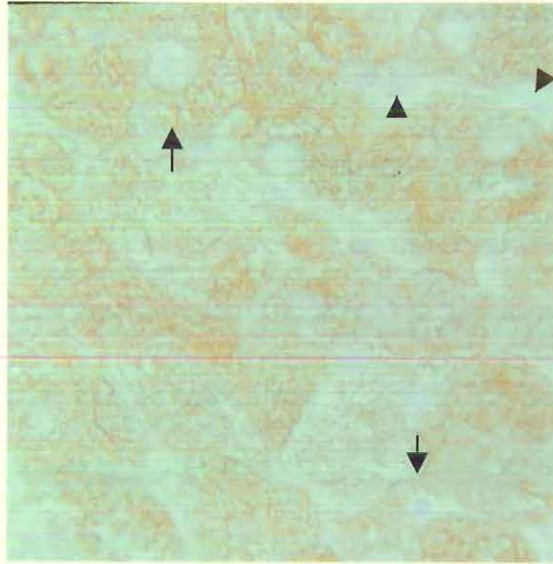


FIGURE 14. Within the corpus luteum, Fas ligand staining was observed within the cytoplasm of luteal cells. Endothelial cells displayed no positive staining for Fas ligand. Rat ovary section incubated with anti-Fas ligand polyclonal antibody and stained with DAB using the immunoperoxidase method. Counterstained with haematoxylin. 100x. Day 1 Post-Partum. Arrows - Luteal Cells. Arrowheads - Endothelial Cells.

5. RELATIVE QUANTITATIVE RT-PCR

5.1. Rationale

Reverse transcription-polymerase chain reaction (RT-PCR) was used to show the regulation of messenger RNA (mRNA) for Fas receptor and Fas ligand during natural regression of the rat corpus luteum.

The expression of genes in cells or tissue is analysed by the detection of mRNA, as only those genes expressed by the cell will be transcribed into RNA. Sensitivity is an important consideration when choosing a method for the detection of mRNA. Procedures commonly used include Northern gels or *in situ* hybridization. These protocols require species-specific probes which were not available at the time of completion of this project. Our laboratory has recently cloned Fas and Fas ligand for rat ovaries. Thus, future studies utilising Northern gels and *in situ* hybridization may be undertaken.

Use of the polymerase chain reaction to amplify complementary DNA (cDNA) produced from mRNA, a method first described by Seeburg *et al* in 1986 (cited in Kawasaki, 1990, pg. 21), provides a rapid method of detection of mRNA with sensitivity comparable to *in situ* hybridization. This method, known as RT-PCR, involves the extraction of RNA from cells or tissue, the generation of cDNA by reverse transcription and amplification of cDNA by specific primers

using PCR. The product can be viewed by fractionating the completed PCR reaction on an ethidium-bromide stained agarose gel. Alternatively, PCR products which were radioactively labelled during the PCR reaction can be fractionated on an agarose gel and quantitated using autoradiography or phosphorimaging.

The first step required for RT-PCR is RNA isolation, which involves separating RNA from other cellular contents, such as DNA and proteins. Tissue samples are homogenized in a commercial preparation containing guanidinium, such as RNAzol B. Chloroform is added to the homogenate and the suspension is centrifuged. RNA forms complexes with guanidinium and water molecules, whilst DNA and proteins are removed from the aqueous phase. The upper aqueous phase (containing RNA) is transferred to a new tube while the lower phases, containing DNA and proteins in chloroform, are discarded. RNA is precipitated by the addition of isopropanol and then centrifuged to form a pellet. The RNA pellet is washed with 75% ethanol, dessicated briefly, resuspended in water and stored at -80°C. The concentration and purity of isolated RNA can be determined by spectrophotometric analysis.

Isolated RNA is used to generate cDNA in a reaction catalyzed by reverse transcriptase. The reverse transcription reaction is initiated either with random hexamers, a specific downstream primer, or oligonucleotide primers

(oligo-dT). Primers bind to mRNA and initiate transcription of the message. Oligo-dT, for example, is a sequence of six thymidylates which bind to the poly adenylate tails of mRNA (see Figure 15). A small amount of RNA is placed in a PCR tube with water and primers and briefly heated at 70°C to unwind RNA. A mixture of the following is then added:

- Reverse transcriptase, the enzyme which transcribes RNA into cDNA;
- RT buffer, a buffer supplied with reverse transcriptase;
- Deoxynucleotides (dNTPs), a mixture of the four nucleotides required for transcription;
- Magnesium chloride (MgCl_2), which catalyzes the reverse transcriptase reaction; and
- Rnasin, which breaks down any RNases present and thus prevents RNA degradation.

The reactants are incubated at 42°C for 30-60 minutes to allow RNA to be transcribed into cDNA (see Figure 15). The reaction is then heated to between 70°C and 95°C for 5 to 15 minutes in order to denature the RNA-cDNA hybrid and to inactivate reverse transcriptase so that it will not interfere with future PCR reactions. The temperature of the tube is quickly dropped to 4°C and the reaction mix is stored at -20°C.

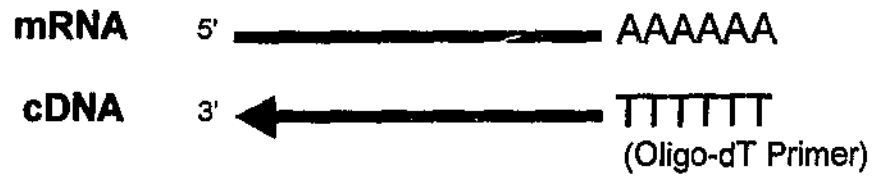


FIGURE 15: Reverse Transcription of RNA into cDNA.

The cDNA obtained from reverse transcription is amplified using PCR technology. The following reagents are required for the PCR reaction:

- cDNA, containing the target message to be amplified;
- DNA polymerase, a heat-stable enzyme which replicates DNA;
- Polymerase buffer, manufacturer's buffer supplied with DNA polymerase;
- $MgCl_2$ or Sodium Pyrophosphate, which catalyses the PCR reaction;
- dNTPs, a mixture of the four nucleotides required for amplification of DNA;
- Primers, specific primers synthesized from DNA sequences on either side of the required message are added to excess and serve to initiate the amplification process;
- Distilled water, as required, to increase reaction volume and dilute reagents.

The reactants are heated to around $94^{\circ}C$ for a short period of time (approximately 4-5 minutes) to ensure that the RNA-cDNA hybrid produced by reverse transcription is denatured and cooled to approximately $58^{\circ}C$ to allow the synthesized primers to anneal to cDNA. Heating the reaction to about $72^{\circ}C$ facilitates replication of cDNA by DNA polymerase. The cDNA-DNA strand is

then denatured by heating to approximately 94°C and the cycles of heating and cooling are repeated to exponentially amplify the DNA.

Each cycle of the PCR reaction theoretically doubles the amount of DNA produced. For example, after one cycle two DNA strands are present, after two cycles four DNA strands are present, after three cycles eight DNA strands are present, and so on. In practice, the actual amount of DNA amplified is less than predicted as the efficiency of PCR reactions vary depending on the conditions used. The number of heating and cooling cycles chosen may range from 15 to 40 cycles, after taking into consideration such factors as the amount of message initially used, the amount of message required for the PCR reaction and the overall efficiency of the reaction. The concentrations of reagents and the temperatures and incubation times for each of the steps may influence reaction efficiency, therefore the PCR reaction conditions should be optimised for each message to be amplified.

Depending on the initial amount of message, amplification usually runs at a constant efficiency of about 70-80% between cycles 15 and 30 of the reaction (Ferre, 1992). After a limited number of cycles the PCR reaction reaches a plateau phase and thereafter the amplification efficiency begins to decrease. Many factors, including a decrease in concentration of dNTPs or primers, reaction inhibition by end-products, competition for reagents by nonspecific products or primer-dimers, and incomplete denaturation of end-

product, contribute to the plateau phase (Innis and Gelfand, 1990, pg. 10). When quantitating without internal controls in PCR, it is important to stop the reaction in the exponential phase, before the efficiency of the reaction becomes variable and the chance of nonspecific products being preferentially amplified increases.

In order to quantitate the amount of DNA produced in each PCR reaction, a radioactively labelled nucleotide ($\alpha^{32}\text{P}$ -dCTP) can be added to the reaction. The nucleotide is incorporated into DNA as it is synthesized and the radioactivity of the amplified product is quantitated. Two methods of quantitation were used in the present study. In the first, unincorporated nucleotides were removed using isopropanol extraction and counts per minute of the sample obtained from a liquid scintillation counter. In the second, each sample was fractionated on an agarose gel (unincorporated nucleotides, weighing less than DNA, travel further along the gel) which was dried and exposed to a phosphorimaging plate. The resultant image produced by the radioactivity of the samples was quantitated using a computer image analysis program.

The first step in making relative comparisons between the amount of message expressed in the rat at various stages of pregnancy and post-partum involved identifying a dilution factor for each time-point at which the PCR reaction was amplifying exponentially. Following reverse transcription, four-fold

serial dilutions of one sample from each time-point were amplified by PCR for a set number of cycles. The samples were then quantitated and the results plotted on a logarithmic graph (see Figure 16).

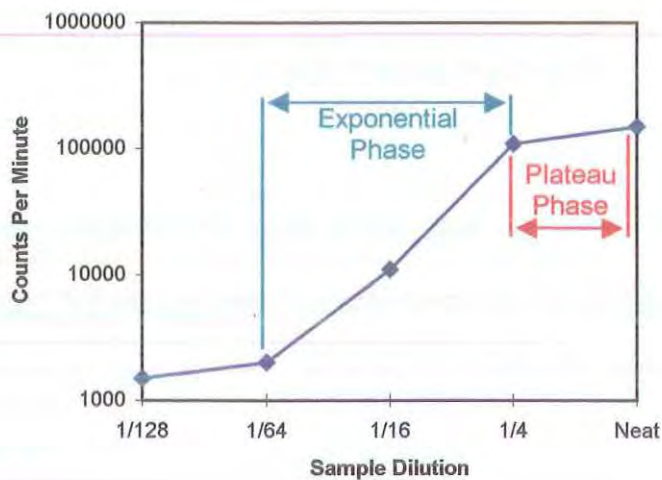


FIGURE 16: Decreasing Dilutions of cDNA Plotted on a Logarithmic Scale Following Amplification by PCR.

A dilution factor for each time-point was chosen where the PCR reaction would be amplifying exponentially when stopped. All samples were then amplified at the specified dilution in one PCR run. The amounts of message expressed at the time-points were compared relative to each other by allowing for the dilution factor used for the cDNA.

Fas mRNA expression has been demonstrated in human granulosa / luteal cells using RT-PCR techniques (Quirk *et al*, 1995), but the expression of Fas ligand mRNA in these cells has yet to be confirmed. The relative amounts

of message expressed at various stages of pregnancy and post-partum in the rat ovary has not previously been investigated. This information may thus assist in providing an insight into the function of the Fas/Fas ligand system in the rat ovary.

5.2. Sample and Tissue Preparation

The animals used for this study were:- four rats from days 1, 8 and 16 of pregnancy and day 3 post-partum, five rats from day 22 of pregnancy and eight rats from day 1 post-partum. The right ovary from each rat was dissected. Immediately following excision each ovary was snap frozen in liquid nitrogen and stored at -80°C until use.

5.3. Method

RNA Isolation. Whole frozen ovaries were homogenised in 1300 μ L RNazol B. 130 μ L of chloroform was added and mixed and the samples were incubated on ice for 5 minutes. The mixtures were centrifuged at 14,000rpm for 15 minutes. Equal volumes of isopropanol were added and mixed, and tubes were incubated at 4°C for one hour. The mixtures were centrifuged at 14,000rpm for 15 minutes and the supernatants were decanted. After adding 1mL 75% cold ethanol, the tubes were vortexed and centrifuged at 6,000rpm for 8 minutes. The supernatants were decanted and dessicated in a biohazard

cabinet for 15 minutes. The resulting pellets were resuspended in 10 μ L of DMDC-treated water. The quantity and purity of the isolated RNA was determined using a Beckman DU-640 Spectrophotometer.

Relative Quantitative RT-PCR for Fas Receptor. Reverse transcription was performed by adding 2 μ L oligo-dT primer (500ng/ μ L), 2 μ g sample RNA and DMDC-treated water to a total volume of 10 μ L. Negative controls contained DMDC-treated water in place of RNA. The reaction mixes were heated to 70°C for 5 minutes and then placed on ice. A master mix of reagents containing 5 μ L reverse transcriptase buffer (5x), 5 μ L dNTPs (5mM), RNasin (40U/ μ L), sodium pyrophosphate (40mM) and AMV reverse transcriptase (23U/ μ L) was added to each tube. The reaction mixes were placed in a thermal cycler and incubated at 42°C for 60 minutes, heated to 95°C for 5 minutes and then cooled to 4°C. The cDNA was stored at -20°C.

PCR for Fas receptor was performed by adding a master mix of reagents to 5 μ L cDNA. Negative controls contained DMDC-treated water in place of cDNA. The master mix contained 11.35 μ L DMDC-treated water, 2.5 μ L Taq. polymerase buffer (10x), 4.0 μ L MgCl₂ (25mM), 0.1 μ L dATP (100mM), 0.1 μ L dTTP (100mM), 0.1 μ L dGTP (100mM), 0.025 μ L dCTP (100mM), 0.075 μ L α^{32} P-dCTP (300mM), 1.5 μ L Fas primers (2pmol/ μ L, Upstream Sequence: 5' CTG CAG ATA TGC TGT GGA TCA 3', Downstream Sequence: 5' TTT GGT GTT GCT GGT TGG T 3') and 0.25 μ L Taq polymerase per reaction. Reaction mixes

were placed in a thermal cycler and heated to 94°C for 4 minutes 30 seconds. The tubes were then incubated at 94°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 minute and this cycle was repeated 35 times. The amplified samples were incubated at 72°C for 5 minutes, cooled to 4°C and stored at -20°C.

PCR products were fractionated on an ethidium bromide stained agarose gel, dried on a slab gel dryer and either exposed to x-ray film or a phosphorimaging plate overnight. Quantitation was performed using an image analysis program. Initially, four-fold serial dilutions (i.e. neat, $1/4$, $1/16$, $1/64$) of one sample from each time-point were amplified by PCR, quantitated and the results plotted on a logarithmic graph. A dilution in the exponential phase of the PCR reaction was chosen for each time-point. One PCR run with all cDNA samples at the specified dilution was completed and quantitated. Data from different time-points were compared after allowing for the dilution factor used in the PCR reaction.

Relative Quantitative RT-PCR for Fas Ligand. Reverse transcription was performed by adding 2.5 μ L of 3'-end primers for Fas ligand (5pmol/ μ L, Sequence: 5' AGT CTC TAG CTT ATC CAT GA 3'), 1 μ g sample RNA and DMDC-treated water to make up the volume to 9 μ L. Negative controls contained DMDC-treated water in place of RNA. Solutions were heated to 70°C for 5 minutes. A master mix of reagents containing 5 μ L reverse

transcriptase buffer (5x), 5 μ L dNTPs (5mM), 1 μ L RNasin (40U/ μ L), 2.5 μ L sodium pyrophosphate (40mM) and 2.5 μ L AMV reverse transcriptase (23U/ μ L) was added to each tube. Reaction mixes were placed in a thermal cycler and incubated at 42°C for 60 minutes, heated to 95°C for 2 minutes, then cooled to 4°C. The cDNA was stored at -20°C.

PCR for Fas ligand was performed by adding a master mix of reagents to 2 μ L cDNA. Negative controls contained DMDC-treated water in place of cDNA. The master mix contained 14.35 μ L DMDC-treated water, 2.5 μ L PLATINUM™ Taq polymerase buffer (10x), 2.5 μ L MgCl₂ (25mM), 0.1 μ L dATP (100mM), 0.1 μ L dTTP (100mM), 0.1 μ L dGTP (100mM), 0.025 μ L dCTP (100mM), 0.075 μ L α^{32} P-dCTP (300mM), 3.0 μ L Fas ligand primers (2pmol/ μ L, Upstream Sequence: 5' AAA GAC CAC AAG GTC CAA CA 3', Downstream Sequence: 5' AGT CTC TAG CTT ATC CAT GA 3') and 0.25 μ L PLATINUM™ Taq polymerase (5.5U/ μ L) per reaction. The reaction mixes were placed in a thermal cycler and heated to 94°C for 4 minutes 30 seconds. The tubes were then incubated at 94°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute, and this cycle was repeated 32 times. The amplified samples were incubated at 72°C for 5 minutes, cooled to 4°C and stored at -20°C.

Isopropanol extraction was used to remove unincorporated nucleotides from PCR products. One tenth volume of sodium acetate, pH 5.2, an equal volume of isopropanol and 1 μ L glycogen were added to each tube. The

solutions were mixed, incubated at 4°C for 15 minutes and centrifuged at 14,000rpm for 15 minutes. The supernatant was removed and the pellet washed with 500 μ L 70% cold ethanol and then vortexed. The samples were centrifuged at 14,000rpm for 5 minutes, the supernatant removed and the samples dessicated for 10-15 minutes in a biohazard cabinet. The pellets were resuspended in 15 μ L ddH₂O and quantitated with a liquid scintillation counter. Initially, four-fold serial dilutions (i.e. neat, $1/4$, $1/16$, $1/64$, $1/128$) of one sample from each time-point were amplified by PCR, quantitated and the results plotted on a logarithmic graph. A dilution in the exponential phase of the PCR reaction was chosen for each time-point. One PCR run with all cDNA samples at the specified dilution was completed and quantitated. Data from different time-points were compared after allowing for the dilution factor used in the PCR reaction.

See Figure 17 for experimental design. Detailed protocols are contained in Appendix B.

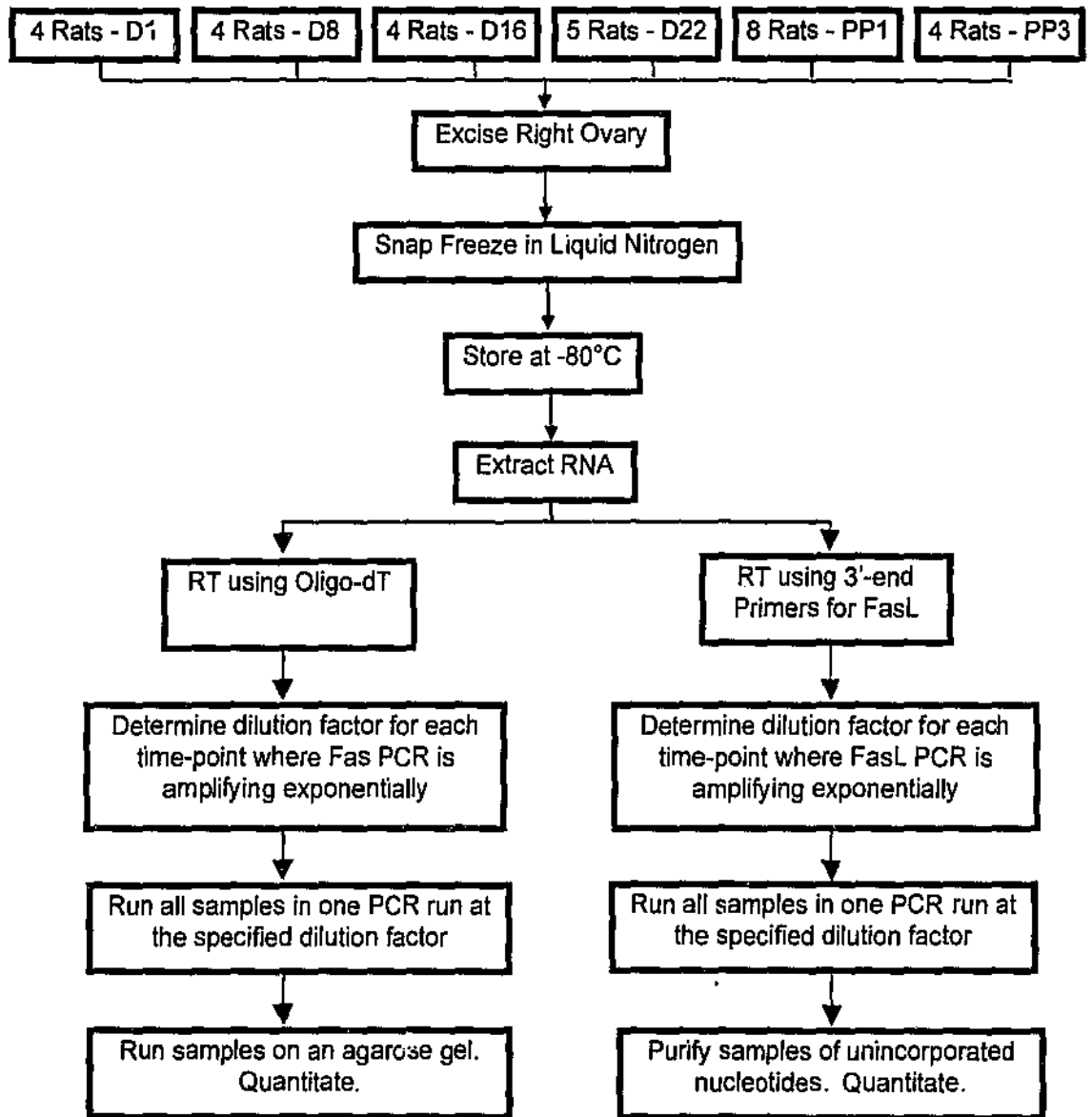


FIGURE 17: Experimental Design for Relative Quantitative RT-PCR.

5.4. Results

Expression of Fas receptor and Fas ligand mRNA in the rat ovary during pregnancy and post-partum was analysed using a relative quantitative method of RT-PCR.

5.4.1. Confirmation of mRNA Expression

Expression of mRNA for Fas receptor and Fas ligand at the various stages of pregnancy and post-partum examined was confirmed by amplifying cDNA of one sample from each time-point by PCR. The PCR product was run on an ethidium-bromide stained agarose gel and viewed under UV light. Both Fas receptor and Fas ligand mRNA were expressed at all stages examined (see Figures 18 and 19 respectively).

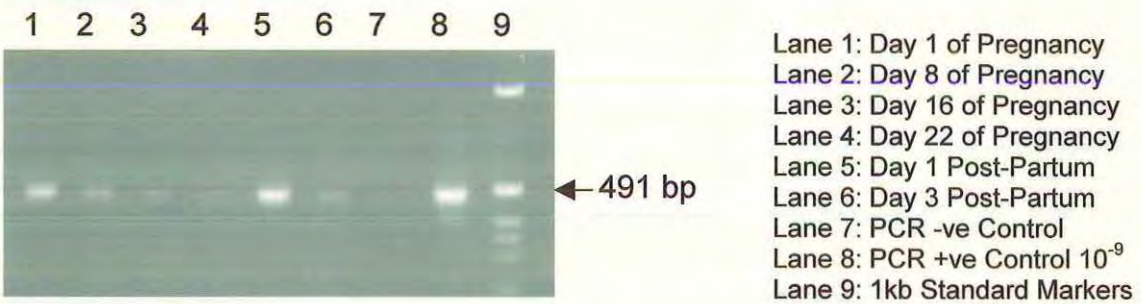


FIGURE 18: Ethidium Bromide Stained Agarose Gel from Fas Receptor RT-PCR. Fas mRNA is expressed at each of the stages of pregnancy and post-partum examined. NOTE: 1.5 μ L of dNTPs (5mM) per reaction were used; α^{32} P-dCTP was not included; Samples were amplified over 38 cycles.

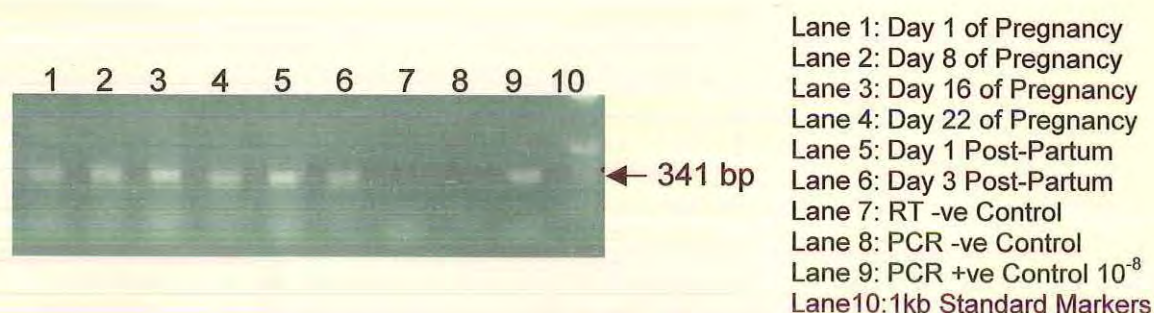


FIGURE 19: Ethidium Bromide Stained Agarose Gel from Fas Ligand RT-PCR. Fas ligand mRNA was expressed at each of the stages of pregnancy and post-partum examined. NOTE: $1.25\mu\text{L}$ of dNTPs (5mM) per reaction were used; $\alpha^{32}\text{P}$ -dCTP was not included; $1\mu\text{L}$ of cDNA per sample was amplified; Samples were amplified over 35 cycles.

5.4.2. Optimal Activity of Radionucleotide in PCR

Samples of cDNA from day 8 of pregnancy and day 1 post-partum were amplified by Fas ligand PCR with varying activities of $\alpha^{32}\text{P}$ -dCTP to determine the optimal activity of radionucleotide to be used in future PCR reactions. The activities tested were $0\mu\text{Ci}$, $0.1\mu\text{Ci}$, $0.25\mu\text{Ci}$, $0.5\mu\text{Ci}$ and $1\mu\text{Ci}$ per reaction. Unincorporated nucleotides were removed from the PCR products using isopropanol extraction and counts per minute obtained with a liquid scintillation counter. The results were plotted on a logarithmic graph (see Figure 20). An activity of $0.75\mu\text{Ci}$ ($0.075\mu\text{L}$) was used in future PCR reactions.

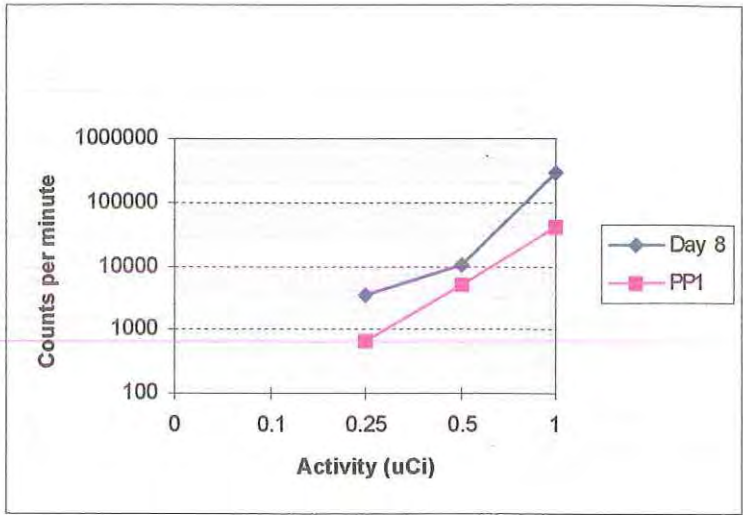


FIGURE 20: PCR using Fas Ligand Primers with Varying Activities of $\alpha^{32}\text{P}$ -dCTP per Reaction. Results at 0 μCi and 0.1 μCi were less than that of negative control and thus are not shown.

5.4.3. Number of Cycles in Fas PCR

To ensure that samples would not be overamplified, four-fold serial dilutions of one sample from day 16 of pregnancy and one sample from day 1 post-partum were amplified by Fas PCR either for 35 or 38 cycles. The PCR products were run on an agarose gel to remove unincorporated nucleotides, exposed to a phosphorimaging plate overnight, and quantitated using the NIH image analysis program. The results were plotted on logarithmic graphs (see Figure 21). Amplification by 35 cycles was used for future Fas PCR reactions as the results did not show a plateau phase. At 38 cycles the reaction efficiency appeared to be slower and a plateau phase was evident, indicating overamplification.

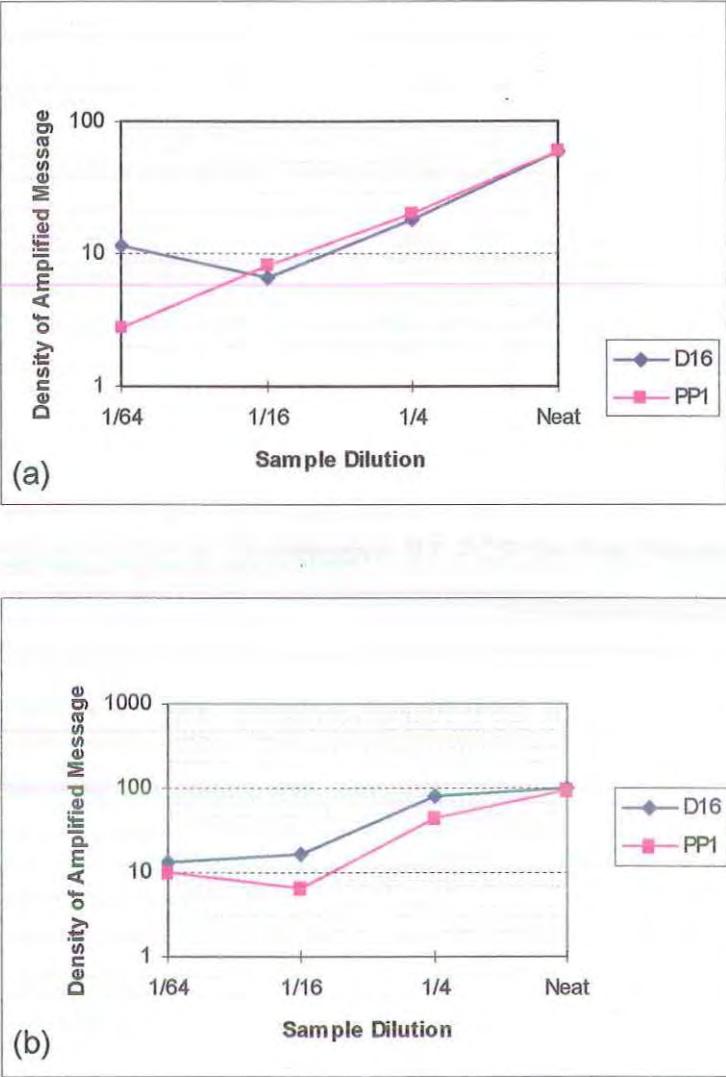


FIGURE 21: Fas PCR Using Varying Sample Dilutions Amplified by either: (a) 35 cycles, or (b) 38 cycles.

5.4.4. Dilution Factors for Fas Receptor

A dilution factor for each time-point was chosen where the PCR reaction would be amplifying exponentially when stopped. This was done by making four-fold serial dilutions of one sample from each time-point and amplifying by

PCR using Fas primers. PCR products were run on an agarose gel to remove unincorporated nucleotides, exposed to a phosphorimaging plate overnight, and quantitated using the NIH image analysis program. The results were plotted on a logarithmic graph (refer to Figure 21 (a) for an example). All samples were amplifying exponentially after 35 cycles at a dilution of 1 in 4, except for day 22 of pregnancy, which was amplifying exponentially at a dilution of 1 in 16.

5.4.5. Relative Quantitative RT-PCR for Fas Receptor

RNA samples were reverse transcribed in one run using oligo-dT primers. cDNA was diluted by the specified factor, and amplified by Fas PCR. Unincorporated nucleotides were removed by running PCR products on an agarose gel. The gel was dried and exposed to a phosphorimaging screen overnight. Samples were to be quantitated using the NIH image analysis program, however the quality of the phosphorimage produced was not adequate for quantitation. The experiment could not be repeated due to time constraints.

5.4.6. Dilution Factors for Fas Ligand

A dilution factor for each time-point was chosen where the PCR reaction would be amplifying exponentially when stopped. This was done by making

four-fold serial dilutions of one sample from each time-point and amplifying by PCR using Fas ligand primers. Isopropanol extraction was used to remove unincorporated nucleotides from PCR products and counts per minute were obtained with a liquid scintillation counter. The results were plotted on a logarithmic graph (for example see Figure 22). Samples at day 1, 8 and 16 of pregnancy were amplifying exponentially when not diluted (i.e. neat), whilst samples at day 22 of pregnancy and day 1 and 3 post-partum were amplifying exponentially at a dilution of 1 in 4.

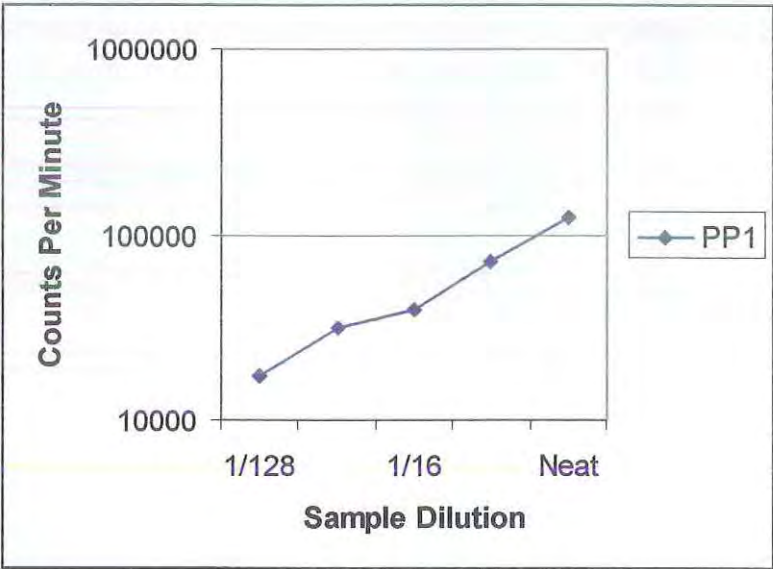


FIGURE 22: Fas Ligand PCR at Day 1 Post-Partum Using Varying Sample Dilutions to Determine Exponential Phase of Reaction.

5.4.7. Relative Quantitative RT-PCR for Fas Ligand

All RNA samples were reverse transcribed using 3'-end Fas ligand primers. cDNA was diluted if required, then amplified by Fas ligand PCR.

Unincorporated nucleotides were removed by isopropanol extraction and PCR products were quantitated by obtaining counts per minute from a liquid scintillation counter. The results were averaged for each of the time-points examined, and plotted as the fold change versus the average of day 1 of pregnancy (see Figure 23).

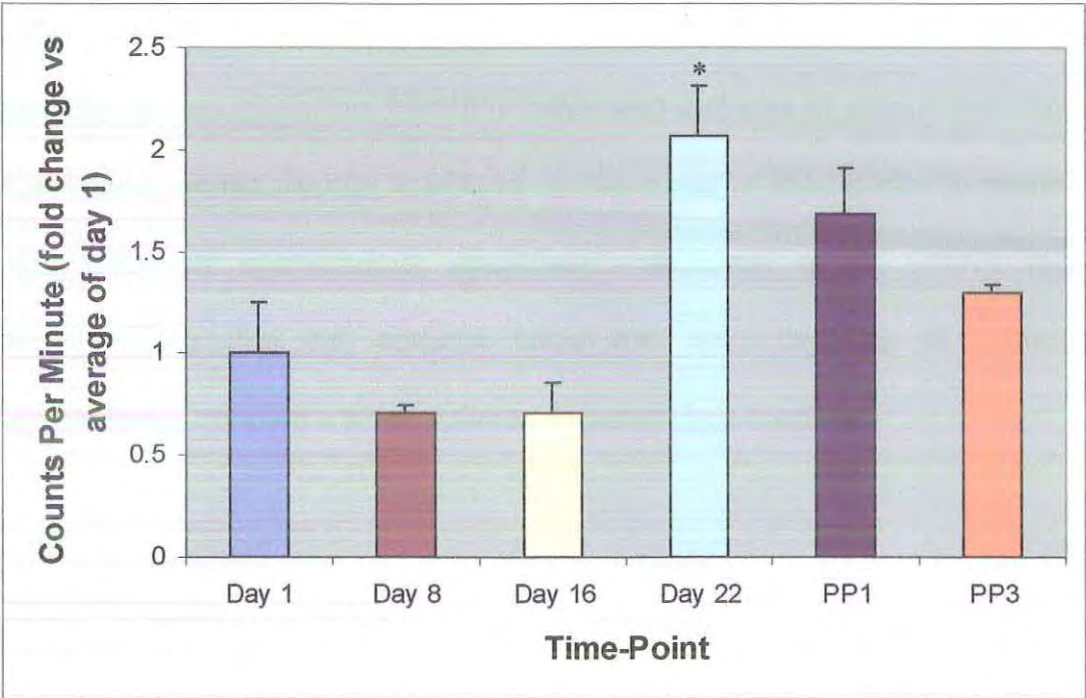


FIGURE 23: Expression of Fas Ligand mRNA at Various Stages of Pregnancy and Post-Partum in the Rat Ovary. * Significant increase compared to D16 values ($P<0.005$, 2-way t test) and to PP3 values ($P<0.05$, 2-way t test).

6. *IN VITRO* CORPUS LUTEUM CULTURE MODEL

6.1. Rationale

The discovery that spontaneous apoptosis occurs in rabbit corpora lutea cultured in serum-free medium led to the development of an *in vitro* corpus luteum culture model (Dharmarajan *et al*, 1994). Corpora lutea functioning at peak levels are dissected from the ovary and cultured in serum-free medium. Treatments added can be assessed on the basis of their ability to reduce the occurrence of spontaneous apoptosis. Sridaran *et al* (1997) recently demonstrated that rat corpora lutea are also capable of undergoing spontaneous apoptosis when cultured in serum-free medium.

Quantitation of cell death by apoptosis was determined by 3'-end labelling of fragmented DNA. The DNA is extracted from samples with chloroform and isoamylalcohol. The 3'-ends of fragmented DNA are then labelled with radioactive $\alpha^{32}\text{P}$ -ddATP in a reaction catalysed by terminal transferase. The reaction is terminated with the addition of ethylenediaminetetraacetic acid (EDTA), and labelled DNA is extracted from unincorporated nucleotides by precipitation with ammonium acetate and ethanol. The labelled DNA is fractionated on an agarose gel which is then exposed overnight to X-ray film to produce an autoradiograph. DNA fragmented at multiples of 180 base pair fragments, as occurs in apoptosis, will

show ladders in multiples of 180 base pairs on the gel, while DNA fragmented randomly, as occurs in necrosis, will show a smear on the gel (Tilly and Hsueh, 1993). Low molecular weight DNA bands from each lane in the agarose gel are excised and counts per minute measured in a liquid scintillation counter.

While some anti-Fas monoclonal antibodies are able to induce apoptosis when bound to Fas receptor (Yonehara *et al*, 1989; Trauth *et al*, 1989), others are capable of blocking the receptor site and preventing transduction of an apoptotic signal (Oshimi *et al*, 1996). In this experiment, rat corpora lutea were cultured *in vitro* in the presence of an anti-rat Fas monoclonal antibody, to determine if spontaneous apoptosis could be blocked via the Fas/Fas ligand system in luteal cells.

Apoptosis can be induced in cultured human granulosa / luteal cells pretreated with interferon gamma when exposed to an anti-human Fas monoclonal antibody (Quirk *et al*, 1995), but studies aiming to block the function of the Fas receptor in luteal cells have not previously been documented.

6.2. Sample and Tissue Preparation

Four rats from day 16 of pregnancy were required. The ovaries were excised and corpora lutea dissected from ovarian stroma. Two corpora lutea were placed in each sterile culture vial. Five vials contained 2mL of serum-free

medium only, five vials also contained 40 μ L antibody storage buffer (50% glycerol, 20mM NaH₂PO₄ pH 7.5, 1.5mM NaN₃, 1mg/mL BSA) and five vials also contained 40 μ L anti-rat Fas monoclonal antibody (250 μ g/mL).

6.3. Method

Sterile culture vials were gassed with a 95% O₂ - 5% CO₂ mixture, their lids sealed with vacuum grease and the vials incubated at 37°C in 95% O₂ - 5% CO₂ for 4 hours. Corpora lutea were placed in sterile polypropylene tubes, snap frozen in liquid nitrogen and stored at -80°C.

To extract DNA, each sample (consisting of two corpora lutea) was first homogenised in 670 μ L of DNA homogenisation buffer and 42 μ L 10% SDS was added and mixed. The tubes were incubated at 65°C for 30 minutes, then 119 μ L 8M potassium acetate was added and mixed. The tubes were placed on ice for 60 minutes and then microcentrifuged at 5,000rpm for 10 minutes. The supernatant was transferred to a new tube, equal volumes of phenol: chloroform:isoamyl alcohol (25:24:1) were added and the tubes vortexed. The samples were then microcentrifuged at 6,000rpm for 5 minutes, the upper phase was transferred to a new tube, equal volumes of phenol: chloroform:isoamyl alcohol (25:24:1) were added and solutions vortexed. The tubes were again microcentrifuged at 6,000rpm for 5 minutes, the upper phase was transferred to a new tube, 2.5x volume of cold 100% ethanol was added

and the reaction mix stored overnight at -80°C . The samples were microcentrifuged at 14,000rpm for 30 minutes and the supernatants decanted. The pellet was resuspended in 100 μL TE, pH 8.0, 2 μL RNase was added and the solutions were vortexed, briefly microcentrifuged and then incubated at 37°C for 60 minutes. The extraction with phenol:chloroform:isoamyl alcohol (25:24:1) was repeated twice and the upper phases transferred to new tubes. 0.1x volume 3M sodium acetate and 2.5x volume cold 100% ethanol were added and the samples stored overnight at -80°C . Tubes were microcentrifuged at 14,000rpm for 30 minutes and the supernatants decanted. 100 μL cold 100% ethanol was added, tubes were placed on ice for 5 minutes and then microcentrifuged at 14,000rpm for 5 minutes. The ethanol layer was decanted and the pellets air dried over a paper towel for 1-2 hours before resuspension in 50 μL ddH₂O. The quantity and purity of the isolated DNA was established using a Beckman DU-640 Spectrophotometer, and the samples stored at -80°C .

3'-end labelling was conducted on 1 μg of DNA from each sample. A sample of DNA from rat corpora lutea, immediately snap frozen after dissection from the ovary (time zero), was included as a baseline measurement against which all samples would be compared. The volumes in each tube were made up to 29 μL with ddH₂O. 10 μL of terminal transferase reaction buffer (5x) and 5 μL CoCl₂ were added, the tubes were vortexed and then microcentrifuged briefly. $\alpha^{32}\text{P}$ -ddATP (3,000Ci/mmol) was diluted 1 in 8 and 5 μL was added to

each tube. Terminal transferase was diluted 1 in 2 and 1 μ L was added to each tube. The samples were vortexed, microcentrifuged briefly and incubated at 37°C for 60 minutes. 5 μ L 0.25M EDTA, 2 μ L tRNA, 12 μ L 10M ammonium acetate and 180 μ L cold 100% ethanol were added and the tubes maintained at -80°C for 60 minutes. The samples were microcentrifuged at 14,000rpm for 20 minutes and supernatant was discarded. The pellet was resuspended in 60 μ L of 1 x TE, 12 μ L 10M ammonium acetate and 180 μ L of cold 100% ethanol were added, the tubes vortexed and then placed at -80°C for 60 minutes. The samples were microcentrifuged at 14,000rpm for 20 minutes, the supernatant was discarded and the pellet was air dried over a paper towel for 20 minutes. The pellet was resuspended in 40 μ L 1x TE and stored at -20°C overnight.

The samples were thawed for 20 minutes and then electrophoresed for 2 hours 30 minutes on an agarose gel. The gel was dried on a slab gel dryer and exposed to X-ray film overnight. The film was developed and viewed for evidence of the laddering of low molecular weight DNA. Using the autoradiograph as a template, low molecular weight DNA from each lane was excised and quantitated using a liquid scintillation counter.

See Figure 24 for experimental design. Detailed protocols are contained in Appendix B.

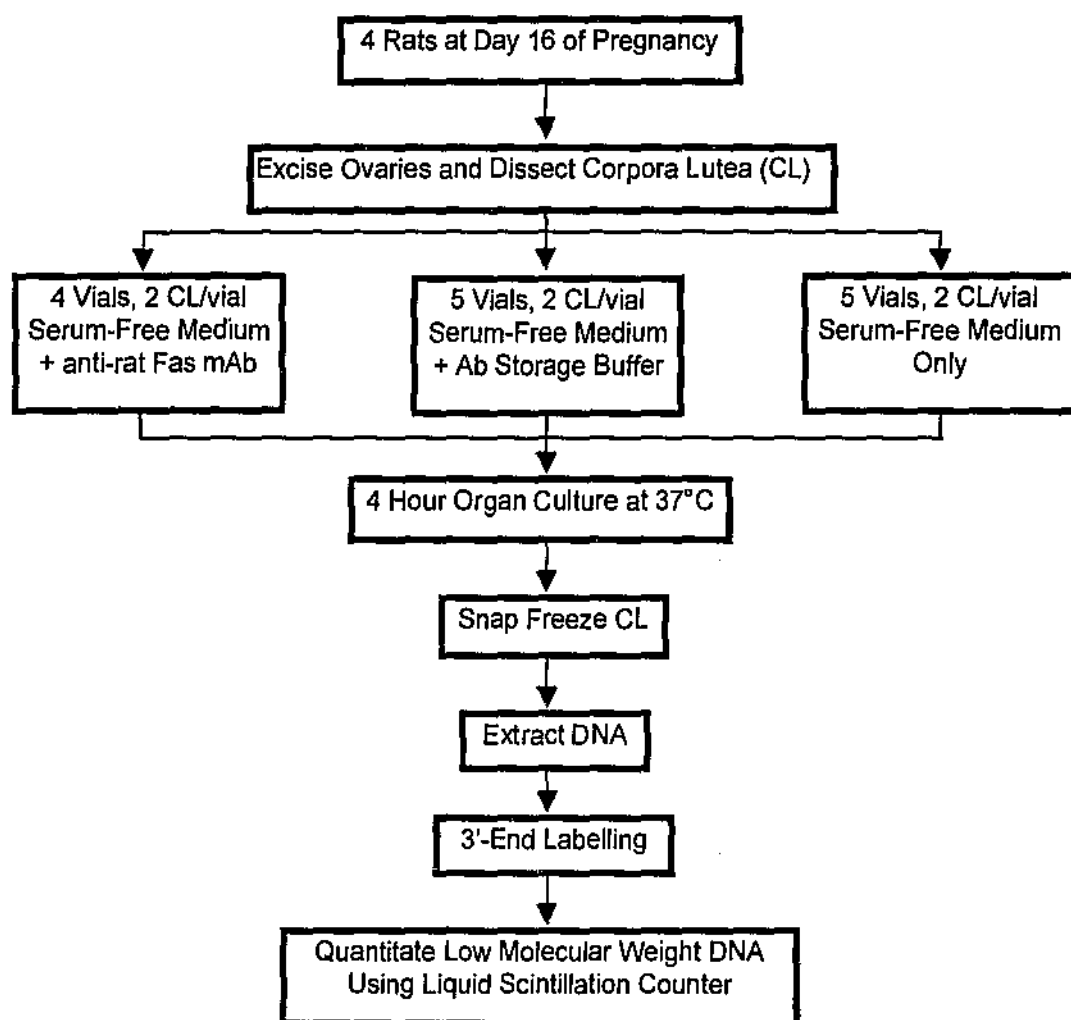


FIGURE 24. Experimental Design for *In Vitro* Corpus Luteum Culture Model.

6.4. Results

6.4.1. 3'-End Labelling

3'-Ends of extracted DNA were radioactively labelled and DNA was fractionated on an agarose gel. Laddering of low molecular weight DNA due to cleavage into 180 base pair fragments, characteristic of apoptosis, was identified (see Figure 25). Low molecular weight DNA was excised from each lane of gel and quantitated with a liquid scintillation counter (see Figure 26). Treatment with the antibody storage buffer demonstrated a trend in reduction in occurrence of spontaneous apoptosis in cultured corpora lutea. A further reduction in apoptosis was apparent when corpora lutea were treated with an anti-rat Fas monoclonal antibody in culture. The results, however, were variable and no significance was found.

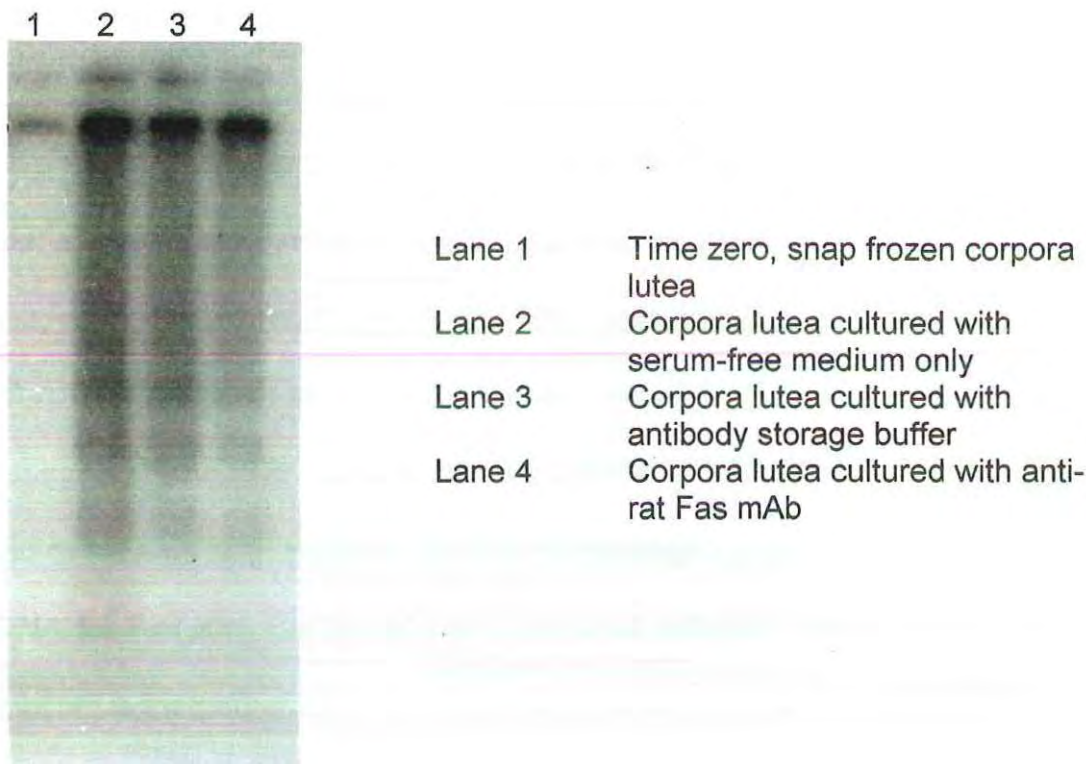


FIGURE 25: Autoradiograph of 3'-End Labelled DNA Extracted from Corpora Lutea Cultured *In Vitro* with Varying Treatments

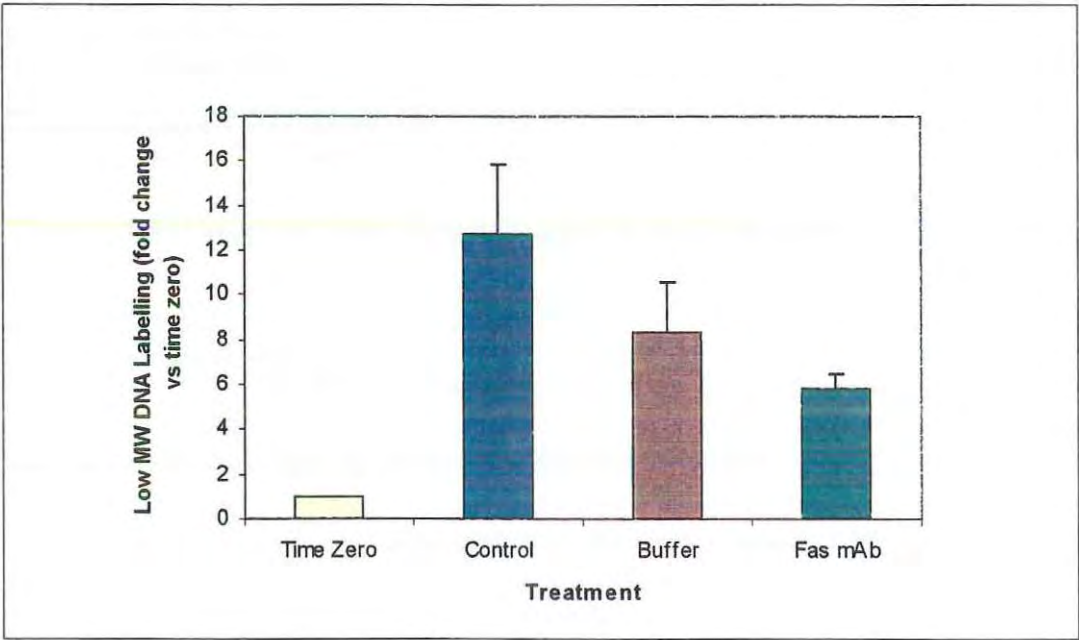


FIGURE 26: Low Molecular Weight DNA Labelling of Corpora Lutea Cultured with Anti-Fas Monoclonal Antibody.

7. DISCUSSION

Previous research has indicated that the Fas/Fas ligand system may have a role in regression of the corpus luteum (Watanabe-Fukunaga *et al*, 1992; Quirk *et al*, 1995; Kondo *et al*, 1996). This study examined the regulation of Fas receptor and Fas ligand in the rat corpus luteum during pregnancy and post-partum to further confirm this hypothesis. Immunohistochemistry was used to localise both proteins at each of the stages examined. Regulation of mRNA for Fas and Fas ligand was compared between stages with a relative quantitative method of RT-PCR. The ability of an anti-Fas monoclonal antibody to block the occurrence of spontaneous apoptosis in corpora lutea cultured *in vitro* with serum-free medium was also assessed.

Whilst immunohistochemistry can be used as a quantitative technique, the evaluation of staining intensity can be subjective, particularly because staining intensity may vary between cells in any one section. Also, the results from different runs should not be directly compared as many factors, such as incubation time and ambient temperature, may affect the intensity of the stain. Immunohistochemistry is most effective when used to confirm the presence or absence of a protein and differences in staining intensity between samples in a single run. The variations which may be seen from run to run were countered by including positive controls of a tissue known to express the protein in question, in this case spleen. These sections were taken from a single tissue

block. The intensity and pattern of staining in positive control slides was compared to evaluate if run to run differences were of concern.

In immunohistochemistry, it is not sufficient that the antigen under investigation is present in the tissue being tested - the epitope to which the antibody binds must be exposed for successful staining. Although the aim of fixation is to maintain the original structure of the tissue, changes do occur during the fixation process, sometimes resulting in epitopes being concealed. Exposure of epitopes may be facilitated by treating the sections with agents such as detergents or the application of heat before the primary antibody is applied. For example, sections were treated with 1% SDS to expose the Fas antigen before treatment with the primary antibody.

Background staining in sections is a common problem encountered when using immunohistochemical techniques. There are many possible causes for background staining, including endogenous enzyme activity, endogenous biotin activity, and hydrophobic or ionic binding of antibodies. A negative control of the same tissue treated in an identical manner except for application of the primary antibody (diluent only is applied) should be run for each section tested. Staining on the negative control section should be compared to that on the tissue section tested, to determine the degree of background staining. Appropriate steps can be taken to reduce the amount of background staining. For example, endogenous peroxidase activity can be

quenched by incubating the sections in 3% hydrogen peroxide diluted in methanol before application of the primary antibody. This step was used when staining for both Fas receptor and Fas ligand. Background staining did not present a problem in either case.

When the Fas receptor is expressed on the cell surface it can be bound and will then transduce an apoptotic signal to the cell. Thus, it is expected that if the Fas/Fas ligand system is involved in cell death occurring during corpus luteum regression, the presence of both proteins would correlate with luteolysis. Fas receptor was localised in the rat corpus luteum at day 1 of pregnancy and days 1 and 3 post-partum. Fas ligand was present in the rat corpus luteum throughout pregnancy and post-partum, with immunohistochemical staining intensity increasing around the time of parturition. Thus, localisation of both proteins within the rat corpus luteum during luteolysis was demonstrated.

The staining of Fas receptor observed at day 1 of pregnancy may relate to natural regression of corpora lutea during the normal estrus cycle of the rat. After formation and growth, rat corpora lutea maintain their maximum size through metestrus of the following cycle and then regress slowly, so that three or more generations of corpora lutea may be seen in the ovary (Hilliard, 1973). Throughout pregnancy rat corpora lutea of previous cycles grow and display characteristics of steroidogenic tissue (Bruce *et al*, 1984). This indicates that even following the onset of luteolysis, regression can be halted and corpora

lutea may be functional. Fas receptor is not present in the corpora lutea of previous estrus cycles at times of peak function in the corpus luteum of pregnancy. This suggests that the factor which maintains the newly formed corpus luteum in the rat may also positively affect the functioning of aged corpora lutea. Also noteworthy is the finding that Fas ligand was present in all but one corpus luteum in each rat ovary at day 1 of pregnancy. Further research will be required to ascertain if those corpora lutea which did not stain for the presence of Fas ligand were newly formed.

Whilst progesterone production in the rat declines by day 22 of pregnancy (Waddell *et al*, 1989), the weight of the corpora lutea of pregnancy does not fall until after parturition (Bruce *et al*, 1984; Taya and Greenwald, 1982). Fas receptor was present in corpora lutea at day 22 of pregnancy, with staining intensity increasing by day 1 post-partum. Thus, it is not clear if the Fas/Fas ligand system is involved in functional regression of the corpus luteum, but a role in structural regression is implicated. The weight of the corpus luteum of pregnancy continues to decline over numerous estrus cycles following parturition (Taya and Greenwald, 1982). Staining intensity for both Fas receptor and Fas ligand decreased by day 3 post-partum, although both proteins were still present. Further research will be required to determine whether Fas receptor and Fas ligand are present in the rat corpus luteum throughout lactation.

Previous research found no evidence of vascular degeneration during natural regression of the pregnant rat corpus luteum (Bruce *et al*, 1984). In agreement with this report, both Fas and Fas ligand were localised in the cytoplasm of luteal cells at the time of luteolysis, but staining was not observed in endothelial cells at any stage of pregnancy or post-partum.

The presence of Fas ligand in rat corpora lutea throughout pregnancy and post-partum indicates that luteal regression may not be its only function. Fas ligand contributes to the immune privilege displayed by the Sertoli cells of the mouse testis (Bellgrau *et al*, 1995) and by the mouse eye (Griffith *et al*, 1995). The ovary has also been identified as an immune privileged site (Streilein, 1995) and it is possible that Fas ligand has a role to play. Furthermore, maintenance of corpora lutea may involve mechanisms of immune privilege. Luteal tissue is not present at the time that the immune system is adapting to "self" (Bukovsky *et al*, 1995) and it may be susceptible to immune attack. Research using gld mice (which express non-functional Fas ligand) is required to establish a role for Fas ligand-mediated immune privilege in the ovary.

The expression of mRNA for both Fas and Fas ligand in the rat ovary was compared at different stages of pregnancy and post-partum using a relative quantitative method of RT-PCR. RT-PCR is a rapid technique and it is also extremely sensitive. Thus mistakes at any of the steps in the protocol can

produce significant errors in the final results. Further, any error will be amplified during the PCR step. For these reasons samples must be treated under the same conditions at all times and, if possible, be processed in a single run in order to reduce between-batch variability. A master mix of reagents is dispensed into each tube for reverse transcription and PCR reactions, so the concentration of reactants in each of the tubes is the same.

One of the advantages of relative quantitative RT-PCR is that the synthesis of internal controls (a time-consuming exercise) is not necessary. However, as reverse transcription and coamplification of samples with exogenous internal controls is not undertaken with this method the possibility of varying reaction efficiencies due to well-to-well variations in the thermal cycler is not controlled. To ensure that allowance was made for variability, at least four samples from each time-point were used and the results were averaged. Standard errors obtained for Fas ligand indicated that the results were reproducible.

Another influence on reaction efficiency is the amount of message in the PCR tube. By making serial dilutions of one sample from each time-point to identify the exponential phase of the reaction, and then using that dilution factor for the final PCR, the amount of message in each tube is similar. Multiplying the result by the dilution factor used permits relative comparisons between

samples. The sample size (at least four for each time-point) assists in accounting for variations in reaction efficiency as results are averaged.

Formation of primer-dimers in PCR reactions can create difficulties when quantitating results. High counts in negative controls were initially obtained from Fas ligand PCRs, indicating that primer-dimers were being formed. The occurrence of primer-dimers can be reduced by developing primers with less complementary sequences, using a hot start PCR method or using a Taq polymerase which is not activated until heated. The development of new primers was beyond the scope and time limitations of this project. Hot start PCRs, which involve adding an essential reagent to each sample tube only after the reactants have been heated to 94°C (Ferre, 1992), was not a preferred solution in this case, given that exposure to radioactivity would be increased and the risk of contaminating samples is high. Thus, a Taq Polymerase which is not activated until heated was used to reduce primer-dimers. PLATINUM™ Taq DNA Polymerase, supplied by Gibco BRL, is bound to an antibody which inhibits polymerase activity. The enzyme regains activity during the denaturation step at 94°C, providing an automatic hot start without the risk of sample contamination and ensuring that all samples receive the same amount of reactants. Use of this enzyme in Fas ligand PCR reduced the counts obtained in negative controls, indicating a reduction in primer-dimer formation.

Although dilution factors for each of the time-points of the Fas PCR were determined by quantitating from an agarose gel using a phosphorimage, results for the final PCR run with all samples could not be obtained. The quality of the phosphorimage produced was not adequate for quantitation. It is likely that formation of primer-dimers influenced PCR reaction efficiency as preparation time was increased in the final run due to the high number of samples. Also, consistency in image quality appeared to vary across the phosphorimage, making accurate quantitation difficult. Time constraints precluded repeating the Fas PCR. My next step will be to repeat the PCR using PLATINUM™ Taq Polymerase to reduce primer-dimer formation. Quantitation will be effected by counts per minute following isopropanol extraction of unincorporated nucleotides, as this method of quantitation was effective for the Fas ligand PCR (as shown in Figure 23).

The expression of Fas ligand mRNA in the rat ovary decreased slightly from day 1 to day 16 of pregnancy. A significant increase in expression was observed from day 16 to day 22 of pregnancy, just prior to parturition. This increase is consistent with the immunohistochemical findings and coincides with luteolysis. Expression appeared to decrease at day 1 post-partum, however the results were not significantly different from those at day 22 of pregnancy. The decrease in expression from day 22 of pregnancy to day 3 post-partum was significant. In summary, Fas ligand mRNA expression

increases significantly at the time of luteolysis and regulation appears to follow a similar pattern to that seen in localisation of the protein.

An *in vitro* corpus luteum culture model was used to evaluate the ability of an anti-Fas monoclonal antibody to block spontaneous apoptosis in corpora lutea placed in serum-free medium. This model does not allow identification of the proportion of cells undergoing apoptosis due to the loss of contact with surrounding cells, which may be caused by the trauma of dissection from the ovary. Care was taken when dissecting corpora lutea from the ovary and all samples were treated in the same way to reduce injury to cells.

The monoclonal antibody chosen for this study was stored in a buffer containing 50% glycerol, a small percentage of BSA, monosodium phosphate and sodium azide. As the storage buffer may have influenced the apoptosis of luteal cells, the effect of serum-free medium with the antibody storage buffer alone on corpora lutea in culture was also examined. The results indicated that the antibody storage buffer may have reduced the occurrence of spontaneous apoptosis.

The anti-Fas monoclonal antibody appeared to further reduce spontaneous apoptosis, although the results were not significant. More conclusive results may be obtained by extracting antibodies from the storage

buffer before culture, thus removing the possible influence of the storage buffer. Dose-response and time-response curves should also be produced to better determine how varying concentrations of antibody and increasing time affect spontaneous apoptosis. With these developments, the *in vitro* corpus luteum culture model should prove to be an excellent system for investigating the pathway(s) involved in Fas-mediated apoptosis.

Whilst a link has been shown between the Fas/Fas ligand system and corpus luteum function, it is important to remember that luteolysis is a multifactorial event. Blocking the Fas receptor *in vivo* may not prevent regression of the corpus luteum. Indeed, I am not aware of any reports of fertility problems in *lpr* or *gld* mice, which carry mutations in Fas receptor and Fas ligand respectively.

Nonetheless, both Fas receptor and Fas ligand appear to have a role in corpus luteum function in the rat. As expression of both proteins increases during natural regression in rat pregnancy and post-partum, a role in luteolysis is implicated. Future research is required in order to further define this role and to relate the function of the Fas/Fas ligand system to the many factors which influence the corpus luteum.

As with many studies, the results of the present project have answered some questions, but generated others. For example, to what extent is the

Fas/Fas ligand system involved in luteolysis? Is the Fas/Fas ligand system involved in corpus luteum regression during the normal estrus cycle of the rat? Are hormonal influences involved in the function of the Fas/Fas ligand system? Can the molecular pathway(s) involved in Fas-mediated apoptosis be identified? Is Fas ligand involved in immune privilege in the corpus luteum and ovary? With development of the *in vitro* corpus luteum culture model and utilisation of gld and lpr mice, it would appear that in future studies the answers to these questions will be within our reach.

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9. APPENDIX A – TABLES, FIGURES AND ABBREVIATIONS

9.1. List of Tables

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9.3. List of Abbreviations

BSA	Bovine Serum Albumin
cm	Centimetres
DAB	3,3'Diaminobenzidine Tetrahydrochloride
ddH ₂ O	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
FasL	Fas Ligand
hr	Hour
HRP	Horse Radish Peroxidase
mAb	Monoclonal Antibody
MEM	Minimal Essential Medium
mRNA	Messenger Ribonucleic Acid
nm	Nanometres
PBS	Phosphate Buffered Saline
RPM	Revolutions Per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP Biotin Nick End Labelling

10. APPENDIX B - DETAILED PROTOCOLS

10.1. Immunohistochemistry

1. Paraffin wax was removed from slides following the protocol shown in Table 3.

Table 3
Protocol for Removal of Paraffin Wax from Sections

SOLUTION	ACTION
Toluene	Incubate 2 minutes
Toluene	Incubate 2 minutes
100% Ethanol	Agitate 10 times
100% Ethanol	Agitate 10 times
70% Ethanol	Agitate 10 times
Tap Water	Agitate 10 times
Tap Water	Agitate 10 times

2. A circle was drawn around sections with a diamond pen, then slides were placed in distilled water.
3. Endogenous peroxidase activity was quenched by covering each section with 250 μ L 3% Hydrogen Peroxide diluted in Methanol. Slides were incubated in a humidified chamber at room temperature for 10 minutes.
4. Slides were washed in fresh PBS three times for 5 minutes each time.
5. Staining for Fas receptor:
 - (a) Sections were covered with 250 μ L 1% SDS to expose Fas antigen. Slides were incubated in a humidified chamber at room temperature for 5 minutes.

- (b) Slides were washed in fresh PBS three times for 5 minutes each time.
 - (c) The primary antibody (Mouse anti-rat Fas monoclonal IgG, 250 μ g/mL) was diluted 1 in 100 with PBS + 1% BSA. Sections on each test slide were covered with 250 μ L of diluted primary antibody. Sections on negative control slides were covered with 250 μ L PBS + 1% BSA. Slides were incubated in a humidified chamber at room temperature for 2 hours.
 - (d) Test slides were washed in PBS for 10 minutes. Negative control slides were left in the humidified chamber. All slides were then washed in fresh PBS twice for 10 minutes each time.
 - (e) The secondary antibody (Sheep anti-mouse immunoglobulins, 0.5mg/mL) was diluted 1 in 100 with PBS + 1% BSA +2% normal rat serum (used to reduce nonspecific staining). Sections were covered with 250 μ L of diluted secondary antibody and incubated in a humidified chamber at room temperature for 45 minutes.
6. Staining for Fas ligand:
- (a) The primary antibody (Goat anti-rat Fas ligand polyclonal IgG, 200 μ g/mL) was diluted 1 in 50 with PBS + 1% BSA. Sections on each test slide were covered with 250 μ L of diluted primary antibody. Sections on negative control slides were covered with 250 μ L PBS + 1% BSA. Slides were incubated in a humidified chamber at room temperature for 2 hours.

- (b) Test slides were washed in PBS for 10 minutes. Negative control slides were left in the humidified chamber. All slides were then washed in fresh PBS twice for 10 minutes each time.
 - (c) The secondary antibody (Biotinylated rabbit anti-goat IgG, 1.5mg/mL) was diluted 1 in 50 with PBS + 1% BSA + 2% normal rat serum. Sections were covered with 250 μ L of diluted secondary antibody and incubated in a humidified chamber at room temperature for 45 minutes.
 - (d) Slides were washed in fresh PBS three times for 5 minutes each time.
 - (e) Streptavidin Horse Radish Peroxidase (HRP) conjugate was diluted 1 in 50 with PBS + 1% BSA. Sections were covered with 250 μ L of diluted Streptavidin-HRP and incubated in a humidified chamber at room temperature for 45 minutes.
7. Staining for both Fas receptor and Fas ligand:
Slides were washed in fresh PBS three times for 5 minutes each time.
 8. 1mL of 10x DAB was added to 9mL PBS. Just before use 5.5 μ L of Hydrogen Peroxide was added to the DAB solution.
 9. Each section was covered with 500 μ L DAB solution. Slides were incubated in a humidified chamber at room temperature for 10 minutes.
 10. Slides were washed by dipping in fresh PBS once, then by placing in fresh PBS twice for 5 minutes each time.
 11. Sections were counterstained with Haematoxylin and dehydrated following the protocol shown in Table 4.

Table 4
Protocol for Counterstaining and Dehydrating Sections

SOLUTION	ACTION
Haematoxylin	Incubate 2 seconds
Tap Water wash	Agitate 10 times
Tap Water wash	Agitate 10 times
70% Ethanol	Agitate 10 times
100% Ethanol	Agitate 10 times
100% Ethanol	Agitate 10 times
100% Ethanol	Agitate 10 times
100% Ethanol	Agitate 10 times
Toluene	Agitate 10 times
Toluene	Agitate 10 times
Toluene	Agitate 10 times

12. Slides were coverslipped using DPX Mountant, then viewed by light microscopy.

10.2. Relative Quantitative RT-PCR

RNA Extraction - RNeasy B Method

1. Homogeniser probe was placed in Dimethyldicarbonate (DMDC)-treated water for one hour to destroy any RNases present.
2. Tissue was kept on ice until ready to homogenise.
3. 650µL of RNeasy B was added to each ovary before homogenising. A further 650µL of RNeasy B was added to each tube and ovaries were again homogenised briefly. Homogeniser probe was rinsed in DMDC-treated water between samples.
4. 130µL of Chloroform was added to each tube and mixtures were shaken vigorously for 15 seconds.

5. Tubes were incubated on ice for 5 minutes.
6. Solutions were transferred to 2mL eppendorf tubes and centrifuged at 14,000rpm for 15 minutes.
7. The upper aqueous phase of each tube was transferred to a new 2mL eppendorf tube and an equal volume of Isopropanol was added.
8. Tubes were mixed, then incubated at 4°C for one hour.
9. Tubes were centrifuged at 14,000rpm for 15 minutes.
10. Solutions were decanted with care taken not to lose pellet.
11. 1mL of 75% cold Ethanol was added to each tube and solutions were vortexed briefly.
12. Tubes were centrifuged at 6,000rpm for 8 minutes.
13. Solutions were decanted. A pipette was used to remove excess solution.
14. Pellets were dried by placing open eppendorf tubes in a biohazard cabinet for 15 minutes.
15. Pellets were resuspended in 10 μ L of DMDC-treated water.
16. Quantitation and purity of isolated RNA was established with a Beckman DU-640 Spectrophotometer.

Reverse Transcription Using Oligo-dT Primer

1. 2 μ L of Oligo-dT Primer, 500ng/ μ L was placed in each tube.
2. 2 μ g of sample RNA was added to each tube. A negative control without RNA was placed in each run.

3. The volume in each tube was made up to 10 μ L with DMDC-treated water.
4. Tubes were placed in thermal cycler and incubated at 70°C for 5 minutes, then placed on ice.
5. 15 μ L of a master mix of reagents was added to each tube.

Reverse Transcription Master Mix (A), 1x

Reverse Transcriptase Buffer (5x)	5 μ L
dNTPs (5mM)	5 μ L
RNasin (40U/ μ L)	1 μ L
Sodium Pyrophosphate (40mM)	2.5 μ L
AMV Reverse Transcriptase (23U/ μ L)	1.5 μ L

6. Sample tubes were placed in thermal cycler on the following program:
 - (a) 42°C for 60 minutes
 - (b) 95°C for 5 minutes
 - (c) 4°C \rightarrow .
7. cDNA was stored at -20°C.

Reverse Transcription Using Synthesized 3'-End Primers for Fas Ligand

1. 2.5 μ L of 3'-end primers for Fas ligand, 5pmol/ μ L was placed in each tube.
2. 1 μ g of sample RNA was added to each tube. A negative control without RNA was placed in each run.
3. The volume in each tube was made up to 9 μ L with DMDC-treated water.

4. Tubes were placed in thermal cycler and incubated at 70°C for 5 minutes.
5. 16µL of a master mix of reagents was added to each tube.

Reverse Transcription Master Mix (B), 1x

Reverse Transcriptase Buffer (5x)	5µL
dNTPs (5mM)	5µL
RNasin (40U/µL)	1µL
Sodium Pyrophosphate (40mM)	2.5µL
AMV Reverse Transcriptase (23U/µL)	2.5µL

6. Sample tubes were placed in thermal cycler on the following program:
 - (a) 42°C for 60 minutes
 - (b) 95°C for 2 minutes
 - (c) 4°C →.
7. cDNA was stored at -20°C.

PCR Using Fas Primers

1. 5µL of cDNA from each sample was amplified. A negative control substituting DMDC-treated water for cDNA and a positive control (diluted 10⁻⁹, developed in the Molecular Biology Laboratory, Dept. of Anatomy and Human Biology, University of Western Australia) were placed in each run.
2. 20µL of a master mix of reagents was added to the cDNA in each tube.

PCR Master Mix (A), 1x

DMDC-Treated Water	11.35 μ L
Taq Polymerase Buffer (10x)	2.5 μ L
MgCl ₂ (25mM)	4.0 μ L
dATP (100mM)	0.1 μ L
dGTP (100mM)	0.1 μ L
dTTP (100mM)	0.1 μ L
dCTP (100mM)	0.025 μ L
α^{32} P-dCTP (300mM)	0.075 μ L
Fas Primers (2pmol/ μ L)	1.5 μ L
Taq Polymerase (5.5U/ μ L)	0.25 μ L

3. Sample tubes were placed in thermal cycler on the following program:
 - (a) 94°C for 4 minutes 30 seconds
 - (b) 94°C for 30 seconds
 - (c) 58°C for 45 seconds
 - (d) 72°C for 1 minute
 - (e) Steps (b) to (d) were repeated 35 times
 - (f) 72°C for 5 minutes
 - (g) 4°C →.
4. Amplified samples were stored at -20°C.

PCR Using Fas Ligand Primers

1. 2 μ L of cDNA from each sample was amplified. A negative control substituting DMDC-treated water for cDNA and a positive control (diluted 10⁻⁹, developed in Molecular Biology Laboratory, Dept. of Anatomy and Human Biology, University of Western Australia) was placed in each run.
2. 20 μ L of a master mix of reagents was added to the cDNA in each tube.

PCR Master Mix (B), 1x

DMDC-Treated Water	14.35 μ L
PLATINUM™ Taq Polymerase Buffer (10x)	2.5 μ L
MgCl ₂ (25mM)	2.5 μ L
dATP (100mM)	0.1 μ L
dGTP (100mM)	0.1 μ L
dTTP (100mM)	0.1 μ L
dCTP (100mM)	0.025 μ L
α^{32} P-dCTP (300mM)	0.075 μ L
Fas Ligand Primers (2pmol/ μ L)	3.0 μ L
Taq Polymerase (5.5U/ μ L)	0.25 μ L

3. Sample tubes were placed in a thermal cycler on the following program:
 - (a) 94°C for 4 minutes 30 seconds
 - (b) 94°C for 30 seconds
 - (c) 58°C for 1 minute
 - (d) 72°C for 1 minute

(e) Steps (b) to (d) were repeated 32 times

(f) 72°C for 5 minutes

(g) 4°C →.

4. Amplified samples were stored at -20°C.

Isopropanol Extraction of Unincorporated Nucleotides from PCR Products

1. 18 μ L of PCR product was placed into a clean eppendorf tube.
2. 1.8 μ L 3M Sodium Acetate, pH 5.2, was added.
3. 20 μ L Isopropanol was added and solution was mixed.
4. 1 μ L Glycogen was added.
5. Samples were incubated at 4°C for 10 minutes.
6. Samples were centrifuged at 14,000rpm for 15 minutes.
7. Supernatant was removed.
8. Pellet was washed with 500 μ L 70% cold Ethanol, then vortexed.
9. Samples were centrifuged at 14,000rpm for 5 minutes.
10. Supernatant was removed.
11. Samples were dessicated for 10-15 minutes in a biohazard cabinet.
12. Pellet was resuspended in 15 μ L ddH₂O.

Running an Agarose Gel to Remove Unincorporated Nucleotides from PCR Products

1. Ends of a gel tray were sealed with autoclave tape and a comb placed with the shorter edge of wells facing forward.

2. 6 μ L Ethidium Bromide per 100mL of a 1.5% Agarose in 1x TAE solution was added and mixed without creating bubbles.
3. Agarose gel was poured into gel tray without creating bubbles and gel was allowed to harden for about 10 minutes.
4. 1x TAE was poured into electrophoresis chamber.
5. Gel tray, with tape removed, was placed in electrophoresis chamber.
6. Gel was just covered with 1x TAE.
7. One drop of 6x Loading Dye was placed onto a sheet of parafilm.
8. 4 μ L of PCR product was mixed with loading dye using a pipette, then loaded into well.
9. Steps 7 and 8 were repeated for each sample.
10. 2 μ L of 1kb Standard with loading dye was used as a marker for each run.
11. Gel was run at 70 - 80 volts for 35 minutes.
12. The gel was viewed under UV light. The Fas PCR product was 491 base pairs and the Fas ligand PCR product was 341 base pairs.
13. Gel was placed on two pieces of blotting paper, which were cut so that the edges extended 2 - 3 cm around the gel.
14. Gel and blotting paper were covered loosely in plastic wrap and dried in a vacuum slab gel dryer for about 1 hour.

15. The radioactive gel was either exposed to x-ray film in an autoradiograph cassette at -80°C overnight, then developed, or exposed to a phosphorimaging plate at room temperature overnight, then quantitated using an image analysis computer program.

Relative Quantitative RT-PCR for Fas mRNA

1. One sample from each time-point was reverse transcribed using the protocol titled "Reverse Transcription Using Oligo-dT Primer".
2. Four-fold serial dilutions (i.e. neat, $1/4$, $1/16$, $1/64$) of each sample were amplified by PCR using the protocol titled "PCR Using Fas Primers".
3. Amplified products were run on an agarose gel and dried using the protocol titled "Running an Agarose Gel to Remove Unincorporated Nucleotides from PCR Products", then quantitated after exposure to a phosphorimaging plate.
4. Results were plotted on a logarithmic graph and a dilution amplifying exponentially when the PCR reaction was stopped was chosen for each time-point.
5. All samples were reverse transcribed in the one run using the protocol titled "Reverse Transcription Using Oligo-dT Primer".
6. The cDNA of all samples was diluted to the factor chosen.
7. All samples were amplified by PCR in one run using the protocol titled "PCR Using Fas Primers".

8. Amplified products were run on one agarose gel using the protocol titled "Running an Agarose Gel to Remove Unincorporated Nucleotides from PCR Products", then quantitated after exposure to a phosphorimaging plate.
9. Data from different time-points was compared after allowing for the dilution factor used in the PCR reaction.

Relative Quantitative RT-PCR for Fas Ligand mRNA

1. One sample from each time-point was reverse transcribed using the protocol titled "Reverse Transcription Using Synthesized 3'-End Primers for Fas Ligand".
2. Four-fold serial dilutions (i.e. neat, $1/4$, $1/16$, $1/64$, $1/128$) of each sample were amplified by PCR using the protocol titled "PCR Using Fas Ligand Primers".
3. The protocol titled "Isopropanol Extraction of Unincorporated Nucleotides from PCR products" was followed and samples were quantitated with a liquid scintillation counter.
4. Results were plotted on a logarithmic graph and a dilution amplifying exponentially when the PCR was stopped was chosen for each time-point.
5. All samples were reverse transcribed in one run using the protocol titled "Reverse Transcription Using Synthesized 3'-End Primers for Fas Ligand".

6. The cDNA of all samples was diluted to the factor chosen.
7. All samples were amplified by PCR in one run using the protocol titled "PCR Using Fas Ligand Primers".
8. Amplified products were purified of unincorporated nucleotides using the protocol titled "Isopropanol Extraction of Unincorporated Nucleotides from PCR Products", then quantitated with a liquid scintillation counter.
9. Data from different time-points was compared after allowing for the dilution factor used in the PCR reaction.

10.3. In Vitro Corpus Luteum Culture Model

Corpus Luteum *In Vitro* Culture Model

1. Four sterile scintillation vials were prepared by adding 2mL serum-free minimal essential medium (MEM) with 1 μ g/mL (a total of 40 μ L) anti-rat Fas monoclonal antibody. Another five sterile scintillation vials were prepared with the addition of 2mL serum-free MEM and 40 μ L of antibody storage buffer (50% glycerol, 20mM NaH₂PO₄ pH 7.5, 1.5mM NaN₃, 1mg/mL BSA). Serum-free MEM only was added to a further five sterile scintillation vials.
2. Two corpora lutea were placed in each vial.
3. The vials were gassed with a 95% O₂ - 5% CO₂ mixture and their lids sealed with vacuum grease.
4. Vials were incubated under 95% O₂ - 5% CO₂ at 37°C for 4 hours.

5. Corpora lutea were placed in sterile polypropylene tubes and snap frozen in liquid nitrogen.
6. Samples were stored at -80°C.

DNA Extraction

1. Each sample (consisting of two corpora lutea) was gently homogenised in 670 μ L of DNA homogenisation buffer.
2. Homogenate was transferred to a 2mL eppendorf tube and 42 μ L of 10% SDS was added and mixed.
3. Tubes were incubated at 65°C for 30 minutes.
4. 119 μ L 8M Potassium Acetate was added and mixed.
5. Tubes were incubated on ice for 60 minutes.
6. Tubes were microcentrifuged at 5,000rpm for 10 minutes.
7. Supernatant was transferred to new tubes and divided in half.
8. Equal volumes of Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to each tube and the solution was vortexed.
9. Tubes were microcentrifuged at 6,000rpm for 5 minutes.
10. The upper phase was transferred to a new tube.
11. Equal volumes of Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to each tube and the solutions were vortexed.
12. Tubes were microcentrifuged at 6,000rpm for 5 minutes.
13. The upper phase was transferred to a new tube.

14. 2.5x volume of cold 100% Ethanol was added and tubes were stored overnight at -80°C.
15. Tubes were microcentrifuged at 14,000rpm for 30 minutes.
16. Solutions were decanted, while taking care not to lose pellet.
17. 100 μ L 1x TE, pH 8.0, was added and the pellet was resuspended.
18. 2 μ L RNase was added and solutions were vortexed.
19. Tubes were microcentrifuged briefly, then incubated at 37°C for 60 minutes.
20. Steps 7 to 12 were repeated.
21. The upper phase was transferred to a new tube.
22. 0.1x volume 3M Sodium Acetate and 2.5x volume cold 100% Ethanol was added and tubes were incubated at -80°C overnight.
23. Tubes were microcentrifuged at 14,000rpm for 30 minutes.
24. Solutions were decanted, taking care not to lose pellet.
25. 100 μ L cold 100% Ethanol was added without disrupting pellet.
26. Tubes were placed on ice for 5 minutes, then centrifuged at 14,000rpm for 5 minutes.
27. Ethanol was decanted without disrupting pellet, then tubes were inverted over a paper towel to allow pellet to air dry for approximately 1 to 2 hours.
28. Pellet was resuspended in 50 μ L ddH₂O.
29. Quantitation and purity of isolated DNA was established with a Beckman DU-640 Spectrophotometer.

30. Samples were stored at -80°C .

3'-End Labelling

1. $1\mu\text{g}$ of each DNA sample was placed into eppendorf tubes. A sample of DNA from rat corpora lutea which were immediately snap frozen after dissection from the ovary (time zero) was included to be used as a baseline measurement against which all samples would be compared.
2. Volumes were made up to a total of $29\mu\text{L}$ with ddH_2O .
3. $10\mu\text{L}$ of 5x Terminal Transferase Reaction Buffer was added.
4. $5\mu\text{L}$ of CoCl_2 was added, then tubes were vortexed well.
5. Tubes were microcentrifuged at $14,000\text{rpm}$ for 10 seconds.
6. $\alpha^{32}\text{P}$ -ddATP ($3,000\text{ Ci/mmol}$) was diluted 1 in 8 and $5\mu\text{L}$ was added to each tube.
7. Terminal transferase was diluted 1 in 2 and $1\mu\text{L}$ was added to each tube.
8. Tubes were vortexed, then microcentrifuged at $14,000\text{rpm}$ for 10 seconds.
9. Tubes were incubated at 37°C for 60 minutes.
10. $5\mu\text{L}$ 0.25M EDTA was added.
11. $2\mu\text{L}$ tRNA solution was added.
12. $12\mu\text{L}$ 10M Ammonium Acetate was added.
13. $180\mu\text{L}$ cold 100% Ethanol was added.
14. Tubes were placed in -80°C for 60 minutes.
15. Tubes were microcentrifuged at $14,000\text{rpm}$ for 20 minutes.

16. Supernatant was discarded.
17. Pellet was resuspended in 60 μ L of 1x TE.
18. 12 μ L 10M Ammonium Acetate was added.
19. 180 μ L of cold 100% Ethanol was added.
20. Tubes were vortexed, then placed in -80°C for 60 minutes.
21. Tubes were microcentrifuged at 14,000rpm for 20 minutes.
22. Supernatant was discarded.
23. Pellet was inverted over paper towel and allowed to air dry for 20 minutes.
24. Pellet was resuspended in 40 μ L 1x TE and stored at -20°C overnight.
25. Samples were thawed for 30 minutes.
26. Ends of a gel tray were sealed with autoclave tape and a comb placed at one end.
27. A 2% Agarose in 1x TAE gel was prepared, poured into the gel tray without creating bubbles and allowed to set.
28. 1x TAE was poured into an electrophoresis chamber.
29. Gel tray, with tape and comb removed, was placed into electrophoresis chamber and gel was just covered with 1x TAE.
30. Samples were vortexed, then microcentrifuged at 14,000rpm for 10 seconds.
31. 5 μ L of DNA loading buffer was added to each sample.
32. Samples were vortexed, then microcentrifuged at 14,000rpm for 10 seconds.

33. 20 μ L of each sample was loaded into a well. The remaining volume of each sample was stored at -20°C.
34. Samples were electrophoresed at 50 volts for 2 hours 30 minutes.
35. The gel was removed and placed onto two pieces of blotting paper, which were cut so that the edges extended 2 - 3 cm around the gel.
36. Gel and blotting paper were covered loosely in plastic wrap and dried in a vacuum slab gel dryer for 3 hours.
37. Blotting paper was removed from gel and gel was covered with a new piece of plastic wrap.
38. The radioactive gel was taped inside an autoradiograph cassette and exposed to X-ray film at -80°C overnight.
39. The film was developed, viewed for laddering of low molecular weight DNA, then placed under the gel in its original position.
40. Each lane was marked using the autoradiograph as a guide.
41. Low molecular weight DNA from each lane was excised using a scalpel, then placed in a scintillation vial.
42. The counts per minute of low molecular weight DNA for each sample was obtained with a liquid scintillation counter.

11. APPENDIX C - SOLUTIONS AND BUFFERS

1.5% Agarose Gel plus 1x TAE

Agarose	1.5g
1x TAE	100mL

Place agarose in TAE and heat in microwave on medium setting until dissolved.

3,3'Diaminobenzidine Tetrahydrochloride (DAB), 10X

DAB	120mg
PBS	10mL

Dissolve DAB in PBS. Store in 1mL aliquots in freezer.

NB: DAB is carcinogenic. Use a biohazard cabinet and wear gloves and a face mask when weighing out powder. Neutralise any item which contacts powder or solution with 1 in 10 dilution of bleach after use.

Dimethyldicarbonate (DMDC) Treated Water

Add one part DMDC to 1000 parts double distilled water (ddH₂O). Autoclave solution.

DNA Homogenisation Buffer

NaCl 1M	50mL
EDTA 0.5M, pH 8.0	10mL
Tris 1M, pH 8.0	150mL
Sucrose 1M	100mL

Make up solution to 500mL by adding 190mL ddH₂O.

DNA Loading Buffer

Glycerol (v:v)	30%
Bromophenol Blue (w:v)	0.25%
Xylene Cyanol (w:v)	0.25%

In Vitro Culture Medium

Minimal Essential Medium (Gibco), 1x (with Earle's salts, without L-glutamine, without Phenol red)	100mL
Bovine Albumin, fatty acid free	100mg
L-glutamine, powdered	29.2mg

Sterile filter, then add:

Penicillin (5000U/mL)	200 μ L
Streptomycin (5000 μ g/mL)	200 μ L

Store at 4°C.

4% Paraformaldehyde

Sodium Phosphate Buffer, pH 7.2	100mL
Paraformaldehyde Powder	4g

Heat 50mL sodium phosphate buffer to 60°C. Add 4g paraformaldehyde and dissolve with constant stirring. Add NaOH drop by drop until solution clears. Cool solution and make up to 100mL with sodium phosphate buffer. Use fresh solution.

NB: Paraformaldehyde is a possible mutagen, teratogen and carcinogen. Use a biohazard cabinet and wear gloves and a face mask when weighing out powder and preparing solution.

Phosphate Buffered Saline (PBS)

NaCl	8.0g
KCl	0.2g
Na ₂ HPO ₄	1.6g
KH ₂ PO ₄	0.2g
MgCl ₂	0.1g
CaCl ₂	0.1g

Dissolve salts individually in the order given in approximately 800mL of ddH₂O. Make up solution to 1 litre with ddH₂O and adjust to pH 7.4 with NaOH. Use fresh solution or autoclave before storage.

PBS plus 1% Bovine Serum Albumin (PBS + 1% BSA)

PBS	100mL
BSA	1g

Dissolve BSA in PBS. Store aliquots at -20°C. Keep at 4°C when thawed.

Sodium Phosphate Buffer, pH 7.2

1M Na ₂ HPO ₄	68.4mL
1M Na ₂ H ₂ PO ₄	31.6mL

Make solution up to 1 litre with ddH₂O.

TAE Buffer, 10x

Tris	48.4g
Glacial Acetic Acid	11.4mL
0.5M EDTA, pH 8.0	20mL

Make solution up to 1 litre with ddH₂O.

TE, 1x

1M Tris-HCl, pH 8.0	5mL
0.5M EDTA, pH 8.0	1mL

Make up solution to 500mL by adding 494mL ddH₂O.

Tris-HCl

Use HCl to buffer Trizma Base to the required pH.

12. APPENDIX D - SUPPLIERS

12.1. Reagents

Reagent	Supplier
$\alpha^{32}\text{P}$ -dCTP	DuPont, USA
$\alpha^{32}\text{P}$ -ddATP	Amersham Life Science, UK
Agarose	Pharmacia Biotech, Sweden
Ammonium acetate	Sigma, USA
AMV reverse transcriptase	Promega, USA
Anti-rat Fas monoclonal antibody	Transduction Laboratories, UK
Anti-rat FasL polyclonal antibody	Santa Cruz Biotechnology, USA
Biotinylated rabbit anti-goat IgG	Vector Laboratories, USA
Bovine serum albumin	Sigma, USA
Bromophenol Blue	Sigma, USA
Calcium chloride (CaCl_2)	Sigma, USA
Chloroform	Sigma, USA
Cobalt chloride (CoCl_2)	Boehringer Mannheim, Germany
dATP	Promega, USA
dCTP	Promega, USA
dGTP	Promega, USA
Diaminobenzidine tetrahydrochloride (DAB)	Sigma, USA
Disodium hydrogen phosphate (Na_2HPO_4)	BDH, Australia
DMDC	Sigma, USA

DPX mountant	BDH, Australia
dTTP	Promega, USA
Ethanol	BDH, Australia
Ethidium bromide	Sigma, USA
Ethylenediaminetetraacetic Acid (EDTA)	Sigma, USA
Glacial Acetic Acid	BDH, Australia
Glycerol	Sigma, USA
Glycogen	Boehringer Mannheim, Germany
Haematoxylin	Australian Biostain, Australia
Hydrogen peroxide	Sigma, USA
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
L-glutamine	Sigma, USA
Magnesium chloride (MgCl ₂)	Sigma, USA
Methanol	BDH, Australia
Minimal essential medium (MEM)	Gibco BRL, USA
Oligo-dT primers	Promega, USA
Paraffin wax	Oxford Labware, USA
Paraformaldehyde powder	Merck, Germany
Penicillin	ICN Pharmaceuticals, USA
Phenol	Sigma, USA
PLATINUM™ Taq polymerase	Gibco BRL, USA
Potassium acetate	Sigma, USA
Potassium chloride (KCl)	Ajax Chemicals, Australia

Potassium dihydrogen orthophosphate (KH_2PO_4)	Ajax Chemicals, Australia
Reverse transcriptase buffer	Promega, USA
RNasin	Promega, USA
RNAzol B	Bresatec, Australia
Sheep anti-mouse immunoglobulins	Amersham Life Science, UK
Sodium acetate	Sigma, USA
Sodium azide (NaN_3)	Sigma, USA
Sodium chloride (NaCl)	BDH, Australia
Sodium dihydrogen orthophosphate (NaH_2PO_4)	BDH, Australia
Sodium dodecyl sulfate (SDS)	Sigma, USA
Sodium pyrophosphate	Sigma, USA
Streptavidin HRP	Amersham Life Science, UK
Streptomycin	ICN Pharmaceuticals, USA
Taq polymerase	Gibco BRL, USA
Taq polymerase buffer	Gibco BRL, USA
Terminal transferase reaction buffer	Boehringer Mannheim, Germany
Toluene	BDH, Australia
Trizma Base	Sigma, USA
tRNA	Boehringer Mannheim, Germany
Xylene Cylanol	Sigma, USA

12.2. Equipment

Equipment	Supplier
Audioradiograph Cassette	Kodak Scientific, Australia
Beckman DU-640 Spectrophotometer	Beckman Instruments Inc., USA
Incubator	Contherm Scientific Co., NZ
Gel Electrophoresis Apparatus, Easy Cast #B2	Owl Scientific, Australia
Mini-Gel Electrophoresis Apparatus	Pharmacia, Sweden
Incubator, Water jacketed, model 3157	Forma Scientific, USA
Light Microscope, Leitz Wetzlar	Henderson Instrument Co., Australia
Liquid Scintillation Counter, Tri-Carb	Packard Laboratory Services, Australia
Microcentrifuge, 5415C	Eppendorf, Germany
Programmable Thermal Controller, PTC-100	MJ Research Inc., USA
P10, P200 and P1000 Pippettes, Biohit Proline	Locus Genex, Finland
Phosphorimager, Mac Bas 1000 Bioimaging Analyser	Fuji Photofilm Co. Ltd., Japan
Power Supply Module, 216A	BWD Electronics, Australia
Tissue Processor, Citadel 1000	Shandon Lipshaw, USA
Slab-gel dryer, Uniequip 3040	Lab Supply, Australia

Spencer 820 Microtome	American Optical Corporation, USA
Ultra-Turrax Homogeniser Probe	IKA®-Werk, Janke and Kunkel GmbH & Co., Germany
Vacuum Embedder	Labec Laboratory Equipment, Australia
Vortex Mixer - 16700 Mixer Thermolyne	Barnstead Thermolyne, USA

13. CONFERENCE ABSTRACT

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ROLE OF FAS AND FAS LIGAND IN APOPTOSIS DURING LUTEOLYSIS

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Whilst apoptosis has been found to occur during structural regression of the corpus luteum (1), the mechanisms involved have yet to be specified. One possible mediator is the Fas (or APO-1 or CD95) receptor, a transmembrane protein which induces apoptosis in the cell when ligated. As the Fas receptor is expressed in the regressing corpus luteum (CL) of the normal adult human ovary (2), and Fas monoclonal antibodies induce apoptosis in cultured human granulosa and luteal cells pretreated with interferon gamma (3), we hypothesized that the mechanism of regression of the CL may involve Fas-mediated apoptosis. The presence of Fas and Fas ligand (FasL) in the rat CL at various stages of pregnancy and post-partum was examined immunohistochemically. Steady state Fas and FasL mRNA levels in the rat ovary at various stages of pregnancy and post-partum were analysed by semi-quantitative RT-PCR. The FasL protein was localised in the rat CL throughout pregnancy and post-partum. In addition, mRNA for FasL was expressed at each time-point examined. The Fas receptor protein was localised in the rat CL at one day post-partum, a period when the CL is undergoing maximal apoptosis. Studies to date indicate that the Fas / FasL system has a role in apoptosis during luteolysis. Further investigations into the mechanisms of Fas-mediated CL apoptosis are currently being undertaken.

1. Juengel, J.L., Garverick, H.A., Johnson, A.L., Youngquist, R.S. and Smith, M.F. (1993). *Endocrinol.* 132: 249-254.
2. Kondo, H., Marou, T., Peng, X. and Mochizuki, M. (1996). *J. Clin. Endocrinol. Metab.* 81: 2702-2710.
3. Quirk, S.M., Cowan, R.G., Joshi, S.G. and Henrikson, K.P. (1995). *Biol. Reprod.* 52: 279-287.