MECHANISMS OF FERTILITY FAILURE IN HIGH YIELDING DAIRY COWS

By

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ABSTRACT

Reproductive tract inflammatory diseases (RTID) of dairy cows are common worldwide and have been associated with a decrease in reproductive performance. The aims of this thesis were: (1) to quantify the effect of RTID on reproductive performance using data from 80 dairy herds across England and Wales, and from the University of Nottingham Dairy Centre; (2) to determine the effect of endometritis on the normality of post-partum milk progesterone profiles; (3) to investigate the association between endometritis and luteal vasculature and protein expression of steroidogenesis enzymes *ex vivo*; (4) to determine the expression of LPS-associated receptors including TLR4, CD14 and MD-2 in bovine CL using multiplex PCR; (5) to investigate the dose dependent effects of LPS on luteal endothelial cell vasculature *in vitro* and protein expression of steroidogenesis enzymes.

Data analysis of 59118 cows from 80 dairy herds across England and Wales showed 12% prevalence of RTID from 2000-2007 *(P<0.001)*. Cows with RTID had significantly longer intervals from calving to both first service and to conception by about 5 days (*P<0.001*) and 22 days (*P<0.001*), respectively. Moreover, cows with RTID had a lower conception rate to 1st service by 14% (*P<0.001*) and required more services per conception about 1 service more (*P<0.001*). In addition, they were 1.2 times more likely to be exited from the herd (*P<0.001*), and had higher 305d milk yield (*P<0.01*). Furthermore, this study analysed data from 708 cows at Nottingham dairy centre showed about 15% of prevalence of RTID form 2008-2014 (*P<0.001*). Similar effects were observed in the Nottingham dairy centre. However, only the day to 1st service was significant (*P<0.001*). The association between endometritis and milk progesterone profiles was investigated to establish the importance of endometritis on postpartum ovarian activity. Endometritis increased the incidence of atypical progesterone profile with prolonged luteal phase being the most affected.

In the *ex vivo* study, there was a negative association between presence of endometritis with luteal vasculature, CL size, luteal progesterone content and quantification of steroidogenic enzyme expression. Endometritis caused significant inhibition in the degree of luteal vasculature, progesterone content and protein expression of steroidogenic enzymes by corpora lutea.

LPS has been implicated in influencing ovarian function. Multiplex PCR showed that LPS receptors (TLR4, CD14, and MD-2) are expressed in bovine CL at all stages of CL development, indicating that CL could be a target for LPS action. A physiological cell culture system was consequently utilised to examine the effect of LPS on luteal endothelial cell network formation, progesterone (P4) production by luteal cells and steroidogenic enzyme expression *in vitro*. Treatment with VEGFA/FGF2 increased progesterone production by luteal cells ($P<0.001$). Moreover, progesterone production increased significantly from day 3-9 of culture. Under both basal condition and/or angiogenic stimulus (VEGFA/FGF-2), LPS (0.01, 0.1, 1 and 10 μg/ml) had no effect on P4 production by luteal cells on day 3, 5, and 9 of culture. LPS significantly inhibited luteal endothelial cells network formation. This was due to inhibition of endothelial cell proliferation and increasing endothelial cell apoptosis ($P<0.001$).

In summary, the adverse impact that endometritis has on dairy cow fertility was identified by this work. This work offers a greater understanding of the effect of endometritis on dairy cow's reproductive performance, early luteal development and function. In particular, this work provides evidence of a novel effect of endometritis on bovine luteal development and adequacy. It also offers further awareness into the role of LPS in terms of its negative effect on luteal endothelial cell angiogenesis and steroidogenesis.

Declaration

The author, except where acknowledged in the text, completed all the work in this thesis. These studies were carried out under the supervision of Dr George Mann, Department of Animal Science, and Dr Bob Robinson, Veterinary School, University of Nottingham.

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This thesis is dedicated to my parents and my wife for all their support over the years.

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Published Abstracts

1. Mohammed A Z, Robinson RS and Mann GE (2015): Impact of endometritis on ovarian cyclicity in dairy cows. The 19th annual Nottingham Cattle Fertility Conference (Oral Presentation). Nottingham University, Sutton Bonington Campus, LE12 5RD. 22 June 2015.

2. Mohammed A Z, Chris D H, Robinson RS and Mann GE (2015): The association between bovine uterine disease and their subsequent reproductive performance in commercial UK dairy herds (Poster Presentation). In the *Society for Reproduction and Fertility*. Annual Conference, Oxford, UK, 20—22 July 2015. Abstract: P015.

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4. Mohammed A Z, Mann GE and Robinson RS (2015): *E.coli* lipopolysaccharide (LPS) adversely affects bovine luteal endothelial cell network formation and survival *in vitro* but has no effect on progesterone production (Poster Presentation). International Conference on *Biology and Pathology of Reproduction in Domestic Animals*. *Endometritis as a Cause of Infertility in Domestic Animals*. The Stanislaw Moniuszko Academy of Music in Gdansk, Lakowa Street 1-2, 80-743 Gdansk, Poland. 28-30 September 2015.

5. Mohammed A Z, Mann GE and Robinson RS R (2016): Post-partum endometritis: its negative impact on luteal vascularisation, progesterone content and steroidogenic enzyme expression in the cow *in vivo*. In the *Society for Reproduction and Fertility*: Annual Conference, University of Winchester, UK, 11-13 July 2016. Abstract. Oral presentation.

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Abbreviations

α	alpha
ACTA2	smooth muscle actin
β	beta
γ	gamma
μg	microgram
μM	micro molar
μm	micrometre
ANGPT	angiopoietins
ANOVA	analysis of variance
BMP-15	bone-morphogenetic protein-15
BSA	bovine serum albumin
CaCl	calcium chloride
cAMP	cyclic adenosine monophosphate
CD14	cluster of differentiation 14
CL	corpus luteum
cm	centimetre
CV	coefficient of variation
CYP11A	cytochrome p450 side-chain cleavage
DAB	3,3-diaminobenzidine tetrahydroxychloride
DF	dominant follicle
DNase	deoxyribonuclease
E2	oestradiol
EC	endothelial cell
ECM	endothelial cell media
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2
Fig	figure
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
g	gram
g	gravitational acceleration
GH	growth hormone
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HIF1A	hypoxic-inducible factor-1α
HSD3B	3β-hydroxysteroid dehydrogenase
IGF1	insulin like growth factor 1
IgG	immunoglobulin G

IL	interleukin
IL-1B	interleukin-1β
IFN-G	interferon-γ
ITCAM-1	Intracellular adhesion molecule-1
ITS	insulin-transferrin-selenium
kDa	kilo Dalton
LDL	low density lipoprotein
LH	luteinising hormone
LHR	luteinising hormone receptor
LLC	large luteal cells
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MD2	myeloid degeneration 2
ml	millilitre
mm	millimetre
MMRs	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NCAM	neural cell adhesion molecule
NEB	negative energy balance
NFkB	nuclear transcription factor kB
ng	nanogram
NO	nitric oxide
OXTR	oxytocin receptor
P4	progesterone
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
PDGF	platelet derived growth factor
pg	picogram
PGE2	prostaglandin E_2
PGF2α	prostaglandin $F_{2\alpha}$
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A_2
PP	post-partum
PRR	pattern recognition receptor
RFM	retained fetal membranes
RTID	reproductive tract inflammatory disease
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean
SER	smooth endoplasmic reticulum
SLC	small luteal cell
SMC	smooth muscle cell

SPC	service per conception
STAR	steroidogenic acute regulatory protein
TGFB	transforming growth factor B
TLR4	toll like receptor 4
TNFA	tumor necrosis factor A
TICAM1	toll like receptor adaptor molecule 1
TICAM2	toll like receptor adaptor molecule 2
TRAF6	tumor necrosis receptor associated factor 6
TRAM	toll-like receptor adaptor molecule
TRIF	toll-like receptor domain adaptor inducing interferon-1B factor
v/v	volume by volume
VEGFA	vascular endothelial growth factor a
VWF	von Willebrand factor
w/v	weight/volume

1 Literature review

1.1 General introduction

Over the past few decades, reproductive performance has decreased in dairy herds in the UK as well as across the globe. This has a negative impact on production and profit of a dairy farm (Lucy 2000, Weigel 2006). Insufficient reproductive performance is one of the most costly problems facing dairy farmers today (Kasimanickam et al. 2004). The decline in fertility over the past 50 years has occurred as milk production has increased (Walsh et al. 2011). The efficiency of production can be assessed by the interval between two calvings. The traditional is 365 days, and it has been estimated that longer than this period, costs up to £3 per cow per day (National Animal Disease Information Service, 2016, Esslemont et al. 2001). In the UK, currently the average calving interval is 425 days.

On the cow level, this cost equates to about £120 per cow and for the UK dairy industry £216 million per-annum. Currently, the dairy industry in the UK supports just under 14,000 dairy herds, with a total of 1.8 million adult dairy cows (DairyCo 2016). Calving interval depends on two components 1) interval from calving to conception and 2) the length of pregnancy (gestation period). As the pregnancy period is relatively constant at 280-285 days, the number of days open (target: 80-85 days) is the main principal determinant of the calving interval. Several factors contribute to this interval, which includes: 1) resumption of ovarian activity; 2) detection of oestrus; 3) ability to conceive and establish pregnancy; 4) abnormal ovarian cycles such as follicular cysts; and 5) management decision when to resume breeding.

The conception rate at first service in the UK is less than 40% (DairyCo 2010), and was estimated to be declining at a rate approaching 1 percentage point per year (Royal et al. 2000). There are two main reasons contributing to the decline of conception rates at first service: 1) ovarian and uterine inadequacy; 2) selection of animals for increased milk production. In early lactation, the dietary intake of cows does not meet their lactation requirements and consequently they enter a period of negative energy balance (NEB). This has a negative effect on both metabolic and endocrine systems. High yielding dairy cows are likely to have extended NEB, which will have further adverse effects on fertility (Webb et al. 2000). Reproductive performance in dairy cattle is dependent on the

interaction between environmental, management (i.e. accuracy of heat detection, use of proper insemination technique and herd health policies) and biological factors. Biological factors include involution of the cervix and uterus post-partum (Mateus et al. 2002, Zhang et al. 2010); age and parity (Hajurka et al. 2005, Melendez et al. 2004); hormone concentrations, nutrition (Wagner and Hansel 1969, Zhang et al. 2010); season (Kasimanickam et al. 2004, Noakes et al. 2009), and uterine infection (Sheldon 2004, Melendez et al. 2004).

Many production diseases have negative influence on the reproductive system in dairy cows. Uterine diseases are common after parturition in cattle and play a role in the reduced fertility of dairy cows (Sheldon et al. 2008). Usually, the uterus becomes contaminated with bacteria shortly after calving. In most cases, these bacteria are eliminated, however in up to 20% of animals, they persist and cause endometritis (Sheldon et al. 2009a). This then has a negative impact on the farm economics due to the direct cost of treatment, sub-fertility and decreased milk production. In the UK, the direct costs of uterine disease treatments and reduced milk yield of a cow were estimated to be approximately £72 per cow (Arnott et al. 2015). The prevalence of metritis/endometritis has been reported to be higher in high-yielding than low-yielding cows (Opsomer and de Kruif 2009). The exact cause for increased occurrence of uterine disease in high-yielding dairy cows is unknown. However, it has been suggested that the increased incidence is associated with greater NEB and the impaired neutrophil function in high-yielding cows (Hammon et al. 2006). This is supported by the observation of increased inflammatory response gene expression in the endometrium two weeks after calving in cows with severe NEB (Wathes et al. 2009). These studies indicate that NEB results in disturbed immune function, which leads to an inability of cows to eliminate bacteria from the uterus.

Uterine infection (i.e. metritis and endometritis) during the postpartum period is regarded as a major health issue in dairy cows. It is demonstrated that uterine disease of various forms contributes to declining fertility (Crowe and Williams 2012). It is likely that it has a direct effect on the uterus, but bacterial endotoxins and immune mediators produced in response to bacterial infection also inhibit pituitary LH secretion, suppress folliculogenesis (Sheldon et al. 2002b), reduce ovarian steroidogenesis and disrupt ovarian cyclicity (Opsomer et al. 2000, Mateus et al. 2002, Huszenicza et al. 1999). Several bacteria are recognised as uterine pathogens are *E.coli*, *Trueperella pyogenes, Fusobacterium*

necrophorum, Prevotella melaninogenica and *Proteus species*. These bacteria are associated with endometrial inflammation and can cause severe clinical uterine infection (Sheldon et al. 2002a, Williams et al. 2005).

Lipopolysaccharide is an endotoxin secreted from the outer layer of Gram-negative bacteria such as *E.coli*. Both LPS and immune mediators can inhibit pituitary LH secretion and suppress folliculogenesis, and reduce ovarian steroidogenesis, (Huszenicza et al. 1999, Mateus et al. 2002, Opsomer et al. 2000, Sheldon et al. 2002b, Williams et al. 2007). It has been reported that post-partum anoestrus and ovarian cysts were associated with uterine infection (Williams et al. 2007, Mateus et al. 2002). A few studies have investigated the relationship between progesterone profiles and clinical signs of uterine inflammation. These have observed an association between uterine infection and abnormal postpartum progesterone profiles (Huszenicza et al. 1999, Mateus et al. 2002, Opsomer et al. 2000, McCoy et al. 2006). This might be related to the ability of the uterine *E.coli* infection within the uterus to impair synthesis of the luteolytic prostaglandin F2α (PGF2α). Thus, it can prolong the luteal phase by switching synthesis away from PGF2α towards luteotrophic PGE2 (Williams et al. 2008a, Herath et al. 2009).

One of the challenges is the correct identification of the inflammatory status in the uterus, particularly in the field. The diagnosis of uterine infection can be performed by different methods such as bacteriological, histological and cytological examination. However, these methods are not always useful under field conditions because diagnosis and effective remedies should be taken immediately. For that reason, vaginal discharge examination is regarded as a simple, non-invasive method is often used under field conditions (Sheldon et al. 2006, LeBlanc et al. 2002). Purulent vaginal discharge can accurately reflect uterine infection and indicate presence of pathogenic bacteria in the uterus (Dohmen et al. 1995, Williams et al. 2005). However, not all vaginal scoring systems can differentiate between the different severities and are also limited to one type of uterine infection such endometritis (Dohmen et al. 1995, Huszenicza et al. 1999, Williams et al. 2005).

1.2 Reproductive tract of the cow

1.2.1 Anatomy of the uterus

The uterus of the cow is bicornuate and is characterised by a small uterine body (5cm) just anterior to the cervical canal and two long uterine horns (20-40cm long). The uterine horns are separated by the septum; they curve downwards and laterally, and then taper at the oviductal end. The uterus is suspended in the body cavity by broad ligaments that also contain the uterine artery that supplies the blood to the uterus (Fig.1.1).
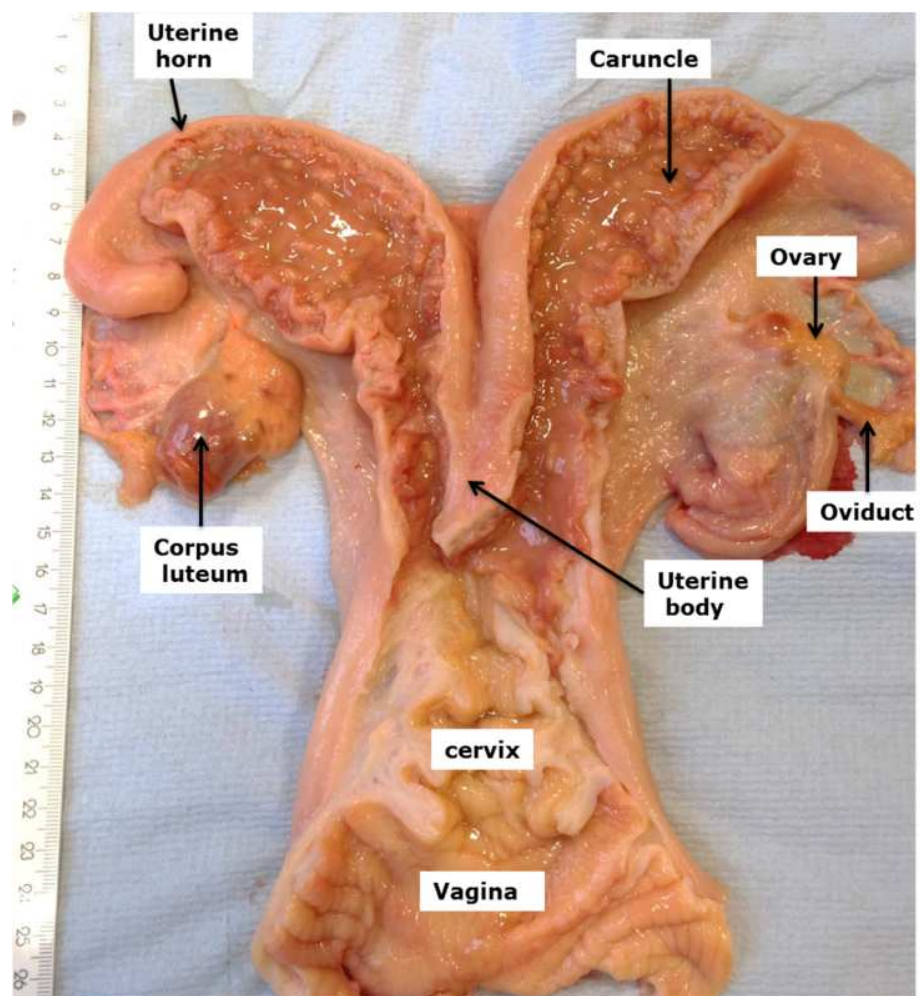
Figure 1.1: The reproductive tract in the cow.

1.2.2 Histology of the uterus

1.2.2.1 Endometrium

Luminal epithelium

The inner layer of the uterus is called the endometrium (Fig.1.2). The luminal epithelium of the endometrium is pseudostratified and/or simple columnar epithelium, adjacent to the uterine lumen. The morphology of these epithelial cells changes during the oestrus cycle. Namely, they become taller at oestrus and are more cuboidal during the luteal phase (Bacha and Bacha 2000).

Glands

Endometrial glands are continuously extensions from the luminal epithelium and penetrate deep into the endometrium. Thus, there are two types of endometrial glands according to their location: superficial glands, which located near to the luminal epithelium and deep glands which are located near to the myometrium. Glands are simple, coiled and branched tubular glands that are lined with both ciliated and non-ciliated simple, columnar epithelium and open out into the lumen (Hafez 1993, Bacha and Bacha 2000).

Caruncle

Caruncles are a particular feature of the ruminant uterus. They are characterised by oval or round thickenings in the uterine mucosa resulting from proliferation of sub-epithelial connective tissue. Caruncles are visible in the non-pregnant uterus (Fig.1.1). Furthermore, they are the sites that form attachments with fetal membranes. There are about 70-120 caruncles in the cow, arranged in 4 or 5 rows.

Stroma

The stroma of the endometrium is composed of tightly packed fibroblasts, which gives the structural support for the endometrium. The stroma also contain, lymphocytes and blood vessels (Hafez 1993, Bacha and Bacha 2000). Immune cells can also be detected in the stroma.

1.2.2.2 Myometrium

The myometrium is composed of two layers of smooth muscle layers of inner, thick circular and outer longitudinal muscle cells. Vascular layer with large

arteries, veins and lymph is located between the two layers (Hafez 1993, Bacha and Bacha 2000).
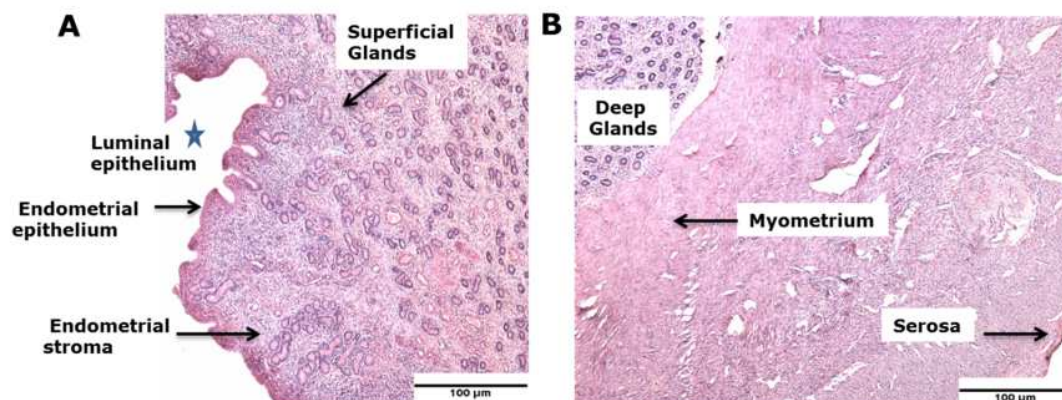
Figure 1.2: The bovine uterine horn cross section.

1.3 Bovine oestrus cycle

1.3.1 General overview

The reproductive cycle of non-primate female mammals is known as the oestrus cycle. One oestrus cycle is the period of the time between two successive oestrus (sexual receptivity) periods and the reproductive system is characterised by numerous physiological changes. Cows in oestrus have many clinical signs such as nervousness, standing to be mounted, vaginal mucus discharge, and acceptance of the bull for mating. Other secondary signs include bellowing, aggressiveness and restlessness (Senger 2003, Ball and Peters 2004). In the cow, the average oestrus cycle lasts 21 days with range 18-24 days, with day 0 is designated oestrus. The oestrus cycle is can be divided into phases as follows: (Noakes et al. 2009).

Pro-oestrus

Proestrus is the period just prior to oestrus, and typically occurs on days 17-20 of the oestrus cycle. It is characterised by declining progesterone concentrations due to corpus luteum regression and extends to the start of oestrus. The main characteristic of this stage is the occurrence of the rapid growth of the pre-ovulatory antral follicle, which is responsible for increasing circulatory oestradiol concentrations. Oestradiol induces numerous changes in the female reproductive

system in preparation for mating as well as induction of the behavioural changes of oestrus (O'Connor 1993).

Oestrus

Oestrus lasts on average 9-12 hours in dairy cows (Noakes et al. 2009). It is a very recognisable stage because of several clinical signs (e.g standing heat; bellowing, restlessness; mucus discharge in the vagina, chin resting and back rubbing) as well as physiological changes (e.g. luteinising hormone (LH) surge). In the cow, ovulation (release of the oocyte for fertilisation) takes place on average 28 hours after the LH surge, which occurs mid-oestrus (Noakes et al. 2009).

Metoestrus (post-ovulation)

It is the period after ovulation while the corpus luteum (CL) is forming. During metoestrus both oestrogen and progesterone are relatively low (Noakes et al. 2009).

Dioestrus (Luteal)

Dioestrus is the longest stage of the oestrus cycle. It starts from day 5-17, when the corpus luteum becomes functional and progesterone concentrations are rising (Senger 2003). This stage lasts until the CL is regressed in the absence of a pregnancy. High progesterone prepares the uterus to create a suitable environment for the conceptus and establish pregnancy. In terms of ovarian function, the oestrus cycle can also be divided into two phases: luteal phase and follicular phase. The luteal phase is about 14-18 days in length and CL is the dominant structure, and is characterised by high progesterone concentrations (Forde et al. 2011, Aerts and Bols 2010). The shorter follicular phase lasts approximately 4-7 days, when increasing pulsatile secretion of gonadotropin hormones stimulate the final maturation of the antral follicle. Oestradiol is the dominant hormone at this stage (Hafez 1993, Bearden and Fuquay 1992).

1.3.2 Hormonal control of oestrus cycle

Hormones are chemical substances that act as a messenger, and often convey the message between organs by the bloodstream controlling their activity. Hormones are secreted from various endocrine glands such as pituitary, thyroid, and adrenal glands. The complex regulation of the oestrus cycle in cows involves

interactions between the hypothalamus, pituitary gland, ovaries with an input from the uterus. The oestrus cycle is regulated by hormones secreted from the hypothalamus (gonadotrophin-releasing hormone; GnRH), anterior pituitary gland (follicle stimulating hormone [FSH] and luteinising hormone [LH]), ovaries (progesterone [P4]; oestradiol [E2] and inhibin) and uterus (PGF2α). These hormones exert their actions through a system of negative and positive feedback that ultimately governs the oestrus cycle (Webb et al. 1991, Forde et al. 2011). The interactions of these hormones are shown in Fig. 1.3.
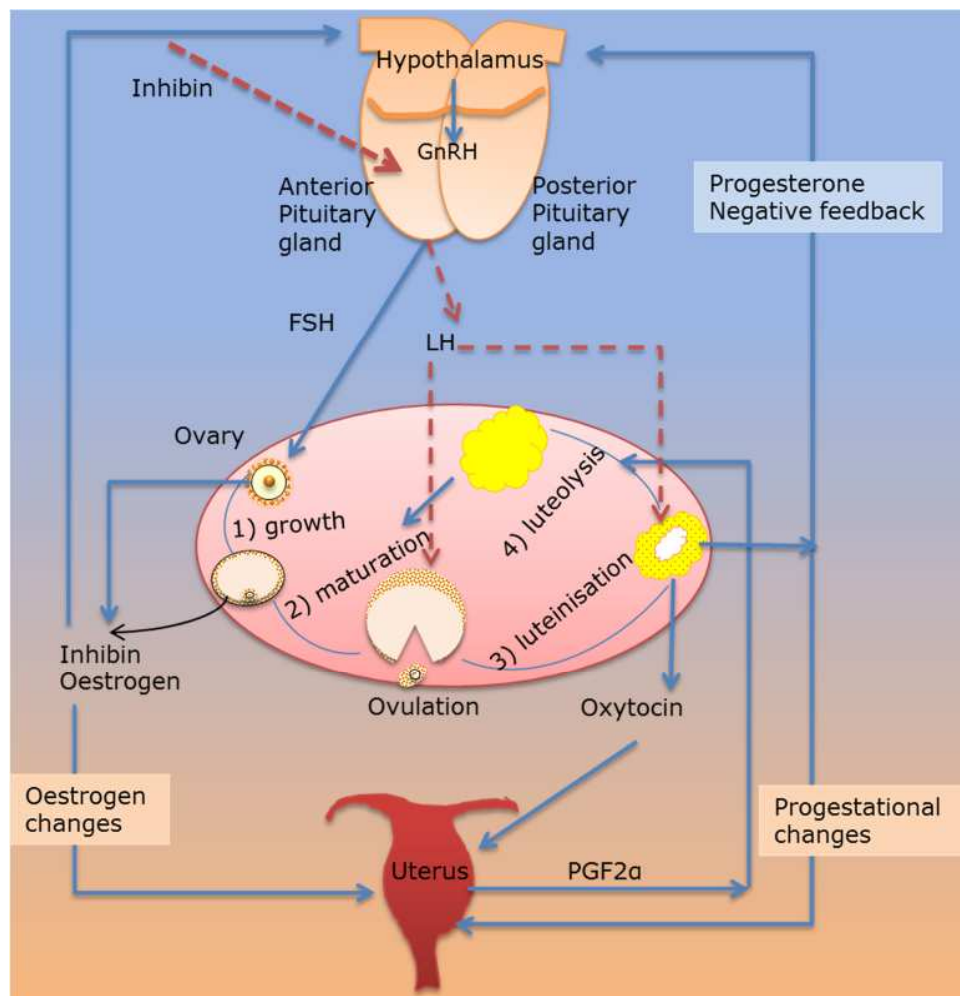
Figure 1.3: The summary of the hormonal control of the ovarian cycle. Adapted from (Ball and Peters 2004).

GnRH is released from the hypothalamus in a pulsatile manner and is responsible for the release of gonadotrophin hormones, FSH and LH from the anterior pituitary gland (Conn and Crowley 1994). FSH stimulates antral follicle

recruitment, which occurs in wave throughout the cycle, typically 2 or 3 per cycle (Fig. 1.4).
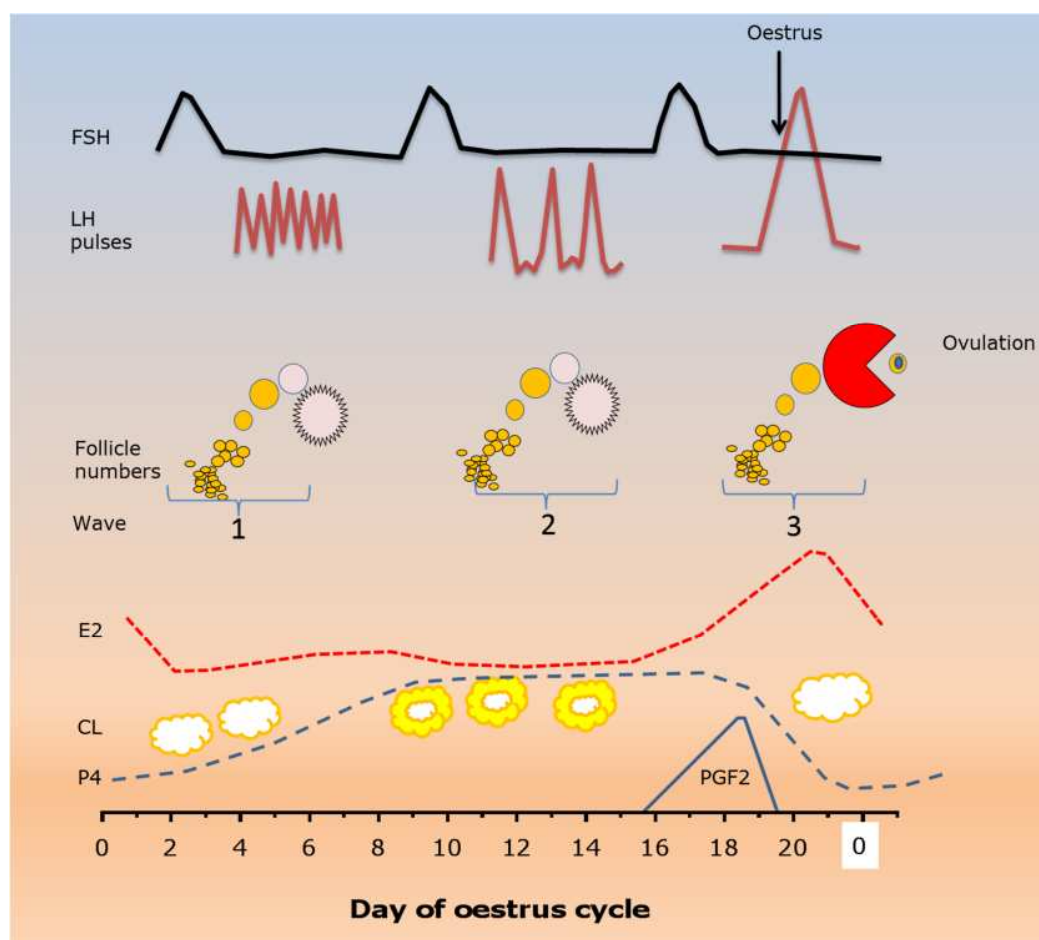
Figure 1.4: Hormonal changes during the bovine oestrus cycle. Follicle stimulating hormone (FSH) stimulates antral follicle recruitment in waves. While pulses of luteinising hormone (LH) stimulate the latter stages of (dominant) follicular development and is responsible for increasing oestradiol (E2) secretion by the dominant follicle. Increasing E2 levels positively feedback on the brain to induce oestrus behaviour and increasing LH pulse amplitude resulting in a surge of LH. This is the trigger for ovulation. Progesterone (P4) is produced by the corpus luteum (CL) during the luteal phase and levels steadily increase post-ovulation. In the non-pregnant cow, uterine derived prostaglandin-F2α (PGF2α) initiates CL regression and thus progesterone concentrations fall. This removes the negative feedback of progesterone on the hypothalamus enabling the cow to re-enter pro-oestrus. Adapted from (Garnsworthy et al. 2008).

During pro-oestrus, the gonadotrophins stimulate final maturation of the pre-ovulatory follicle, resulting in increased release of oestradiol. Oestradiol acts on oestrogen receptors in the surge and tonic centres of the hypothalamus to trigger the release of LH, when progesterone levels are low (Peter et al. 1985). The action of LH, is mediated by a membrane G protein-coupled receptor (LHCGR) present in the theca and granulosa cells of the ovulatory follicle (Ireland and

Roche 1982). LH binding to the LHCGR activates a G-protein (Gibori 1993), which leads to the production of cyclic AMP (cAMP) and phosphorylation of regulatory intracellular proteins (Catt and Dufau 1976). The pre-ovulatory LH pulses continue to stimulate the final development of the dominant follicle, which is associated with further increased oestradiol production. Above a certain threshold, positive feedback of oestradiol on the hypothalamic surge centre leads to increased GnRH pulsatile release and ultimately an LH surge that causes ovulation (Fig. 1.4). During the luteal phase of the oestrus cycle, the CL produces P4 whose concentrations increase post ovulation before reaching a peak around day 8-10. During the luteal phase, there is a negative feedback mechanism through which progesterone inhibits LH secretion via suppression of GnRH. In non-pregnant cows, the CL will regress as a result of PGF2α production from the uterus which causes a decrease in P4 concentration. This enables the final stages of follicular maturation to occur and the animal to return to oestrus (Webb et al. 1991, Webb and Campbell 2006, Garnsworthy et al. 2008). It is important to note that in the cow, both dominant follicles and CL can be present (at various stages of their development) on the ovary at the same time.

1.4 Folliculogenesis

There are approximately 450,000 primordial follicles present in each bovine ovary at birth, this number has declined to about 3000 when the cow reaches 15-20 years of age (Erickson 1966, Webb et al. 1991). The central dogma is that no new primordial follicles are produced after birth. Each primordial follicle composed of an oocyte (egg) surrounded by a single layer of flattened precursor- of granulosa cells. The process of follicular development and growth is called folliculogenesis (Fig. 1.5) and is controlled by gonadotrophins and local ovarian factors. The earliest stages are gonadotrophin independent and appear to be largely regulated locally.
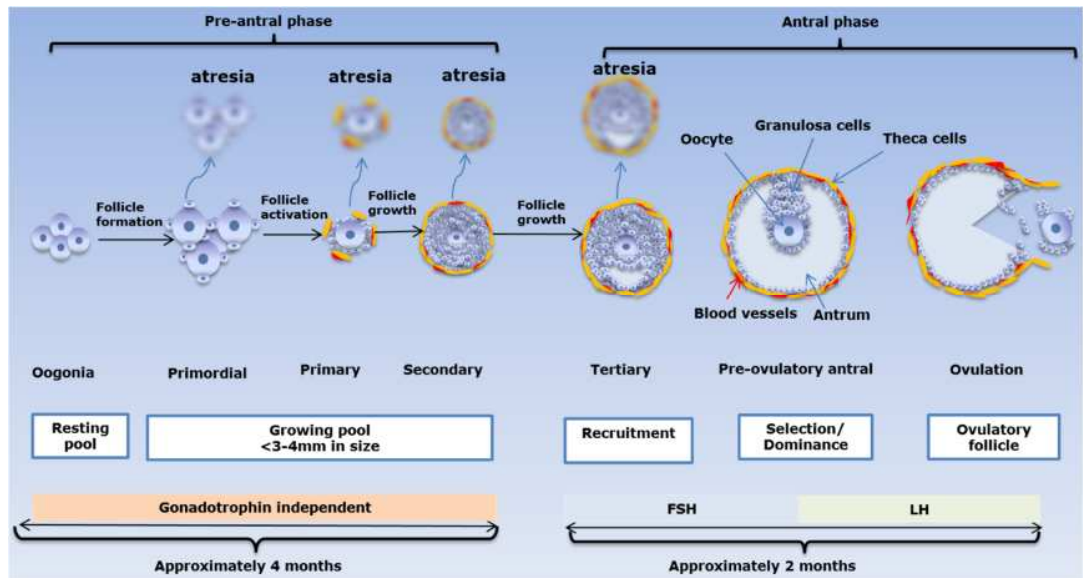
Figure 1.5: Schematic representation of the growth and maturation of the bovine follicle throughout folliculogenesis.

A cohort of primordial follicles from the resting pool is stimulated by intra-ovarian signals. This cohort will develop into primary (granulosa cells become cuboidal in shape), then pre-antral (increasing numbers of granulosa cell layers and recruitment of a theca layer from the stroma), and ultimately antral (formation of an antral cavity) follicles. It is these antral follicles that become pre-ovulatory and release the oocyte. In cattle, sheep and humans, this process takes about 6 months (Campbell et al. 2003). However, the majority of follicles will not develop to pre-ovulatory size but will become atretic and be lost. The process of atresia will involve apoptosis of both follicular cells as well as the oocyte (Chun and Hsueh 1998, Quirk et al. 2004). It has been indicated that the development of the pre-antral follicle is independent of gonadotropic stimulation, with paracrine control mechanisms having a primary role (Gong et al. 1996). However, FSH receptors (FSHR) have been detected in primary follicles and FSH stimulated growth of medium-sized pre-antral follicles *in vitro* (Gutierrez et al. 2000, Webb et al. 2002).

There are several factors that have been identified as important regulators for the development of pre-antral and antral follicles including: fibroblast growth factor 2 (FGF2), intra-ovarian peptides (activin and inhibin), epidermal growth factor (EGF), stem cell factor (SCF or kit-ligand: KL), transforming growth factor B (TGFB), bone morphogenetic protein-15 (BMP-15 or GDF-9B), vascular endothelial growth factor A (VEGFA) and insulin-like growth factor 1 (IGF-1) (Mihm et al. 2000, Berisha et al. 2000, Juengel et al. 2004).

1.4.1 Antral follicle

As follicular growth continues, granulosa cells increase in number and once about 6 layers thick, follicles will undergo the transition to the antral phase (Braw-Tal and Yossefi 1997). Follicles at this stage will develop a fluid-filled cavity known as an antrum, which then surrounds the oocyte. Further follicular growth is dependent on increasing granulosa cell number and follicular fluid volume. Granulosa-derived factors generate an osmotic gradient which draws in fluid from the thecal vasculature, resulting in the formation of follicular fluid and an antral cavity (Rodgers and Irving-Rodgers 2010). Follicular fluid has a similar composition to serum and is composed of steroid hormones, protein, proteogylcans, peptide hormones, insulin like growth factors (IGF) and their binding proteins and several other factors that determine the fate of the oocyte and follicle (Bao and Garverick 1998, Webb et al. 1998, Fortune 1994, Rodgers and Irving-Rodgers 2010). Moreover, it has been reported that follicles selected to grow to ovulatory size possess a more advanced microvascular network (Ferrara 2004).

1.4.2 Endocrine regulation of ovarian antral follicle development in cattle

In cattle, antral follicle growth occurs in two different phases; the first 'slow' growth phase spans the period from antrum acquisition to a size of roughly 3mm and the second 'fast' phase which is gonadotrophin-dependent. This phase includes cohort recruitment, selection and growth of dominant follicle, (DF) (Mihm and Bleach 2003) (Fig. 1.5), and the complete acquisition of ovulatory capacity (Mihm and Bleach 2003).

Increased circulating FSH concentrations constitute the essential stimulation for recruitment of an antral follicle cohort and emergence of a follicular wave (in bovine, two or three waves per cycle; (Adams et al. 1992, Fortune 1994). In cows, only one follicle is selected which then exerts dominance over the other antral follicles. It achieves that by secreting oestradiol and inhibin, which causes a decrease in plasma FSH concentrations, that is still required for the growth of any subordinate follicles (Ginther et al. 1996). In so doing, their support is removed and thus subordinate follicles will undergo atresia. When LH binds to LHCGR, it influences different types of activities in the follicle such as

steroidogenesis, follicular growth, maturation of oocyte and ovulation (Hyttel et al. 1997). For that reason, the presence of LHCGR on granulosa cells is essential for continued folliculogenesis from the acquisition of follicular dominance as well as ovulation (Beg et al. 2001, Ginther et al. 2001, Barros et al. 2009).

1.4.3 LH surge and ovulation

In the non-pregnant cow, uterine PGF2α initiates corpus luteum regression and a reduction in P4 concentrations. Subsequently it initiates the increased frequency of GnRH pulsatile secretion, which in turn initiates the production of low amplitude, high frequency LH pluses. These LH pulses support not only the continued growth of the large follicle but continued oestrogen production. The production of oestrogen is regulated under the activities of both LH and FSH. Namely androstenedione production by theca cell is stimulated by LH; while, FSH stimulates the aromatisation of androstenedione to oestradiol-17β act in granulosa cells. Subsequently, oestradiol acts on the hypothalamus to increase the release of GnRH pulse frequency, which produces high level of FSH and LH surge (Fig. 1.3). Furthermore, LH surge stimulates oocyte maturation and ovulation of the dominant follicle (Gordon 1996).

1.5 Development of the corpus luteum

Following ovulation, the corpus luteum (CL) develops from the ruptured follicle and forms a temporary endocrine gland. Critical to the formation of the CL is the LH surge. Proper development of this gland is essential for the maintenance of early pregnancy in mammals. Its main function is to produce and secrete progesterone. This acts on the uterus to support embryo development and the establishment of pregnancy (Mann et al. 1998, Mann and Lamming 2001, Green et al. 2005).

The CL is composed of luteinising steroidogenic theca and granulosa cells called small and large luteal cells, respectively. Immediately following ovulation, the tissue of the collapsed follicle folds in and mainly consists of luteinising granulosa cells adjacent to theca cells (Amselgruber et al. 1994). There are two major simultaneous process involved in luteinisation: extensive remodeling of the tissue leading to CL formation and the acquisition of luteal function. The first step involves cell proliferation and hypertrophy of the steroidogenic cells. Theca cells from the ovulated follicle undergoing proliferation and become small luteal cells

(Farin et al. 1986). The basement membrane between the theca interna and granulosa cells breaks down with the theca and vascular cells migrating into the luteinising granulosa cell layers. Eventually, they will be completely dispersed among luteinised granulosa cells. Simultaneously there is growth of an extensive vascular network (Amselgruber et al. 1994). Formation of these blood vessels occurs due to an increase of angiogenic factors, which are secreted form during the ovulatory process (Niswender and Nett 1994).

Corpus luteum formation involves the proliferation of non-steroidogenic cell populations such as endothelial cells (required for the formation of the luteal vascular network) alongside other cells like fibroblasts, pericytes and macrophages (Alila and Hansel 1984, Schams and Berisha 2004). On the other hand, the luteinisation of granulosa cells yields large luteal cells, which generally cease dividing but undergo massive hypertrophy (Alila and Hansel 1984, Schams and Berisha 2004). Hypertrophy of luteinising granulosa cells results in an increase in cellular size through altered expression of cytoskeletal components. The resulting structure usually protrudes from the ovary and is red-tan in colour (Peters and Ball 1995). Different studies have reported that the ability of luteinising granulosa cells transiently change tubulin expression and acquire the ability to express cytokeratin, vimentin and desmin (Khan-Dawood et al. 1996).

The mature CL is composed of about 30% steroidogenic cells and 30-40% are endothelial cells (Devoto et al. 2002). In domestic ruminants, other cell types have been identified including: fibroblasts, vascular smooth muscle cells, macrophages, lymphocytes, leukocytes and pericytes (Hansel et al. 1990, O'Shea et al. 1989). Pericytes are characterized as perivascular cells attached to endothelial cells and their branched cytoplasm surrounds newly formed blood vessels (Amselgruber et al. 1994).

In addition to the morphological changes seen during luteal development, numerous biochemical changes occur during luteinisation (Niswender and Nett 1994). The cytoplasm of both large and small steroidogenic cells accumulate abundant smooth endoplasmic reticulum and numerous mitochondria (Niswender and Nett 1994). This is associated with the initial increase of circulating progesterone (Niswender and Nett 1994). LH is the most important hormone that stimulates synthesis of progesterone in the bovine CL (Niswender and Nett 1994). The inhibition of LH pulses during metoestrus caused inhibition of luteal

development and reduced its ability to secrete progesterone. Both large and small steroidogenic cells contain LHCGR, which increased during CL development. However, only small luteal cells respond to LH in regards to progesterone production (Quintal-Franco et al. 1999).

The large luteal cells produce large amount of progesterone in the absence of LH stimulation (Quintal-Franco et al. 1999). However, LH is critical for progesterone production from the CL (Hansel and Blair 1996). This is partially due to LH stimulating the expression of *cytochrome P450 side chain cleavage* (CYP11A) mRNA which is then maintained in the absence of cAMP-stimulating agents (Juengel et al. 1994). Large luteal cell also highly express *steroid acute regulatory protein* (STAR) mRNA. STAR expression was stimulated by IGF-1 (Amselgruber et al., 1994), through an autocrine and endocrine manner (Sauerwein et al. 1992).

1.5.1 Cellular content of the corpus luteum

The array of different cell types in the CL interact together to achieve normal function during growth, differentiation and regression of the CL (Niswender et al. 2000, Grazul-Bilska et al. 1997, Reynolds et al. 2000). These interactions are mediated by both contact-dependent (e.g. via gap junctions) (Reynolds and Redmer 1998), Notch signaling (Vorontchikhina et al. 2005) and contact-independent mechanisms, e.g. secretion of growth factors (Schams and Berisha 2002). This section will provide basic knowledge on these different luteal cell types.

1.5.1.1 Steroidogenic cells

Large luteal cells

Large luteal cells (LLC) range from 24 to 38 μm in diameter (O'Shea et al. 1989, Lei et al. 1991) and represent about 40% of the total volume of the luteal tissue (O'Shea et al. 1989). However, these cells only represent about 10% of the total number of luteal cells (O'Shea et al. 1989). Morphologically, these cells are characterised by a spherical shape with a spherical nucleus and contain extensive rough and smooth endoplasmic reticula, abundant mitochondria and secretory granules (Wiltbank 1994).

Small steroidogenic cells

Small luteal cells (SLC) represent about 25-27% of the total number of cells and about 20-30% of the luteal volume. These cells differ from large luteal by their much smaller diameter (16-18 µm) (O'Shea et al. 1989). These cells also contain abundant mitochondria and smooth endoplasmic reticulum, but contain less rough endoplasmic reticulum and secretory granules compared to large luteal cells (Wiltbank 1994). In addition, their nuclei are irregular with cytoplasmic inclusions and more lipid droplets compared to large luteal cells (O'Shea et al. 1989).

1.5.1.2 Non-steroidogenic cells

Endothelial cells

Endothelial cells (EC) represent the vast majority of non-steroidogenic cells in the CL (50% of the total cell number) (O'Shea et al., 1989). These cells express neural cell adhesion molecule (NCAM), which could mediate interaction between EC and LLC, which express NCAM. This functional adhesion between EC and LLC has been suggested to stimulate progesterone production by large luteal cells (Girsh et al. 1995). Endothelial cells are much smaller than large and small luteal cell with a diameter of 10-11 µm. Morphologically, EC are characterised by an elongated and slender shape, with a large nuclear to cytoplasmic ratio, small number of organelles and a distinct basal lamina (Wiltbank 1994). The cells are involved in vasodilation, vasoconstriction, angiogenesis and immune cell passage into tissue (Michiels 2003).

Pericytes

Pericytes interact with EC through discontinuities in the shared basement membrane. Morphologically, pericytes are composed of a cell body, prominent nucleus, and small content of cytoplasm with many long processes. They are located along the basement membrane of micro vessels (Mandarino et al. 1993). Pericytes communicate with endothelial cells by direct physical contact and paracrine signaling pathways. Pericytes stabilise newly formed endothelial tubes, modulate blood flow and vascular permeability as well as EC migration, differentiation and branching (Carmeliet 2003).

In the CL, pericytes produce angiogenic factors such as vascular endothelial growth factor A (VEGFA) (Redmer et al. 2001). Interestingly, ECs degenerate during luteal regression but the number of pericytes remain high (Redmer and Reynolds 1996). They play a role in both regulation of tissue remodeling and in maintaining the integrity of large blood vessels by allowing structural luteolysis to occur.

Vascular smooth muscle cells (SMC)

In contrast to pericytes, SMCs are not embedded in the basement membrane and might not have direct contact the endothelium (Gerhardt and Betsholtz 2003). During angiogenesis, SMCs exhibit very high rates of proliferation, migration and synthesis of extracellular matrix components including: collagen, elastin and proteoglycans that compromise a major portion of the blood vessel wall. At the same time, these cells acquire contractile abilities (Owens et al. 2004). Likewise, in response to a vascular wound, the SMC undergo a high level of cell proliferation, migration, and synthetic capacity. Thus, they play a fundamental role in wound and vascular repair (Owens et al. 2004).

Fibroblasts

Fibroblasts are 10-12 µm in diameter and represent about 6-9% of the luteal volume and 10-20% of the total number of cells (O'Shea et al. 1989). These cells play an important role in the maintaining the structural framework of the corpus luteum.

Immune cells

Immune cells, lymphocytes and macrophages are all observed in the bovine CL (Lobel and Levy 1968) particularly evident from 14 days onwards around the time of luteal regression. The function of immune cells during CL regression is thought to be destruction of the luteal tissue through different processes, including phagocytosis (Paavola 1979). However, immune cells may have another active role in controlling the lifespan and function of the CL (Adashi 1990). There has been specific attention to tumor necrosis factor α (TNFA), because it is cytotoxic for tumors cells. Similar processes occur during luteal tissue regression (Ji et al. 1991). Indeed, TNFA has also been detected in the bovine CL undergoing regression (Shaw and Britt 1995).

1.6 Angiogenesis

Angiogenesis is defined as a complex process by which new blood vessels grow from pre-existing ones. In addition, it is a physiological process involving the proliferation and migration of endothelial cells, and plays an important role in normal growth and tissue development (Klagsbrun and D'Amore 1991). In fact, rapid CL growth is associated with intense vascular growth (Zheng et al. 1993). Angiogenesis is a complex process, which requires the breakdown of walls of existing blood vessels, which starts the migration, and proliferation of endothelial cells to form the new blood vessels. For this to occur, the extracellular matrix requires to be degraded (e.g by matrix metalloproteinases (MMPs)) that consequently facilitates EC migration and proliferation (Klagsbrun and D'Amore 1991, Bendeck 2004).

New blood vessels are composed of endothelial cells with one or more surrounding layers of supporting cells including pericytes or smooth muscle cells (Chantrain et al. 2006). These supporting cells (pericytes) have actually been found at the end tips of endothelial sprouts and once the new vessels are formed these cells then start to extend along the length of these vessels and stabilize them (Bergers and Song 2005, Hall 2006, Raza et al. 2010, Robinson et al. 2009). Angiogenesis also involves the creation of a capillary lumen and differentiation of these newly formed vessels into arterioles or venules (Klagsbrun and D'Amore 1991). Angiogenesis occurs infrequently in the normal adult, except in the female reproductive system. This includes during follicular development, the formation of the CL, and in the placenta during pregnancy. It also occurs as a part of the body's repair process, for instance, wound healing which has parallels to the ovulatory response. However, uncontrolled neovascularization is often pathological. For example, in tumours, the growth of tissue depends on neovascularisation while in diabetes, blindness can result from retinopathy and vascularisation of the retina (Folkman 1972).

1.6.1 Angiogenesis and the function of corpus luteum

During early luteal development, the blood flow starts slowly but new blood vessels are highly permeable until a new basement membrane is formed and pericytes cover the length of the proliferated endothelial cells (Amselgruber et al.

1994). Capillary sprouting depends on the coordinated growth of both pericytes and endothelial cells (Amselgruber et al. 1994).

The rate of vascular growth in the luteal tissue is greatest in metoestrus and by the mid-luteal phase the mature CL is highly vascularized (Redmer and Reynolds 1996). Up to 85% of proliferating cells during luteal development are endothelial cells (Redmer and Reynolds 1996). Vascular development of the luteal tissue during its development has been quantified using immunlocalisation for von Willebrand Factor (VWF) (Robinson et al. 2008). It has been observed that most microvessels were found in the thecal-derived areas in the early CL but numerous capillary sprouts were observed among luteinising granulosa derived steroidogenic cells (Zheng et al. 1993). The mid-CL was composed of numerous endothelial cells forming capillaries, which form a dense network surrounding luteal steroidogenic cells (Zheng et al. 1993). Indeed, many luteal steroidogenic cells were immediately adjacent to at least one capillary. Similar observations have been reported in the marmoset CL (Young et al. 2000). However, Young et al. (2000) also observed endothelial cell proliferation during luteal regression, suggesting that these proliferate to from the large blood vessels in order to remove away the cellular debris of regressed particles. They observed that luteal angiogenesis did not change until functional regression was terminated and progesterone concentrations have decreased to follicular phase levels (Young et al. 2000).

Luteal angiogenesis is rapid, being established within days of ovulation (Amselgruber et al. 1999) and is summarized in Fig. 1.6. After ovulation, the blood vessels of the ruptured follicular wall rapidly grows in the CL, which reaches its maturity from about 10 to 12 days. The main function of the CL is to produce progesterone, with plasma progesterone concentrations used as an indicator of luteal function (Niswender and Nett 1994, Reynolds et al. 1994). The establishment and remodelling of a complex ovarian vascular system plays a crucial role in the development and function of the CL (Robinson et al. 2009).
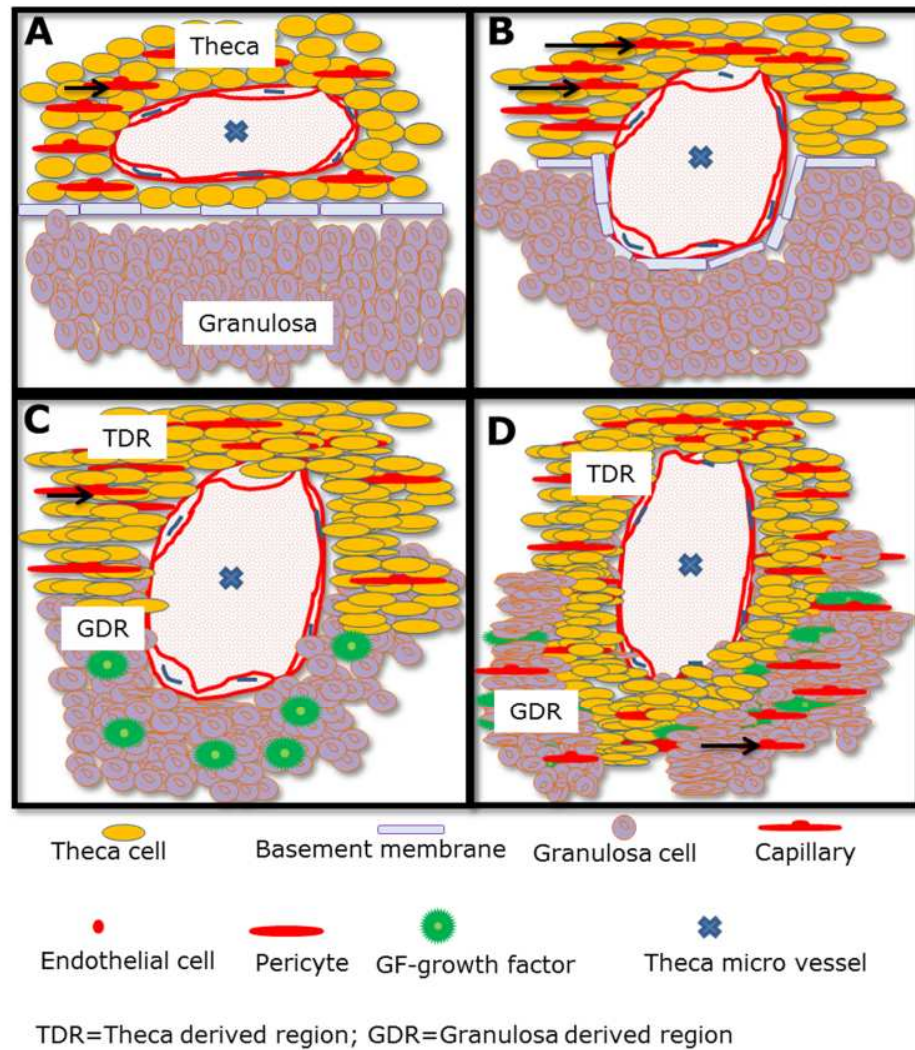
Figure 1.6: Corpus luteum development and its vascularisation. A) In the pre-ovulatory follicle, the extensive vascular network (endothelial cells, pericytes) is localised to theca layer (arrow). The thecal micro-vessels are separated from the granulosa layer by a basement membrane. B) Prior to ovulation, theca microvessels dilate, invaginate into the basement membrane and expand into the granulosa layer. C) During ovulation, the endothelial cell basement membrane degrades due to action of proteases. Pericytes start to migrate from thecal area towards the granulosa region under the influence of granulosa-derived growth factors. D) During CL development, pericytes stimulate migration and sprouting of endothelial cells from thecal area (Reynolds et al. 2000, Fraser and Wulff 2003, Robinson et al. 2009).

This is critical for the supply of nutrients, oxygen, hormonal support and facilitation of the release of steroid hormones (Robinson et al. 2009). In addition, the vascular network will facilitate the transport of progesterone precursors to

the luteal cells (Reynolds et al. 1994). The differentiation and maturation of a functional CL is based on a well-developed capillary bed (Amselgruber et al. 1994). During the early luteal phase, progesterone concentrations in the blood mirror luteal blood flow increases (Acosta et al. 2003) which highlights the importance of angiogenesis for CL function.

1.6.2 Stimulation of angiogenesis

Several studies have indicated that in many tissues (e.g tumours), hypoxia is the stimulus for the synthesis of pro-angiogenic factors, in particular vascular endothelial growth factor A (VEGFA). This then subsequently initiates angiogenesis. However, there is evidence that luteal angiogenesis is, at least in part, up-regulated by LH (Fraser and Wulff 2003, Berisha et al. 2006, Robinson et al. 2009). For example, in cows, follicular fibroblast growth factor (FGF) 2 mRNA and protein concentrations were significantly increased after LH surge (Berisha et al. 2006). In most *in vitro* studies, VEGFA production by granulosa cells was stimulated by LH or hCG in cows (Schams et al. 2001) and primates (van den Driesche et al. 2008, Martinez-Chequer et al. 2003). In addition, IGF-1 has also been shown to simulate VEGFA secretion by bovine granulosa cells (Schams et al. 2002).

There are several factors that regulate angiogenesis with the FGF family (Presta et al. 1989), VEGFA (Ferrara et al. 2003) and PDGF (Presta et al. 1989) being the most important. However, other modulatory factors have also been shown to stimulate angiogenesis including angiopoietins (ANGPT1 and ANGPT2), hypoxia-inducible factor-1α (HIF1A), and inhibitory factors including TNFA and TGFB (Yancopoulos et al. 2000, Folkman and Shing 1992).

1.6.2.1 Fibroblast growth factor 2 (FGF2)

In the bovine CL, fibroblast growth factor (FGF2) seems to be more dominant factor at stimulating endothelial cell growth and is most likely the major pro-angiogenic factor (Klagsbrun and D'Amore 1991). Expression of FGF2 protein was increased during follicular-luteal transition in the cows, suggesting that FGF2 plays a key role in the initiation of angiogenesis (Robinson et al. 2007). While VEGFA expression was unaffected over the same time period. FGF2 has been shown to induce angiogenesis by stimulating endothelial cell production of collagenase and plasminogen activator (Klagsbrun and D'Amore 1991). The

release of these proteases which are able to degrade the basement membrane and induce migration of capillary endothelial cells. However, this factor is different from most other angiogenic factors, in that FGF-2 is not a typical secreted protein and must, therefore, be released by alternative mechanisms, perhaps from cellular storage as a result of cell lysis (Klagsbrun and D'Amore 1991).

FGF2 has a fundamental role in the stimulation of endothelial cell sprouting and formation of luteal EC networks *in vitro* (Laird et al. 2013). This was demonstrated utilising an *in vitro* luteal angiogenesis culture system, in which endothelial cell network formation was inhibited by adding FGF receptor inhibitor (Woad et al. 2009). Furthermore, FGF2 was regulated during the follicular–luteal transition, which localised in thecal endothelial cells and vascular pericytes before the LH surge (Berisha et al. 2006). However, after the LH surge for about 2 days, FGF2 was localised dominantly in the luteinising granulosa cells, this is may be important for maintenance of granulosa cells (Berisha et al. 2006). The most striking effect of angiogenesis was seen when VEGFA and FGF2 were used together to induce angiogenesis *in vitro* (Pepper et al. 1992).

1.6.2.2 Vascular endothelial growth factor A (VEGFA)

Formerly, VEGFA was originally known as vascular permeability factor due to its capability to cause leakage and induce fenestrations in the endothelial cells of capillaries (Senger et al. 1983), vascular endothelial cell protease production, proliferation and migration. Moreover, it is a potent stimulator of angiogenesis in the follicle and CL in many species. Furthermore, *VEGFA* mRNA increased dramatically during both normal and pathological angiogenesis such as wound healing, fracture (Reynolds and Redmer 1998) and tumour growth (Ferrara and Davis-Smyth 1997).

It has been demonstrated that the *VEGFA* mRNA expression was temporally and spatially related to angiogenesis in the ovary (Phillips et al. 1990, Ravindranath et al. 1992). It is a potent mitogenic for endothelial cells (Senger et al. 1993, Connolly 1991). The action of VEGFA is mediated through two receptors, VEGFR-1 and VEGFR-2. It has been shown that CL development is related to intense angiogenesis which is influenced by VEGF-A and its receptor VEGFR-2 (Hünigen et al. 2008). In the cow, local immunoneutralisation of VEGFA decreased luteal development and progesterone production (Yamashita et al. 2008) and the

inhibition of VEGFA signalling resulted in inhibition of the formation of endothelial networks *in vitro* (Woad et al. 2009).

1.6.2.3 Influence of bacterial endotoxins

Bacterial lipopolysaccharides have been shown to induce angiogenesis in several tissue systems. LPS directly stimulated endothelial cell sprouting and increased angiogenesis *in vitro* through the activation of tumour necrosis receptor associated factor-6 (TRAF6) dependent signaling pathways (Pollet et al. 2003). Moreover, VEGFA protein and mRNA expression in macrophages was markedly increased by LPS in cirrhotic patients (Pérez-Ruiz et al. 1999). However, other studies found that bacterial LPS causes downregulation of VEGFA in endothelial cells and delayed wound healing (Power et al. 2001).

1.7 Progesterone synthesis

Progesterone is the principal steroid hormone produced by the bovine CL. There are several source of cholesterol (the precursor of progesterone) within luteal cells including free cholesterol found in the membrane (Wiltbank et al. 1990) and esterified cholesterol deposited in cytoplasmic lipid droplets (Farin et al. 1986, Rennert et al. 1993) (Fig. 1.7). Esterified cholesterol is delivered to the bovine luteal cell via high density lipoproteins (HDL) and to a smaller extent via low density lipoproteins (LDL) (Savion et al. 1982). The HDL/LDL cholesterol complex attaches to specific receptors present on the outer membrane of the luteal cells before entering them. Production and secretion of progesterone by the CL involves two key enzymes and other regulatory proteins. The key rate-limiting step in synthesis of progesterone is the conveyance of cholesterol from the outer to the inner mitochondrial membrane. This is facilitated by steroid acute regulatory protein (STAR). Cytochrome P450 side chain cleavage (CYP11A) is located in the inner mitochondrial membrane and converts cholesterol to pregnenolone. While, 3β-hydroxysteroid dehydrogenase (HSD3B), which is found in smooth endoplasmic reticulum, converts pregnenolone to progesterone (Rekawiecki et al. 2008). In the cow, the luteal secretion of progesterone is stimulated by LH and PGE2 *in vitro* (Rekawiecki et al. 2005). This was explained by the increased STAR, CYP11A, and HSD3B protein expression after treatment. Furthermore, LH is responsible for an increase in the number of intracellular organelles including mitochondria, smooth endoplasmic reticulum and lipid droplets (Gibori 1993).
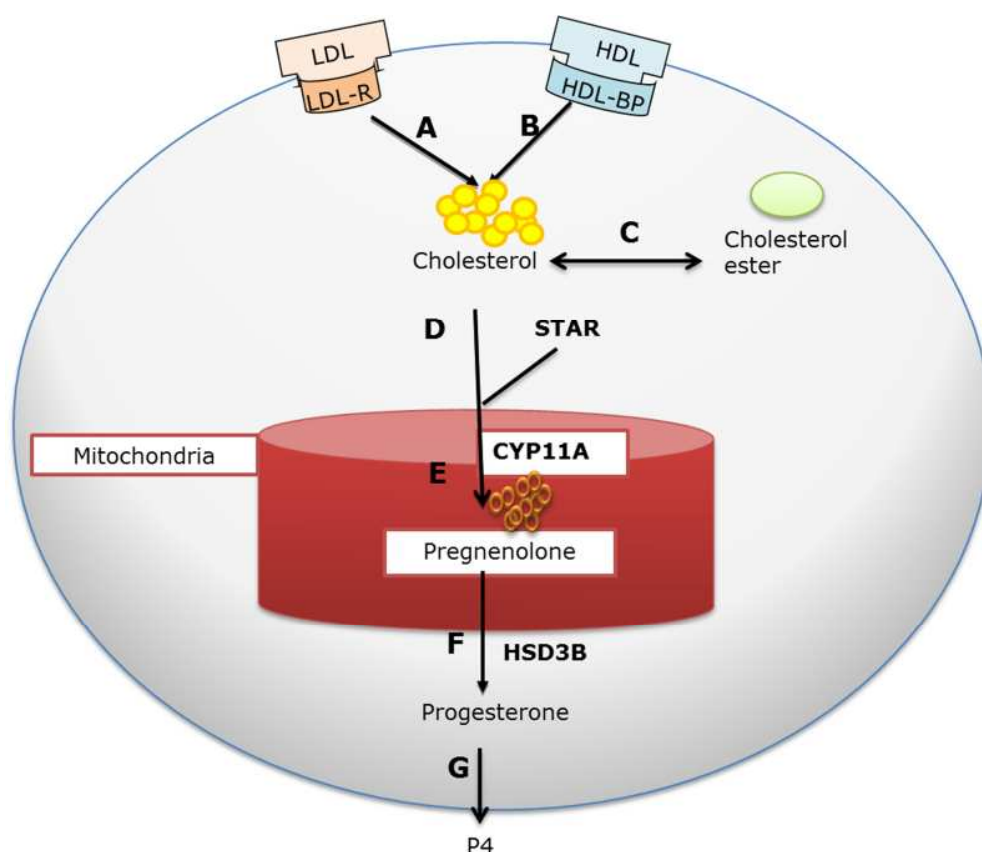
Figure 1.7: Progesterone synthesis pathway in a luteal cell. There are three sources of cholesterol: A) High density lipoprotein (HDL), B) Low density lipoprotein (LDL), C) Hydrolysis of stored cholesterol esters by cholesterol esterase. D) Free cholesterol then transported to inner mitochondrial membrane, which is stimulated by steroidogenic acute regulatory protein (STAR), E) cholesterol is converted to pregnenolone by cytochrome P-450 chain cleavage enzyme, CYP11A before being transported back out of mitochondria, F) In the smooth endoplasmic reticulum, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B); G) Then progesterone appears to diffuse from luteal cell. Adapted from (Niswender et al. 1994).

Steroid acute regulatory protein (STAR)

The action of STAR is mediated thorough the interaction between of its C-terminus with the relatively sterol-rich outer mitochondrial membrane. This causes cholesterol to pass from the outer membrane and transfer to inner membrane (Fig. 1.7). STAR remains within the mitochondria during this process therefore, continued synthesis of STAR is important and is required for sustaining steroidogenesis (Alpy and Tomasetto 2005). In addition, it has been suggested that post-or co-translational modification of STAR may increase the action of newly formed STAR protein (Strauss et al. 2003). LH-induced increases in

intracellular cAMP concentrations are important for protein kinase A (PKA) activation and increasing STAR activity through phosphorylation of STAR and this will lead to increased progesterone production (Rekawiecki et al. 2005).

Cytochrome P450 side chain cleavage enzyme (CYP11A)

The cleavage of the side chain from cholesterol to form pregnenolone on the inner mitochondrial membrane is catalysed by CYP11A. CYP11A is induced during luteinisation (Meidan et al. 1992) but its transactivation is repressed by activation of the protein kinase C (PKC) pathway (Moore et al., 1990). PGF2α appears to inhibit secretion of progesterone by ovine large luteal cells *in vitro* through the protein kinase-C (PKC) pathway (Wiltbank et al. 1989), Fig. 1.7.

3β-hydroxysteroid dehydrogenase (HSD3B)

3β-hydroxysteroid dehydrogenase is located in the endoplasmic reticulum and mitochondria, where it catalyses 3β-hydroxysteroid dehydrogenation and Δ^5- to Δ^4-isomerisation of the Δ^5-steroid precursors (Lorence et al. 1990). Pregnenolone formed by CY11A diffuses out of the mitochondria and then is transported to the smooth endoplasmic reticulum (SER), where pregnenolone is converted to progesterone by the action of 3β-HSD (Niswender et al. 1994), Fig. 1.7.

1.8 Luteolysis

In the cow, luteolysis is initiated around day 16 of the oestrus cycle in the absence of a sufficiently developed embryo. Oxytocin secreted from the CL and/or posterior pituitary gland acts on oxytocin receptors (OXTR) in the endometrium to induce pulsatile PGF2α secretion. Then, PGF2α enters the uterine vein, and as a result of counter current is transferred to the uterine/ovarian artery and PGF2α diffuses to the ovary. It is then when it causes luteolysis (McCracken et al. 1999) by acting on large luteal cells (Wathes and Lamming 1994, Flint et al. 1994). Luteolysis involves apoptosis, the infiltration of immune cells, and ultimately reduction in the blood flow to the CL (Niswender et al. 1994). There are some histological changes, which occur in the regressing CL including thickening of the arterial wall, a decreased number of granules in the cytoplasm and peripheral vacuolation of the large luteal cells. When the luteal cells decrease in size, the CL decreases in size, becomes pale in colour and is

known as a corpus albicans (Jainudeen and Hafez 1993). This is associated with a rapid decline in progesterone concentrations.

1.9 Post-partum reproductive performance

The productivity and sustainability of any dairy cattle enterprise depends on efficient reproductive performance (Radostits et al. 2006). To maintain this enterprise, dairy cows should have regular oestrus cycles, conceive at an optimal time and deliver a healthy calf each year (Hare et al. 2006). Any fertility and other reproductive problems during the postpartum period are crucial factors in cull making decisions (Radostits et al. 2006). In the USA, about 20% of dairy cows are culled yearly due to poor and inadequate reproductive performance (McDougall 2006). Dairy herd reproductive performance indices provide an indication of reproductive abnormality or problems and make it possible for strategic treatments to be introduced (Radostits et al. 2006). Unfortunately, reproductive performance indices and their targets vary from country to country and local trends should be considered when interpreting reproductive targets in dairy enterprises (McDougall 2006, Radostits et al. 2006).

Inadequate reproduction in dairy cows results in excessively long lactations where milk production steadily declines, or prolonged periods with no milk production at all. Such situations are costly to the dairy farmer, and may require extensive replacement heifers to maintain a stable herd size (McDougall 2006, Radostits et al. 2006). Establishing a successful reproductive monitoring programme requires the veterinarian to work with the dairy producer. It also requires intense monitoring of farm's management e.g. feeding, housing, cow comfort and keeping records (McDougall 2006, Radostits et al. 2006). The design of this programme can generate successful breeding in cows and a diminished involuntary culling rate due to reproductive inefficiency (Hare et al. 2006, Radostits et al. 2006). There are numbers of indices been used to measure fertility and reproductive efficiency of dairy enterprise, but the most commonly been used is calving interval (Hare et al. 2006, Radostits et al. 2006).

1.9.1 Uterine and cervical involution

Involution of the uterus and cervix is the process of returning these structures to a state capable of supporting a new pregnancy. The exact time required for uterine involution is difficult to evaluate (Kindahl et al. 1999). Generally, it takes

place within three weeks after calving, but usually it is not completed until 4-5 weeks postpartum. It can be assessed by rectal palpation and ultrasonography (Kindahl et al. 1999). After calving, pregnancy re-establishment depends on the system returning to its pre-gravid condition (Mateus et al. 2002). Delayed uterine involution has negative impacts on the economy of dairy farms (Zhang et al. 2010). The activity of the ovaries during the postpartum period requires the resumption of ovarian follicular activity. In dairy cows, a delay in resumption in ovarian activity has adverse effects on the calving to conception interval (Ramakrishnan and Dhami 2012). In contrast, a delay in uterine involution was associated with the endometrial damage and a negative impact on the ability to conceive (Gilbert et al. 2005). The mechanism of uterine involution varies among domestic species and mainly depends on the type of placentation. The placentation in cattle is cotyledonary (Hafez 1962). Several events happen after uterine involution including continued contraction of the myometrium (Hirsbrunner et al. 2002, Kindahl et al. 1999, Noakes et al. 2009), physical shrinkage, atrophy of glandular tissue and muscle cells, tissue necrosis and shedding it and regeneration of new endometrium (Levkut et al. 2002). The size of the uterus rapidly decreases due to constriction of the blood vessels and contractions of the myometrium (Leslie 1983). At the same time as uterine involution, the uterus is often contaminated with bacteria (Azawi 2008). This will stimulate the local innate immunity for clearing and repairing the uterine infection.

Casida et al (1968) defined uterine involution as complete when the uterus returned to its normal non-pregnant position and when the two horns were similar in diameter and showed normal consistency and tone. However, for many cows the uterus cannot be palpated easily during the first three weeks post-partum, so diameter of the cervix may be a more useful indicator of the involution process. The involution of the cervix is slower and it is completed by day 30 postpartum (Leslie 1983).

1.9.2 Factors that delay uterine involution

It has previously been reported in many studies that the time of uterine involution can be affected by several factors. Generally, uterine involution takes a few days longer in multiparous cows than in primiparous cows (Hajurka et al. 2005, Leslie 1983, Melendez et al. 2004, El-Din Zain et al. 1995, Noakes et al.

2009). However, the biggest factor is endometritis, which can have a large negative impact on the time for uterine involution. This is discussed in more detail in the following sections.

1.9.3 Pathogenic bacterial infection of the uterus

After calving, the uterine environment enhances the growth of a variety of aerobic and anaerobic bacteria. However, the most pathogenic bacteria causing uterine infection are believed to be *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum* and *Prevotella species*. It has been show that some bacteria, including *T. pyogenes, F. necrophorum* and *Prevotella* act synergistically to increase the severity uterine disease (Ruder et al. 1981, Olson et al. 1984). The most prevalent pathogenic bacteria in the reproductive tract during postpartum period in cows are *E.coli* (37%) and *T. pyogenes,* (49%) (Williams et al. 2005). Often infections with *E.coli* appear to precede and pave the way for *T. pyogenes* infection (Williams et al. 2007). The endometrial pathogenic *E. coli* are different from diarrhoeic or extra-intestinal pathogenic *E. coli* (Sheldon et al. 2010). This is because these bacteria are more adherent and invasive for endometrial epithelial and stromal cells, compared with *E. coli* isolated from the uterus of clinically unaffected animals (Sheldon et al. 2010).

1.9.4 Contamination and elimination of bacteria from the postpartum uterus

During pregnancy, the vulva, vestibule, and vagina and in particular the cervix act as anatomical barriers to prevent bacteria entering the uterus. During parturition, the cervix will dilate which allows for the entrance of bacteria into the uterus (Sheldon 2004, Azawi 2008), for that reason, bacterial contamination of the uterus postpartum is common. It has been reported that 80–100% of animals have bacteria in their uterine lumen during the first 2nd weeks after parturition. While immune responses increasingly remove the bacteria, still more than 40% of animals still have bacterial infection in the 3rd week after parturition (Sheldon et al. 2008).

1.10 Postpartum uterine infection

1.10.1 Metritis

Metritis is the severe inflammation of all layers of the uterus (endometrium, submucosa, muscularis and serosa). It is characterized clinically by delayed uterine and cervical involution, pyrexia (≥39.5°C) and a fetid watery purulent discharge. It occurs within the first 21 days postpartum (Sheldon and Dobson 2004). The incidence of metritis varies between studies often due to different exact definitions of the disease. In North America, the incidence of metritis in dairy cattle ranged from 10 to 20% (Dubuc et al. 2010, Overton and Fetrow 2008). The main risk factors for metritis included retained fetal membrane, dystocia, stillbirth, twins, NEB, abortion, milk fever, and deficiency in hygiene (Correa et al. 1993, Dubuc et al. 2010). Out of these, retained placenta was the major risk factor with 30 to 50% of metritis cases also having retained placenta (LeBlanc 2008). It is likely that the fetal membrane becomes necrotic and provides an ideal media for bacterial growth, if it remains inside the uterus for more than 24 hours (Sheldon 2004).

1.10.2 Endometritis

Endometritis is regarded as an inflammation of the superficial layers of the endometrium, and only involves the epithelial surface and underlying glandular tissues (Azawi 2008). Endometritis is often not associated with clinical systemic signs (Gunduz et al. 2010). It can be further divided into (1) Clinical endometritis which is characterized clinically either by the presence of purulent vaginal discharge at three weeks or more postpartum, or mucopurulent discharge 26 days after calving (LeBlanc 2008, Sheldon et al. 2008); (2) Subclinical endometritis has no clinical signs and is only diagnosed in the laboratory by endometrial cytology (Sheldon et al. 2008). Namely, 21 to 33 days after calving, when there is >18% of neutrophils detected in the endometrium or >10% of neutrophils during 34-47 days post-calving. Both clinical and subclinical endometritis are sequel to delayed uterine involution (Sheldon et al. 2008). In terms of effect reproductive performance, endometritis has been shown to have a negative on the calving to first service interval; from calving to conception interval and decreased conception rates (Kasimanickam et al. 2004).

The reported prevalence of endometritis varies between studies and countries. For example, in the UK the prevalence of endometritis was 10% in dairy herds (Borsberry and Dobson 1989). Similar rates have been reported in Denmark [6.3%] (Bruun et al. 2002), Spain [2.6-4.5%] (Lopez-Gatius 2003) and Australia [5.6-10.9%] (Moss et al. 2002). However, much higher prevalence has been reported in Korea [47.6%] (Kim and Kang 2003) and the USA [53%] (Gilbert et al. 2005).

1.10.3 Pyometra

The accumulation of the pus in the uterine lumen is called pyometra and is often associated with a persistent CL (Sheldon et al. 2008). It mainly develops in cows that have their first ovulation after calving before bacterial contamination of the uterus has been eliminated. At the same time, the cervix is closed due to progesterone production from the corpus luteum (Chapwanya et al. 2012, Földi et al. 2006). It usually occurs from 3 weeks postpartum onwards.

1.10.4 Risk factors for uterine infection

It has been reported that the risk factors for uterine infection include retention of the placenta, the calving environment, twins, dystocia, and nutrition. Retained placenta is regarded as an important predisposing factor for uterine infection (Potter et al. 2010). Moreover, retained placenta is related to a substantial decrease in milk yield (Sheldon et al. 2004) and this can persist even after the problem has been corrected.

1.11 Mechanism through which uterine disease affects fertility

1.11.1 Uterine function

There is no doubt that a healthy endometrium is necessary to support the development of the embryo and successful pregnancy. Indeed uterine infection with pathogenic bacteria will drastically reduce conception. The embryonic mortality rate is increased when uterine infection with pathogenic bacteria after conception occurred (Semambo et al. 1991). The presence of microbes or pathogen-associated molecules appears to provoke a considerable immune response (Sheldon et al. 2008).

The uterine immune response is mediated through immune cells infiltrating the endometrial epithelial and stroma layers. Certainly, epithelial cells are the first endometrial cells in the line of defense against microbes. In the genital tract, the innate immunity is highly dependent on the expression of pattern recognition receptors (PRR) to detect pathogen-associated molecular patterns (PAMPs). The PRR family includes the Toll-like Receptors (TLR), which are highly conserved across phyla and detect a range of PAMPs associated with bacteria, viruses and fungi (Fig.1.8) (Beutler 2004, Akira and Takeda 2004, Akira et al. 2006). Binding of PAMPs to PRR stimulates signal transduction pathways for mitogen-activated protein kinase (MAPK) and the nuclear factor-kappa B (NFκB) transcription factor pathways. This results in altered secretion of prostaglandins, and cytokines such as TNFA, IL6 and IL8 (Ghosh et al. 1998, Li and Verma 2002, Akira and Takeda 2004).

The bovine endometrial epithelial and stroma cells express Toll like receptor-4 (TLR4) and its co-receptor MD-2 and CD14. This complex, forms the innate immune receptor for bacterial endotoxin lipopolysaccharide (LPS), the key PAMP of the common uterine pathogen *E.coli* (Fig. 1.8) (Herath et al. 2006b). The pathogen-associated molecules acting on uterine cells not only cause inflammation but also affect endocrine function. The endometrium produces two principle hormones $PGF_{2\alpha}$ and PGE_2, respectively. Endometrial explants and cells treated with LPS, resulted in a shift towards PGE_2 from $PGF2_\alpha$ production (Herath et al. 2009) (Fig.1.8E). This though was reversed when co-treated with oxytocin (Herath et al. 2009). This switch in prostaglandin accumulation was associated with an increased level of phospholipase A_2 (PLA2) protein in epithelial cells (Herath et al. 2009). This concept could provide a mechanism to explain prolonged luteal phases that has been reported in animals with uterine disease. Equally, PGE_2 may play an important role in regulating inflammatory responses in the endometrium (Herath et al. 2009).
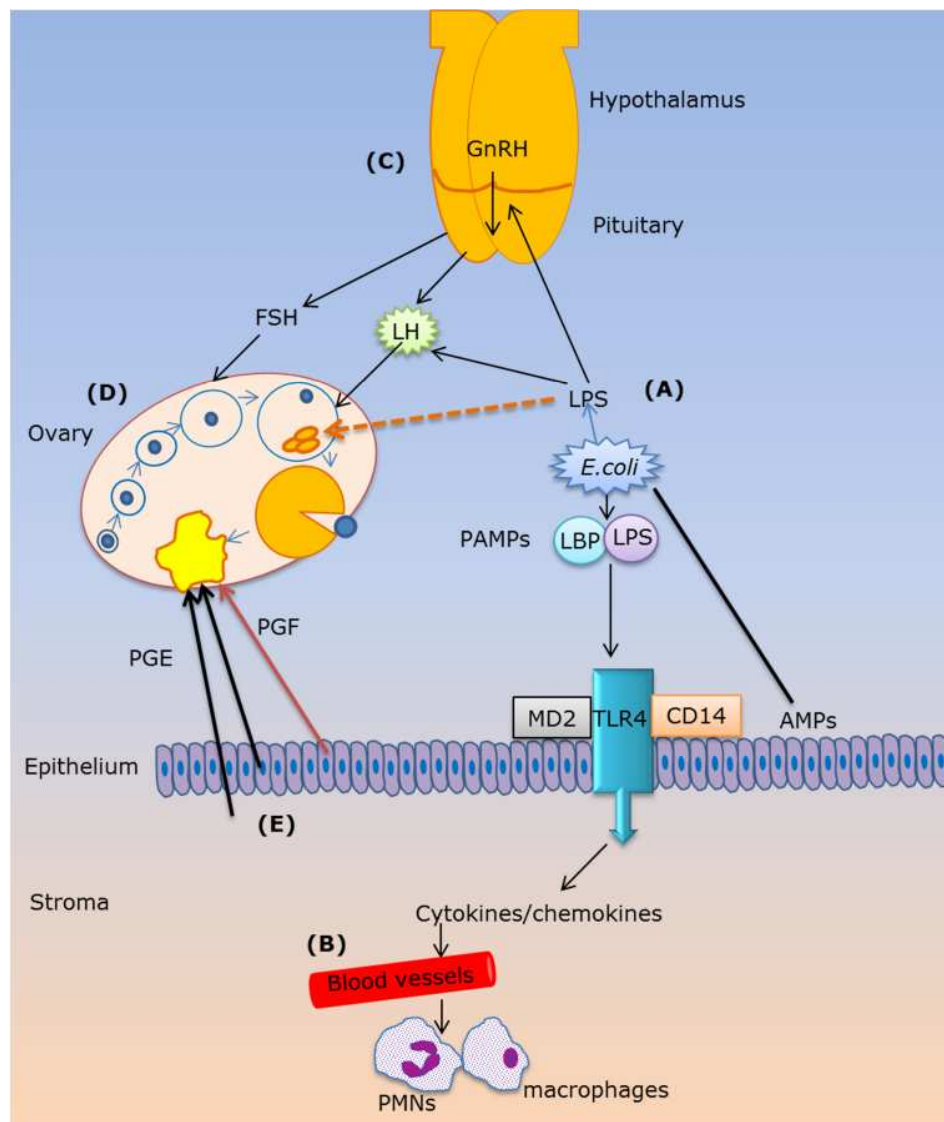
Figure 1.8: The molecular interplay between uterine infectious disease and ovarian function. A) Toll-like receptor 4 (TLR4) detect pathogen-associated molecules (PAMPs) such as lipopolysaccharide (LPS) when the endometrium is infected with *E. coli*. LPS requires LPS binding protein (LBP) for it to be recognised by TLR4. Consequently, the innate immune system is alerted stimulating the release of cytokines and chemokines and then infiltration of immune cells and production of anti-microbial peptides(AMPs). B) Polymorphonuclear neutrophils (PMNs) and macrophages to remove bacteria. C) LPS inhibits secretion of gonadotrophin releasing hormone [hypothalamus] and luteinising hormone (LH) [anterior pituitary] thereby decreasing the ability of the dominant follicle to ovulate. Follicle stimulating hormone (FSH) concentrations are however, less affected thus emergence of follicular waves still occurs in the first weeks post-partum. D) LPS can accumulate in follicular fluid and interact with TLR4 in granulosa cells disturbing oestradiol secretion through the downregulation of aromatase gene. Thus dominant follicle growth is reduced by endometritis with a decreased likelihood of ovulation. E) There is also a shift in prostaglandin secretion to prostaglandin E_2 (PGE) rather than prostaglandin $F_{2\alpha}$ (PGF) which would disturb luteolysis and lead to an extension of the luteal phase. Adapted from (Sheldon et al. 2009a).

1.11.2 Uterine disease and ovarian function

1.11.2.1 Follicular function

Uterine infections not only cause subfertility by disrupting endometrial health but also affect ovarian function. It is assumed that a follicle at ovulation needs to be a minimal size, healthy and oestrogenic in order to establish a successful pregnancy (Perry et al. 2005). Cows with uterine disease had slower growth of first post-partum dominant follicle, and lower peripheral plasma oestradiol concentrations at the time of maximal follicle diameter and plasma progesterone levels were lower 7 days after ovulation (Sheldon et al. 2002b, Williams et al. 2007). Bacteria are rarely found in the ovary, thus the effects are likely to be indirect. Indeed, LPS has been detected in the peripheral plasma as well as the follicular fluid of postpartum cows with endometritis (Mateus et al. 2003, Herath et al. 2007). The concentration of LPS in follicular fluid ranged from 0.04-0.8μg/ml in animals with clinical disease but was not detected in normal animals (Herath et al. 2007). The reduced ovulation rate might be due to associated effects of the uterine infection on hypothalamus, pituitary as well as ovary (Fig.1.8). At the level of the hypothalamus and pituitary, LPS can suppress hypothalamic GnRH secretion and plasma LH levels (Battaglia et al. 2000, Rivest et al. 1993, Williams et al. 2001). However, not all studies have shown that LH is impaired in animals treated with intrauterine infusion of LPS (Williams et al. 2008a). However, peripheral plasma FSH concentration profiles and ovarian follicle wave emergence were not affected by uterine infection (Sheldon et al. 2002b), Fig.1.8.

Regardless of the follicular stage, LPS had no effect on cell survival or androstenedione production by theca cell *in vitro*; however, oestradiol production in granulosa cells from medium and dominant follicles was decreased by LPS treatment (Herath et al. 2009). The additional evidence that LPS directly affected granulosa cells included: 1) cell cultures were confirmed to be free from contaminating leucocytes (Herath et al. 2007); 2) No immune cells are able to cross an intact follicular basement membrane *in vivo* (Petrovská et al. 1996); 3) Granulosa cells express TLR4, CD14, MD2 receptor complex required for binding LPS (Herath et al. 2009, Bromfield and Sheldon 2013), and expression of *aromatase* mRNA was reduced by LPS treatment of granulosa cells collected from dominant follicles (Herath et al. 2007). This all provides evidence that there is a

mechanism by which LPS can directly exert an effect on granulosa cells to disturb follicular function and ovulation (Fig. 1.8D).

1.11.2.2 Corpus luteum

Prolonged luteal phase has been commonly observed in cows with uterine infection, which might reflect attenuated luteolysis. Indeed, the switch in endometrial PGs from PGF2α to PGE2 would explain this since PGF is luteolytic, while PGE is luteotropic in ruminants (Poyser 1995, Arosh et al. 2004, Sheldon et al. 2009a). Another important aspect that is more obscure is how LPS effects progesterone production by the CL. It has been reported that peripheral plasma progesterone concentrations were lower in cows with high uterine pathogen growth density than in cows with low uterine pathogen growth density (Williams et al. 2007). The timing of the post-ovulatory rise in progesterone is important for embryonic survival (Mann and Lamming 1999), and low progesterone concentrations could lead to the loss of embryos. Bovine luteal cells treated with LPS had increased progesterone production *in vitro*, however, cell survival was decreased under higher concentration of LPS [3μg/ml] (Grant et al. 2007). Whether LPS exerts its action directly on luteal cells, or indirectly (e.g. mediated by cytokines) is unclear. It has been shown that the CL appears to have an association with the immune system. For instance, in the bovine CL parenchyma components of the antigen presentation pathway were expressed and these aided interactions with T cells (Cannon et al. 2007). Furthermore, there is evidence that macrophages and lymphocytes are present in the bovine corpus luteum throughout the luteal phase, particularly at luteolysis (Townson et al. 2002, Bauer et al. 2001). These cells could play a major role in the immune response of the CL to LPS and possible mediatory role for cytokines production (Penny et al. 1999, Townson et al. 2002). It should be noted that endothelial cells, could also have a role in any response to LPS (Faure et al. 2001). It has been demonstrated that TLR4 expression by endothelial cells could be regulated at the site of infection and inflammation, either directly by LPS or indirectly by inflammatory cytokines such as TNFA and IFNG (Faure et al. 2001). It has been recognised that LPS directly exerts action on endothelial cells. For example LPS binding to endothelial cell surface increased expression of pro-inflammatory cytokines, adhesion molecules and triggered endothelial cell apoptosis (Hack and Zeerleder 2001). Also LPS-activated macrophages and monocytes stimulate the

production of pro-inflammatory mediators that modulated endothelial cell function (Aird 2003).

1.12 Bacterial endotoxin and their effects on mammalian cells

1.12.1 Lipopolysaccharides

Bacterial lipopolysaccharides (LPS) are glycoproteins that form the major component of the outer layer of the cell wall in Gram negative bacteria. These give structural integrity and defense of the bacterial cell wall (Erridge et al. 2002). LPS also creates a negative charge to the cell membrane, and covers up to 75% of the bacterial cell area (Le Brun et al. 2013). LPS is important for many biological interactions between bacteria and the external environment including adhesion and recognition. In addition, LPS are also endotoxins as once cell-bound, they are secreted by the bacteria and, trigger the immune system in many eukaryotic living organisms (Alexander and Rietschel 2001). In this way, LPS plays a major role in the pathogenesis of Gram-negative bacterial infections. It is important to clarify the chemical structure of LPS to understand its function. LPS is an amphiliphic macromolecule consisting of three various structures (Fig. 1.9):

1- Lipid A (a lipophilic domain)
2- Core (sub-divided into inner and outer cores; hetero-oligosaccharide denominated)
3- O-polysaccharide chain or O-chain (a hydrophilic hetero-polysaccharide)

Structure of Lipid A

Lipid A (endotoxin) can cause severe fever and septic shock in mammals (Akira et al. 2006). It is a glycolipid, directed towards the interior of the bacterial cell, and it is the component responsible for its endotoxic activity (Kirikae et al. 1994, van der Poll and Opal 2008, Alexander and Rietschel 2001). The lipid A molecule from *E.coli* consists of a disaccharide backbone to which four fatty acid (acyl) chains are attached (Alexander and Rietschel 2001). A further two fatty acid chains are then added to these primary chains creating a total of six chains. The disaccharide moiety is a β-1,6-linked glucosamine unit (Alexander and Rietschel

2001). Usually, lipid A consists of a mixture of glycoforms, which differ from both acylation and phosphorylation pattern. The subtle variation of these chemical structures is recognised as being responsible for different degrees of bacterial virulence and their adaptation to the host environment.
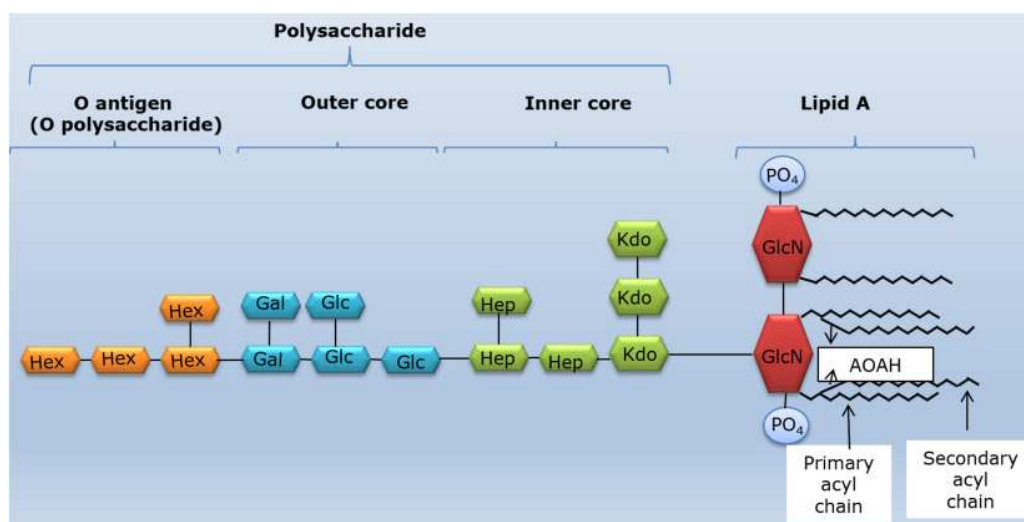
Figure 1.9: Chemical structure of lipopolysaccharide: Abbreviation include: Hex=Hexose; Gal=Galactose; Glc=Glucose; Hep=Heptose; Kdo= 2-keto-3-deoxyoctonate; AOAH= Acyloxyacyl Hydrolase; PO$_4$=Phosphate; GlcN= Glucosamine backbone.

Core-oligosaccharide

The core-oligosaccharide is divided into two regions, the inner core that attaches to the lipid A and the outer core that attaches to O-chain side. The inner portion of the core-oligosaccharide is structurally less similar and composed of 3-deoxy-D-manno-octulosonic acid (Kdo) and peculiar monosaccharides like heptose (L-glycero-D-manno heptose and D-glycero-D-manno-heptose). The outer core is composed of hexose residues (e.g. D-glucose, D-mannose, D-galactose) and this is bound to the last heptose residue in the inner core (Alexander and Rietschel 2001).

O-antigen (O-side, or O-polysaccharide)

The O antigen is the most variable part of LPS between bacterial species. It is composed of many repeats of O units, typically between 10 and 25 units that are attached to the core oligosaccharide. It is made up of 1-8 monosaccharide constituents (Raetz 1990). It stimulates production of antibodies in the host that are capable of identifying specific O-chains. The composition of the O chain varies from strain to strain. This variation depends on the arrangement and composition

of the sugars, as well as the binding between them within the O unit, and the linkages between O units. There are more than 160 different O antigen structures formed by different *E.coli* strains (Raetz and Whitfield 2002).

1.12.2 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are a class of proteins that have been identified in mammals (Medzhitov et al. 1997). In mammals, the TLR family contains at least 10 members (Table. 1.1, Beutler 2004a). TLRs play major roles in the innate immunity. Generally, TLRs are transmembrane glycoproteins and are composed of two important domains: an extracellular domain containing leucine rich repeat (LRR) motif and Toll/IL-1R (TIR) domain is the cytoplasmic (intracellular) signaling domain (Fig.1.10). LRRs are an extracellular domain, known in all structures to adopt an arc (horseshoe) shape of repeating molecular sequences (Matsushima et al. 2007). These are rich in the hydrophobic amino acid, leucine. The extracellular domain of TLR4 consists of 23 LRR and these play a central role in the binding LPS as well as the co-receptor molecules, CD14 and MD-2 (Matsushima et al., 2007). The sequence of cytoplasmic TIR domain is similar across many species and within the TLR family. It is characterised by docking intracellular adaptor molecules, which define the specificity of downstream signaling (Werling et al. 2009).
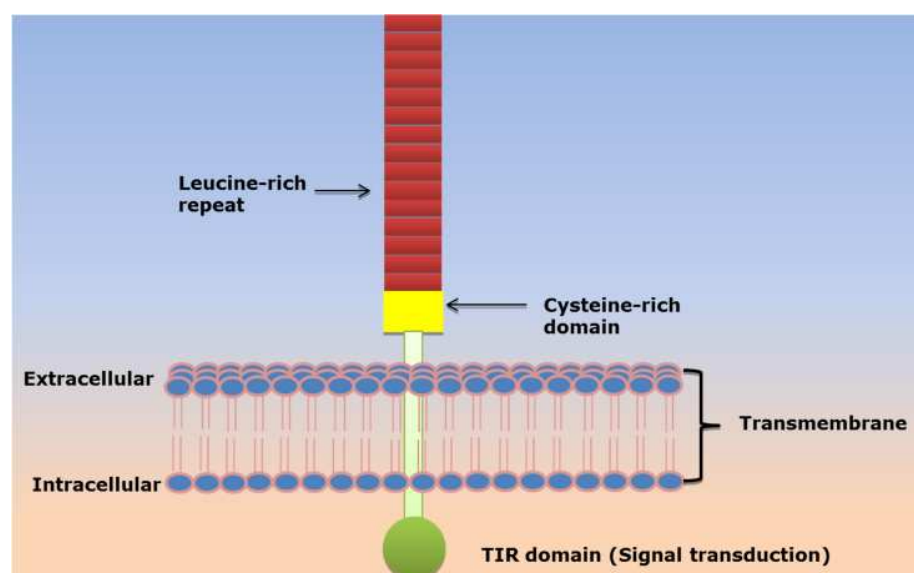
Figure 1.10: Structure of toll-like receptosr (TLRs): TLRs consists of an extracellular domain of leucine rich repeats (LRRs), which are responsible for ligand recognition and binding. There is an intracellular Toll/IL-1R (TIR) domain which acts as a downstream adaptor molecule.

Table 1.1: Overview of toll like receptors and their ligands

TLRs	Location*	Origin of ligand**	Ligand**
TLR1	Cell membrane	Bacteria and mycobacteria	Tri-acyl lipopeptide
TLR 2	Cell membrane	Gram-positive bacteria	Lipoprotein/lipopeptides
TLR 3	Endosome	Virus	Double-stranded RNA
TLR 4	Cell membrane	Gram-negative bacteria; Virus	Lipopolysaccharides (LPS) Heparin sulphate Heat shock protein
TLR 5	Cell membrane	Bacteria	Flagellin
TLR 6	Cell membrane	Mycobacteria	Di-acyl lipopeptides
TLR 7	Endosome	Synthetic compounds Virus	Imidazoquinoline Single-stranded RNA
TLR 8	Endosome	Synthetic compounds; Virus	Imidazoquinoline; Single-stranded RNA
TLR 9	Endosome	Bacteria and viruses	CpG-oligodeoxynucleotides
TLR 10	Cell membrane	Not defined	
(Kannaki et al. 2011, Kawai and Akira 2010)*; (Chen et al. 2007)**			

1.12.3 TLR4

This study focused on TLR4 as it has been established that TLR4 is the signaling receptor for LPS (Poltorak et al. 1998, Zhang and Ghosh 2000). In addition, TLR4 also recognises the endogenous mammalian ligands, such as heat shock proteins (HSP60 and HSP70) (Gao and Tsan 2003); fibronectin (Okamura et al. 2001); heparin sulphate (Akbarshahi et al. 2011), but only at high concentrations (Gao and Tsan 2003). Importantly, LPS does not bind to the surface of cell by itself. Instead, there are different molecules involved in the recognition of LPS by the cell including LPS binding protein and CD14.

LPS-binding protein (LBP)

LPS-binding protein (LBP) is an acute-phase protein that is the first host protein involved in the recognition of LPS (Schumann et al. 1990). LBP is produced primarily by hepatocytes as a 50-kDa protein, which circulates in the bloodstream as a 58-kDa protein following glycosylation (Schumann et al. 1990). The primary role of LBP appears to be presenting LPS to the cell surface, where it recognised by TLR4 through the amphipathic lipid A region of LPS with high affinity. In so doing, it forms a ternary complex with the LPS receptor molecule, CD14 (Schumann et al. 1990). This complex facilitates LPS transfer to the signaling LPS receptor complex which is composed of TLR4 and MD2 (da Silva Correia et al. 2001, Tobias et al. 1995; Fig 1.11). Additionally, LBP serves to enhance LPS uptake in endothelial cells (Dunzendorfer et al. 2004).

Cluster of differentiation 14 (CD14)

CD14 is a glycoprotein that exists in two forms: The first form is a membrane-bound glycosylphosphatidylinositol (GPI)-anchored protein (mCD14) that functions in LPS mediated cell activation (Jersmann et al. 2001). mCD14 is attached to the surface of myeloid cells through its GPI tail, enabling CD14 to be proximal to the membrane proximal despite lacking a transmembrane domain. The second form (sCD14) is a soluble proteolytic fragment without a GPI anchor, and this is present in plasma where it aids to transfer LPS in cells devoid of membrane-bound CD14, such as endothelial and epithelial cells (Wright 1995, da Silva Correia et al. 2001; Fig 1.11). Thus sCD14 has to be cleaved from mCD14 bearing cells such as macrophages and monocytes for its activity in endothelial cells (Arditi et al. 1993).

There is though some evidence that CD14 is active in endothelial cells since early passage human umbilical vascular endothelial cells (HUVECs) express

functional CD14 on the cell surface and blockade of mCD14 with a specific anti-mCD14 antibody prevented endothelial cell detection of LPS and subsequent leukocyte recruitment (Jersmann et al. 2001, Lloyd and Kubes 2006). As CD14 lacks an intracellular domain, it is unlikely that CD14 could induce an intracellular signal in response to LPS (Schumann et al. 1990), for that reason, the complex of TLR4/MD-2 has a vital role in conveying intracellular signaling.

TLR signaling

The intracellular TIR domain plays an important role in TLR4 dimerisation and the binding of lymphocyte gene 96 (MD-2) to the ectodomain by facilitating this homotypic interaction (Miguel et al. 2007). The binding of the ligand (LPS) to TLR4 leads to stimulation of homodimerisation, and this causes concerted structural alteration in the receptor leading to activation of the cytoplasmic TIR signaling domain (Miguel et al. 2007). Following ligation of TLR4, one of two cytoplasmic signaling pathways become activated, depending on the identity of the specific adaptor molecule recruited to TIR domain (O'Neill and Bowie 2007). Such adaptor molecules are: myeloid differentiation 88 (MYD88), TIR-domain consisting of adaptor protein (TIRAP; also known as My88 adaptor-like protein), TIR-containing adaptor inducing IFNB (TRIF; also known as TICAM-1) and TRIF associated with adaptor molecule (TRAM; also called TICAM-2) (Fig. 1.11).
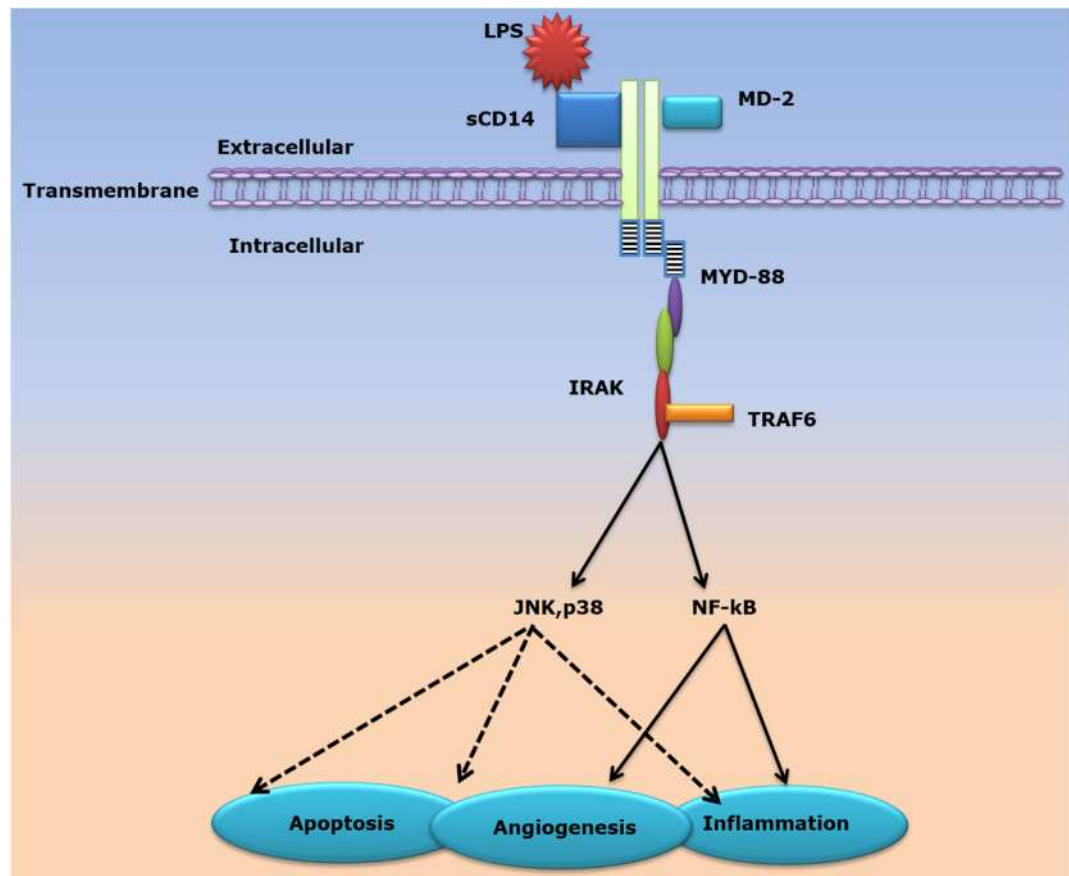
Figure 1.11: Proposed Toll-like receptor 4 (TLR4) signalling pathway events in endothelial cells in MYD88- dependent pathway. Stimulation of endothelial cells with lipopolysaccharide (LPS) activates tumour necrosis factor receptor associated factor-6 (TRAF6), resulting in the activation of several downstream signalling pathways, including activation of Jun N-terminal kinase (JNK) and nuclear transcription factor-κB (NF-κB) and subsequent production of pro-inflammatory molecules. TRAF6 activation induces endothelial sprouting and leading to a proapototic state through JNK and NF-κB-dependent pathways. Abbreviations include interleukin-1 receptor-associated kinase (IRAK). Adapted from (Dauphinee and Karsan 2006).

1.13 Aims of the PhD thesis

The aims of this thesis were to investigate the mechanisms involved in the failure of fertility in dairy cows during post-partum period with a particular focus on the impact of endometritis. This thesis concentrated on four aspects:

1. The association between reproductive tract inflammatory disease (RTID) and reproductive performance in a large-study retrospective study that incorporated multiple commercial dairy herds across England and Wales. This quantified the impact of RTID on dairy cow fertility under field conditions.

2. A prospective study that investigated the association between uterine infection post-partum, ovarian activity (resumption of postpartum cyclicity and subsequent progesterone profiles) and reproductive performance at the University of Nottingham dairy centre. This study provided insights into how endometritis affected ovarian activity and thus fertility.

3. *Ex vivo* investigation to characterise progesterone production, steroidogenic enzyme protein expression and vascularization of the bovine corpus luteum in cows with endometritis. It also investigated the presence of mRNA encoding TLR4 and it co-receptors in the bovine CL throughout the oestrus cycle.

4. Determination of the dose-dependent effects of *E.coli* lipopolysaccharide (LPS) on bovine luteal endothelial cell network formation and steroidogenesis *in vitro.*

2 The association between uterine disease and subsequent reproductive performance in commercial UK dairy herds

2.1 Introduction

Good reproductive performance in dairy cows is a crucial part of any successful farming enterprise. Numerous studies have determined reduced reproductive performance as a major cause of impaired production efficiency in the dairy industry, caused by greater costs of herd replacement, veterinary intervention, and ultimately decreased milk yield per year. The reproductive performance of dairy herds can be monitored using different measurements and indicators. Time interval measurements are frequently used as reproductive performance indicators and many of these indicators are based around the cows' individual calving date (e.g. day to first service (DFS) and calving interval (CI)). The different parameters have various advantages and limitations. For example, calving interval depends on two components 1) interval from calving to conception (or days open), and 2) the length of pregnancy which is relatively constant (~283 days). Thus, days open is the main principal determinant of the calving interval and incorporates the number of days from calving to first service (DFS), and services per conception (SPC). However, this is also influenced by environmental factors, management (accuracy of heat detection, use of proper insemination technique and appropriate herd health policies) as well as biological factors. Biological factors include involution of the cervix and uterus (Mateus et al. 2002, Zhang et al. 2010); age and parity (Hajurka et al. 2005, Melendez et al. 2004); concentrations of key reproductive hormones, nutrition (Zhang et al. 2010, Wagner and Hansel 1969); season of the year (Kasimanickam et al. 2004, Noakes et al. 2009); uterine infection (Sheldon and Dobson 2004, Melendez et al. 2004, Noakes et al. 2009).

Dairy cow subfertility has been associated with uterine infection. As well as a direct effect on the uterus, infection has been shown to cause disturbance in the hypothalamus, anterior pituitary and ovary. Moreover these effects can persist even after clinical resolution of the disease (Sheldon and Dobson 2004). Uterine diseases such as metritis and endometritis are highly prevalent in high yielding dairy cows. Often, these diseases are combined with cervicitis and vaginitis and collectively termed reproductive tract inflammatory disease

(RTID). The incidence of metritis affects about 25-40% of cows in the first 14 days after calving (Sheldon and Dobson 2004). The observed variation is likely to be due to different diagnostic methods and time of examination relative to calving. The prevalence of clinical endometritis is about 20% within a herd (McDougall et al. 2007, Goshen and Shpigel 2006). Among all types of uterine disease, subclinical endometritis is the most prevalent one, which affects up to 50% of the animals with uterine disease (Sheldon et al. 2008, Gilbert et al. 2005, Kasimanickam et al. 2004). Numerous of aerobic and anaerobic Gram positive and Gram negative bacteria have been detected in the uterus in more than 90% of cows in the first 10-14 days post-partum (Földi et al. 2006, Williams et al. 2005, Sheldon et al. 2002b). Vaginal discharge during the postpartum period can accurately reflect uterine infection (Williams et al. 2005). However, vaginal scoring systems cannot accurately differentiate the actual severity of the case and the actual location of the infection e.g. endometritis *vs* cervicitis (Dohmen et al. 1995, Huszenicza et al. 1999, Williams et al. 2005). It has been reported that there is not always agreement between purulent vaginal discharge and endometritis as defined by cytology. This questions the source of the pus in the vagina, and states that it is not always from the uterus (Dubuc et al. 2010). Typically, the risk factors for RTID include retained fetal membrane, assisted calving, stillbirth, vulval discharge and parity (Potter et al. 2010). Various studies have characterised the effect of uterine infection on reproductive performance in dairy herds. However, this study could be the first large scale, multi-herd study to characterise the effect of uterine infection on a variety of reproductive parameters. Namely, this chapter incorporates 80 dairy herds from England and Wales.

2.1.1 Aim

To quantify the association and potential influence of reproductive tract inflammatory disease (RTID) on various reproductive performance parameters in multiple commercial dairy herds across England and Wales.

2.1.2 The objectives of the study were:

In order to achieve this aim, the following objectives were developed:

1. To quantify the prevalence of reproductive tract inflammatory disease in different commercial herds across England and Wales

2. To compare various reproductive performance between cows that experienced RTID during their lactation and control cows. The parameters chosen included the following parameters:

a. Day to first service (DFS)

b. Calving to conception interval (CCI)

c. Number of services per conception (SPC)

d. Conception rate at first service

e. Calving interval (CI)

f. Milk yield 305d

3. To assess the prevalence of animals exiting the herd (e.g. due to culling) between cows that had or did not have RTID, on a herd level and between calving years.

2.2 Materials and methods

2.2.1 Experimental animals

The database was kindly provided by Dr Chris Hudson, University of Nottingham as previously described (Hudson et al. 2012). The retrospective data were collected from routine dairy herd records. This was inputted and managed the herd manager. No personal data was stored as part of this project and all herds were coded anonymously by a number. This study incorporated 80 commercial Friesian-Holstein dairy herds from England and Wales. The data was collected from 2000 to 2007 and contained 59,118 lactations from 29,157 cows. The average parity was 3. There were a number of cows (n=12,805) within a particular lactation that exited the herd. The exact reason is unknown but the most likely cause was culling. Animals that exited the herd were included in the analysis where possible. All animals were classified into two experimental groups according to their uterine health status. This was determined by the vet(s) assigned to that particular herd. The first group included 52,014 control lactations (i.e. no reported clinical signs of endometritis or vaginal discharge). The second group involved 7,104 lactations where clinical uterine infectious disease was reported by the herd manager. In this study, cases of vulval discharge and endometritis (as defined by the on-farm veterinarian) are considered together under one category that was called RTID. Animals were diagnosed with RTID were treated by the veterinarian in charge as deemed appropriate for that individual case.

2.2.2 Dependent variables/ Reproductive performance indices

The definitions of the reproductive parameters are described in Table 2.1.

Table 2.1: Definition of the key fertility parameters

Fertility traits	Definition
Day to first service (DFS)	Number of days from calving to the day of first insemination after calving
Calving to conception interval (CCI)	Number of days from calving to subsequent first reported conception (based on positive pregnancy diagnosis) after calving
Number of services per conception (SPC)	Number of inseminations required for a cow to become pregnant defined by transrectal ultrasound/rectal palpation
Conception rate at first insemination	The number of cows at 1st insemination that become pregnant divided by the total number of cows inseminated.
Calving interval (CI)	Number of days between two consecutive calvings.

2.2.3 Data management

Data for these parameters was calculated in Excel based on the reported insemination, pregnancy and calving dates. While the majority of the data values were in the expected ranges (Table 2.2), there were data values that did not fit these ranges. These were considered non-physiological or atypical management and were excluded for that particular end-point only.

Table 2.2: The accepted values for reproductive performance indices were used to evaluate the postpartum reproductive performance of dairy cows in 80 commercial dairy herds across England and Wales (2000-2007).

Independent variables	Accepted range	Number of accepted data points	Number of excluded data points	Total
Days to first insemination (days)	20-150	46890	12228	59118
Number of services per conception	1-12	46890	12228	59118
Calving to conception interval (days)	20-300	43049	16069	59118
Calving interval (days)	305-585	40015	19103*	59118
Milk yield 305d (Kg)	4000-20000	47266	11852	59118
*= Number of cows without CI due to exited =3233; number of cows with no data=12683; number of cows out of range=3187				

The prevalence of RTID in the UK dairy herd

Data from each herd, calving year and lactations number were entered and stored in Microsoft Excel 2010 (Microsoft Corporation, 2010). Dichotomous

values were used to indicate lactation positive with RTID; however lactation without RTID coded into "0".

 Comparisons between "control" cows with different reproductive performance

In order to interrogate the fertility of the "control" cows, three sub-groups were created depending: 1) on the cows' day to first service (DFS) interval as follows: <50, 50-60 or >60 DIM; 2) calving interval as follows: <400, 400-425 and >425 DIM. For these sub-groups, reproductive parameters analysed were calving to conception interval (CCI), conception rate at 1st service, number of service per conception and 305d milk yield.

RTID and its association with subsequent reproductive performance

Data from each lactation were entered and stored in Microsoft Excel 2010 (Microsoft Corporation, 2010). Dichotomous values were used for pregnancy outcome at 1st insemination, RTID status and RFM where "1" indicated they were pregnant/had RTID and RFM or were coded into "0" when animals did not conceive at 1st service and/or free from disease and RFM. Lactations with with one case of RTID were coded as RTID1, those with two cases were coded as RTID2 and those recorded 3 cases were coded as RTID3.

2.2.4 Statistical analysis

Before performing analysis, data were checked for normality and homogeneity of variance using residual plots and Bartlett's test, respectively. If the data did not satisfy this, then it was transformed appropriately, usually by log transformation. For all experiments $P<0.05$ was significant and all data are quoted as mean±SEM

The prevalence of RTID

The prevalence of RTID between calving years, herds and lactation number were analysed using logistic regression in GenStat 16th Edition (VSN International Ltd, Hemel Hempstead, UK).

Comparisons between control cows with different reproductive performance

In order to compare the reproductive performance of cows with different DFS values (<50, 50-60, >60DIM), unbalanced one-way ANOVA was performed with the DFS category as the factor to compare reproductive indices including

(DFS, CCI, CI) and 350d milk yield between DFS categories. Logistic regression was performed to compare the conception rate at 1st service between these DFS categories. A non-parametric Kruskal-Wallis one-way ANOVA was performed to compare SPC. Similarly, one-way ANOVA was conducted to compare reproductive performance of cows with different CI values (<400, 400-425 and >425 DIM). Logistic regression was performed to compare the conception rate at 1st service between CI category. One-way ANOVA was performed to compare SPC between CI categories.

The association between RTID and subsequent reproductive performance

An unbalanced ANOVA was performed to determine the association between RTID status (included as the factor) and reproductive performance (DFS, CCI, SPC, conception rate at 1st service, and CI). Kaplan-Meier survival analysis was performed to compare calving to conception interval between cows that experienced RTID and those that did not. The proportion of non-pregnant cows represented the survival probability, which was plotted against the time (calving to conception interval). Only cows that became pregnant were included in this analysis. Within the database the number of reported RTID cases/incidences for a particular lactation was recorded within the spreadsheet.

Influence of multiple RTID cases on subsequent reproductive performance

An unbalanced ANOVA, followed by multiple comparisons, was performed to determine the differences of reproductive performance (DFS, CCI, SPC, conception rate at 1st service, and CI) between different RTID cases report (including as the factor). RTID cases means that number of RTID has been reported per each cows.

2.3 Results

2.3.1 Prevalence of RTID across England and Wales dairy herds from 2000-2007

The overall prevalence of RTID increased over the time period (P<0.001, Table 2.3). The mean prevalence of RTID over eight years was 12.0%. There was a wide variation in the reported prevalence of RTID between herds, with a range of 5-33% (Fig 2.1). Frequency distribution of prevalence of RTID between

herds showed that out of 80 dairy herds, 47 herds had a low prevalence of RTID (1-10%), 23 herds had a prevalence of 11-21% and 10 herds had 21-≥33 (Fig 2.2). In addition, prevalence of RTID was much greater in cows with reported RFM compared to cows without RFM ($P<0.001$, Fig 2.3)

Table 2.3: The changes in the prevalence of reproductive tract inflammatory disease (RTID) between years 2000 and 2007 in multiple-commercial dairy herds across England and Wales ($P<0.001$).

Year	Total number of calvings	Lactations with RTID (n)	Lactations with no RTID (n)	% of RTID
2000	2352	183	2169	7.8
2001	2841	232	2609	8.2
2002	4159	431	3728	10.4
2003	6502	743	5759	11.4
2004	7275	922	6353	12.7
2005	11322	1303	10019	11.5
2006	11976	1481	10495	12.4
2007	12691	1809	10882	14.3
Total	59118	7104	52014	12.0
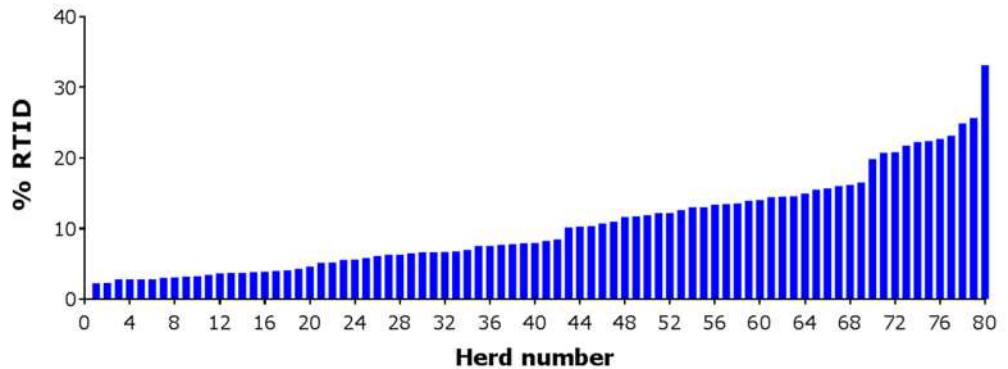
Figure 2.1: Prevalence of reproductive tract inflammatory disease (RTID) in the different 80 herds in England and Wales. The prevalence of RTID varied from 5-33%.
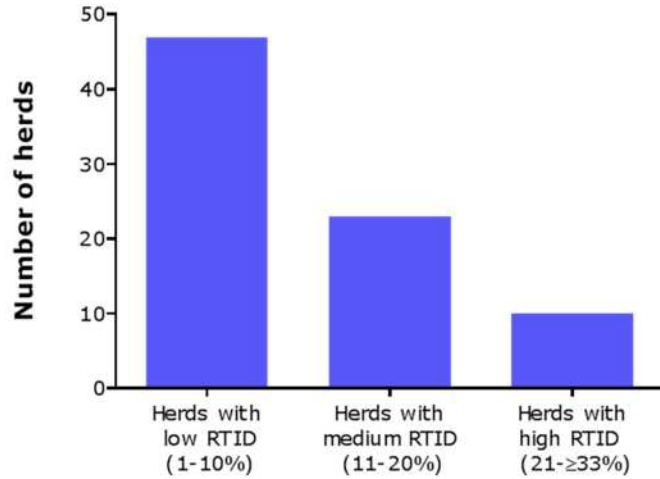
Figure 2.2: Frequency distribution of the prevalence of reproductive tract inflammatory disease (RTID) between the different herds. The majority (~45%) of herds had relatively low prevalence but about 10% had a prevalence >20% RTID.
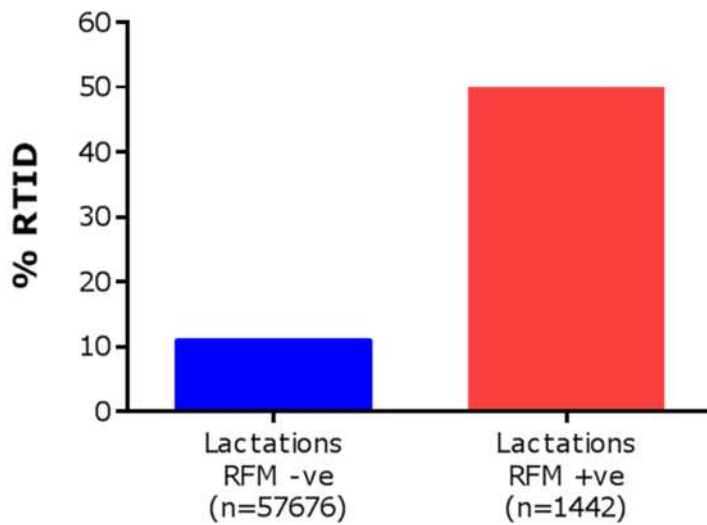
Figure 2.3: The impact of retained fetal membranes (RFM) on the prevalence of RTID. Lactations that were RFM+ve had higher prevalence of RTID (50.0%) compared to those that were RFM-ve (11.1%; *P*<0.001).

2.3.2 Comparison of reproductive performance of control lactations with reproductive indices

The overall mean DFS was 76.4 days, mean calving to conception interval was 124.1 days, mean number of services per conception (SPC) was 2.1 services, mean calving interval was 405.6 days, conception rate at 1st service was 36.3% and mean milk yield was 8425 kg (Table 2.4).

Table 2.4: Descriptive statistics for production variables for 59118 control lactations in 80 commercial dairy herds across England and Wales.

Variable	Minimum	Maximum	Mean	SEM
Day to first service	20	150	76.4	0.1
Calving to conception interval (days)	20	300	124.1	0.3
Service per conception (n)	1	12	2.14	0.01
Calving interval (days)	305	585	405.6	0.3
Conception rate at 1st service (%)	-	-	36.3%	-
305d milk yield (L)	4000	20218	8425	0.3

Lactations with a low mean DFS (<50 days) had more services per conception compared to lactations with mean DFS 50-60 day and >60 days by 0.1 and 0.4 services, respectively ($P<0.001$, Table 2.5). In addition, lactations with mean DFS <50 days had shorter CCI compared to lactations with greater DFS intervals ($P<0.001$, Table 2.5). However, the highest conception rate at first service ($P<0.001$) and least services per conception ($P<0.001$) were observed in the lactations with a DFS interval of >60days. This group of lactations also had higher milk production compared to herds with mean DFS more than 50 days, 50-60 days groups ($P<0.001$; Table 2.5). Lactations with longest CI (>425 days), not unexpectedly, had the longer DFS ($P<0.001$) and CCI ($P<0.001$) than those the shortest CI (<400 days) (Table 2.6). However, large differences between the groups were found regarding number of services per conception ($P<0.001$) and conception rate at 1st service ($P<0.001$) with the lactations in >425 day CI group having a conception rate at 1st service as low as 12% and requiring 3.3 services per conception. Also this model found that lactations with mean CI <400 days produced 348kg and 532kg less milk compared to lactations with mean CI 400-425, >425 days groups, respectively ($P<0.001$).

Table 2.5: Reproductive performance of lactations with differing day to first service intervals from 80 commercial dairy herds across England and Wales. Data are mean ±SEM.

Reproductive performance parameter	Days to first service			*P* value
	<50 days	50-60 days	>60 days	
Days to 1st service	41.6±0.3 n=5721	56.2±0.2 n=8235	87.1±0.1 n=32934	<0.001
Services per conception (n)	2.77±0.03 n=5721	2.67 ±0.02 n=8235	2.48 ±0.01 n=32934	<0.001
Calving to conception interval (days)	98.0±0.8 n=4834	103.7±0.7 n=7072	129.3±0.3 n=27457	<0.001
Conception rate at 1st service (%)	29.4%±0.6	35.3%±0.5	37.1%±0.2	<0.001
Calving interval (days)	381.4±0.9 n=4459	385.6±0.7 n=6529	410.6±0.4 n=25533	<0.001
305d milk yield (L)	8244±24.19 n=5721	8367±19.6 n=8235	8572±10.8 n=32934	<0.001

Table 2.6: Reproductive performance of lactations with differing calving intervals from 80 commercial dairy herds across England and Wales. Data are mean ±SEM.

Reproductive performance	Calving interval (CI)			P value
	<400 days	400-425 days	>425 days	
Days to 1st service	69.8±0.2 n=21047	86.6±0.2 n=5042	82.3±0.3 n=10432	<0.001
Services per conception (n)	1.35±0.00 n=22224	2.17±0.02 n=5282	3.31±0.01 n=12509	<0.001
Calving to conception interval (days)	81.4±0.2 n=22171	129.0±0.4 n=5277	196.0±0.3 n=12331	<0.001
Conception rate at 1st service (%)	65.8%±0.3	25.8%±0.6	11.9%±0.4	<0.001
Calving interval (days)	361.0±0.2 n=20432	411.6±0.4 n=4625	481.0±0.3 n=10592	<0.001
305d milk yield (L)	7256±11.1 n=22224	7604±22.7 n=5282	7788±14.8 n=12509	<0.001

2.3.3 The association between lactations experiencing RTID and their reproductive performance

2.3.3.1 Days to first service (DFS)

Lactations that experienced RTID had a longer mean DFS compared to control lactations by 5 days ($P<0.001$; Fig 2.4A). The mean DFS for control lactations was 75.9±1.0 days in comparison with 81.0±0.3 days for lactations that had RTID ($P<0.001$; Fig 2.4A). The distribution of DFS between in these groups can be seen in Fig 2.4B.
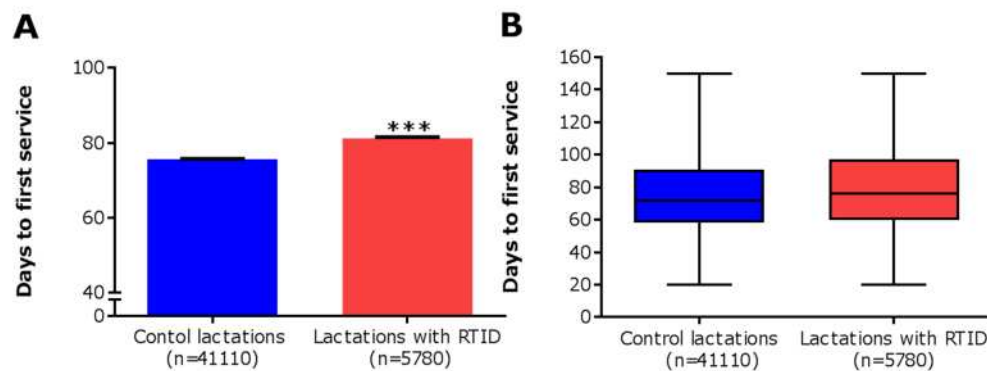
Figure 2.4: The impact of lactations experiencing reproductive tract inflammatory disease (RTID) on days to first service. (A) Days to first service in control lactations was 5 days shorter than those lactations than those lactations that experienced RTID (***;$P<0.001$). Data are mean±SEM (B) Box and whisker plots comparing the variation in DFS for control lactations and experienced RTID. The mid line within the box represents the median of DFS for control lactations and lactations experienced RTID were 72 and 76 days, respectively, the whiskers represent minimum and maximum values.

In addition, lactations with an increased number of RTID cases was associated with an increased number of days to 1st service ($P<0.001$, Fig 2.5) with the longest DFS observed in lactations with >3 reported RTID cases. Namely, lactations with RTID 1, 2 and 3 cases had DFS longer by 3, 5 and 9 days, respectively compared to control lactations.
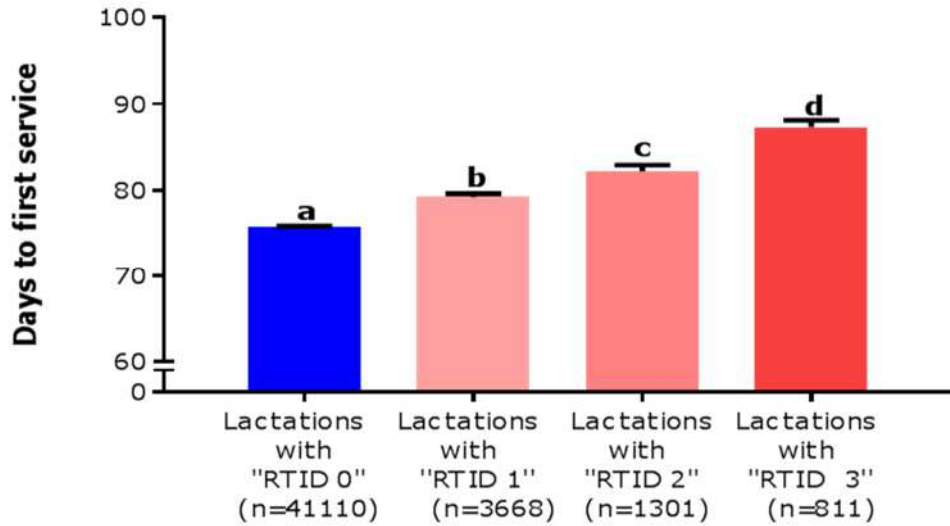
Figure 2.5: The association between days to first service (DFS) and number of RTID observations. Mean of DFS was significantly different ($P<0.05$) between lactations with (RTID 0) and lactation with RTID 1, 2 and 3. Differences between lactations are indicated by different letters a<b<c<d. Data are mean±SEM.

2.3.3.2 Conception rate at first service

The 1st service conception rate was lower in the RTID group compared to the control lactations by 14 percentage points ($P<0.001$, Fig.2.6).
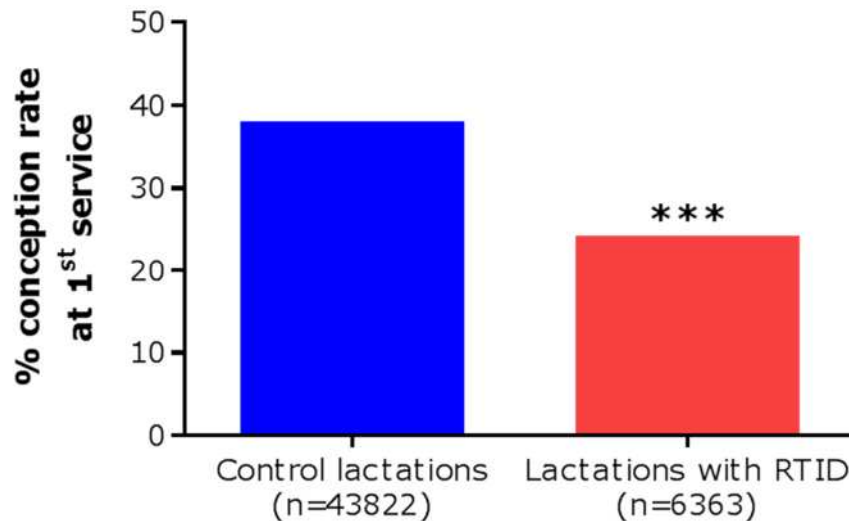
Figure 2.6: Comparison of conception rates at first service between control lactations and those that experienced RTID. Control lactations had higher first service conception rate (38.0±0.02) compared to lactations with RTID (24.4±0.06), ***;$P<0.001$. Data are mean.

In addition, a higher number of RTID observations was associated with a lower conception rate at 1st service ($P<0.001$, Fig 2.7). Namely, the conception rates in the RTID 1, 2 and 3 groups were 12, 17.1 and 18 percentage points, respectively lower than those found in control lactations (CR=38%).
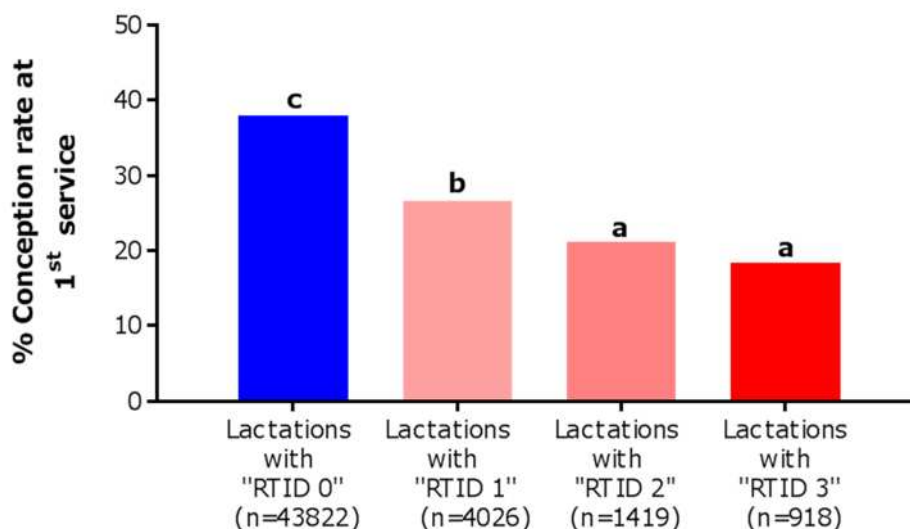
Figure 2.7: The comparison of the conception rate at first service between lactations with different number of incidence of RTID during that lactation. There was a difference (P<0.001) in the conception rate at first service between lactations with different number of RTID observations (P<0.05). Differences between lactations are indicated by different letters c>b>a. Data are mean.

2.3.3.3 Calving to conception interval (CCI; days open)

Lactations that experienced RTID took longer to establish a pregnancy by 22 days compared to control lactations ($P<0.001$, Fig 2.8A). This difference of CCI between the groups was much bigger than that observed for DFS. Kaplan-Meier survival analysis showed that the rate at which RTID positive lactations became pregnant was lower than RTID negative lactations (P<0.001, Fig 2.8B). By 105 days in milk, half the control lactations were pregnant; however, this was increased in cows that experienced RTID to 131 days.

Additionally, the CCI was affected by the number of RTID observations that the lactations had, with increasing number being associated with longer CCI ($P<0.001$, Fig 2.9). Namely, lactations with no RTID had shorter CCI compared to those with RTID 1, 2 and 3 observations by about 19, 26 and 35 days, respectively.
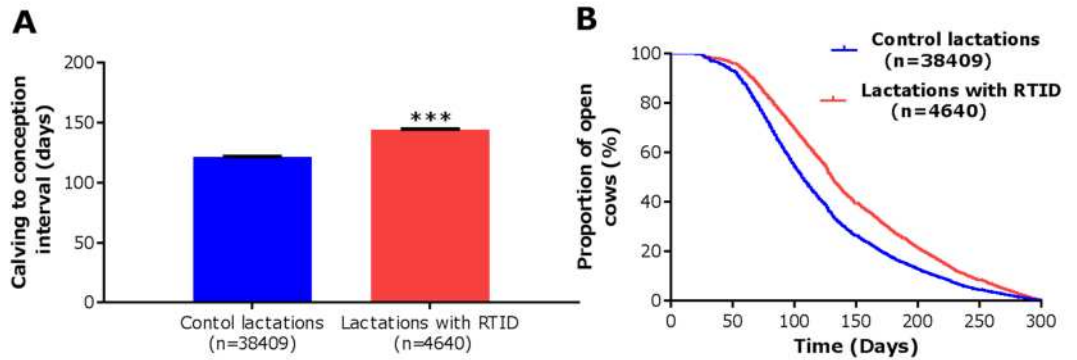
Figure 2.8: The association between lactations experiencing RTID and calving to conception interval (CCI). (A) Lactations with RTID had longer CCI (144.0 days) compared to control lactations (121.7 days; ***;$P<0.0001$). (B) Kaplan–Meier survival curves for the proportion of animals that became pregnant over time. Lactations positive for RTID during the lactation took longer to get pregnant compared to lactations without RTID. Median days were 105 and 131 days for control and RTID lactations , respectively (Log rank test: $\chi^2= 384.2$, $P<0.001$). Data are mean±SEM.
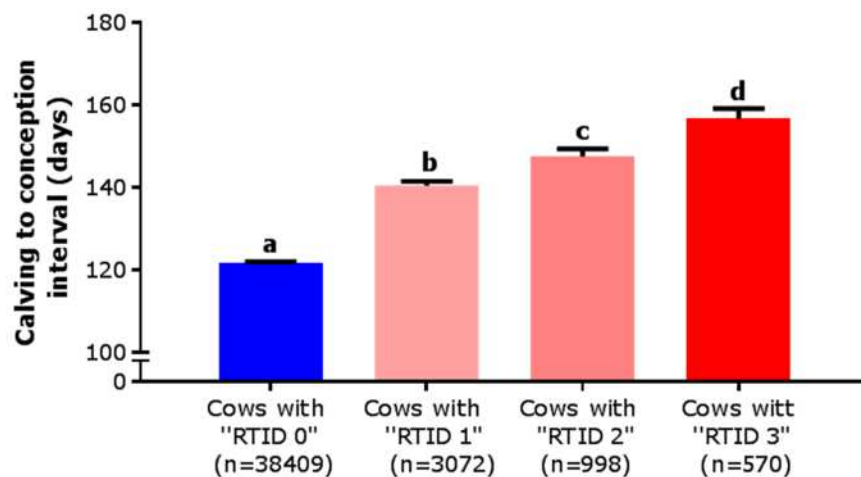
Figure 2.9: The association between calving to conception interval (CCI) and incidence of RTID. Mean of CCI was different between lactations with (RTID 0) and lactations with RTID 1, 2 and 3 ($P<0.05$). Differences between lactations are indicated by different letters a<b<c<d. Data are mean±SEM

2.3.3.4 Number of services per conception (SPC)

The lactations that experienced RTID required more services to become pregnant ($P<0.001$; Fig 2.10A). The mean for control lactations was 2.0±0.01 services in comparison to 2.9±0.02 services for lactations positive for RTID (Fig 2.10A). The variation in the distribution of SPC between control lactations and lactations with RTID can be seen in Fig 2.10B. This showed that there was a significant proportion of lactations in the RTID group took more than 3 services in order to become pregnant.
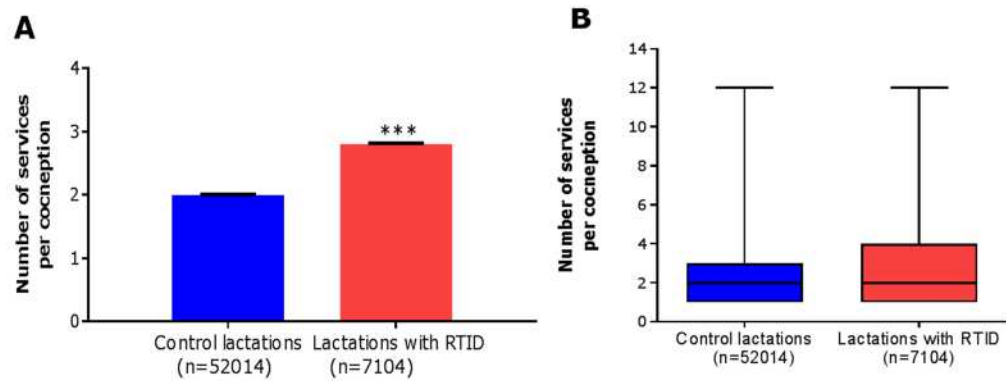
Figure 2.10: The association between lactations experiencing RTID during lactation and number of services per conception (SPC). (A) Service per conception in control cows was 0.9 services lower than those cows that experienced RTID (***;*P*<0.001). Data are mean±SEM. (B) Box and whisker plot comparing the variation in SPC for control and cows that experienced RTID. The mid line within the box represents the median of SPC and the whiskers represent minimum and maximum values.

Furthermore, a higher number of RTID cases were associated with an increased number of services per conception (*P*<0.001, Fig 2.11). Lactations in the RTID2 and 3 groups had similar number services per conception (~3.0 services) that was greater than both RTID 1 and control groups.
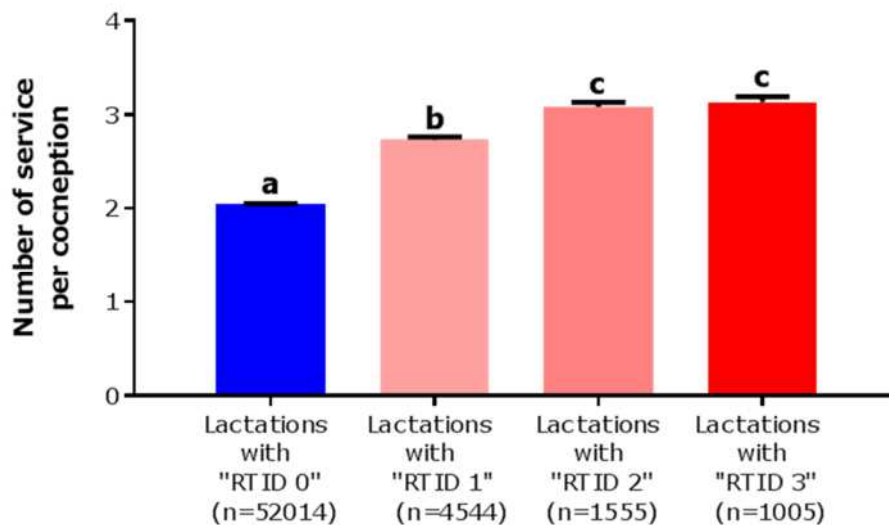
Figure 2.11: The association between number of services per conception (SPC) and incidence of RTID. Mean of SPC was different (*P*<0.001) between lactations with (RTID 0) and lactations with RTID 1, 2 and 3 (*P*<0.05). Differences between lactations are indicated by different letters a<b<c. Data are mean±SEM.

2.3.3.5 Calving interval (CI)

Lactations that experienced RTID during the lactation had a significantly longer calving interval compared to control lactations (*P*<0.001, Fig 2.12A) with 403.2 days for control lactations vs 425.6 days for lactations with RTID. The difference between the groups was 22.4±0.6 days. The distribution of CI in control lactations and lactations with RTID can be seen in Fig 2.12B and

revealed that the 25% and 75% quartile were greater in the RTID group. A higher number of RTID cases was associated with longer CI (P<0.001, Fig 2.13). Lactations with 1, 2 or 3 RTID cases had longer CI than control lactations by 19, 26 and 38 days, respectively.
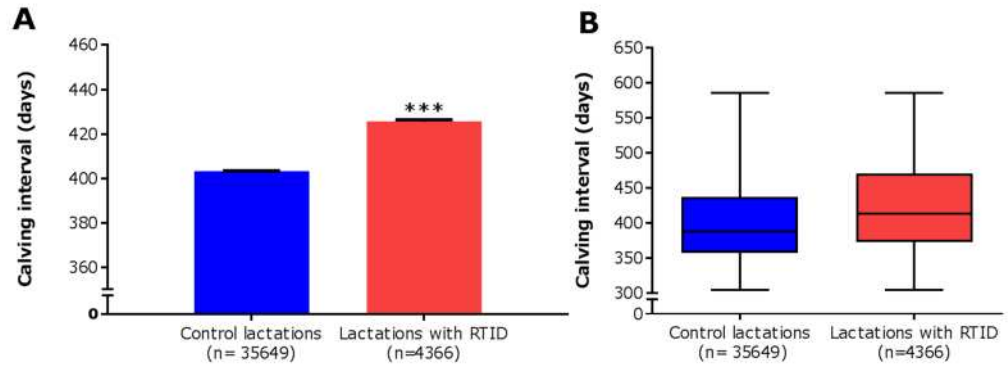
Figure 2.12: The impact of lactations experiencing RTID on calving interval (CI). (A) Calving interval in control lactations was 22.4 days lower than those lactations that experienced RTID (***;*P*<0.001). Data are mean±SEM (B) Box and whisker plot comparing the variation in CI for control lactations and lactations experienced RTID. The mid line represents the median CI which was 388 and 414 days for control and RTID groups, respectively. The whiskers represent minimum and maximum values.
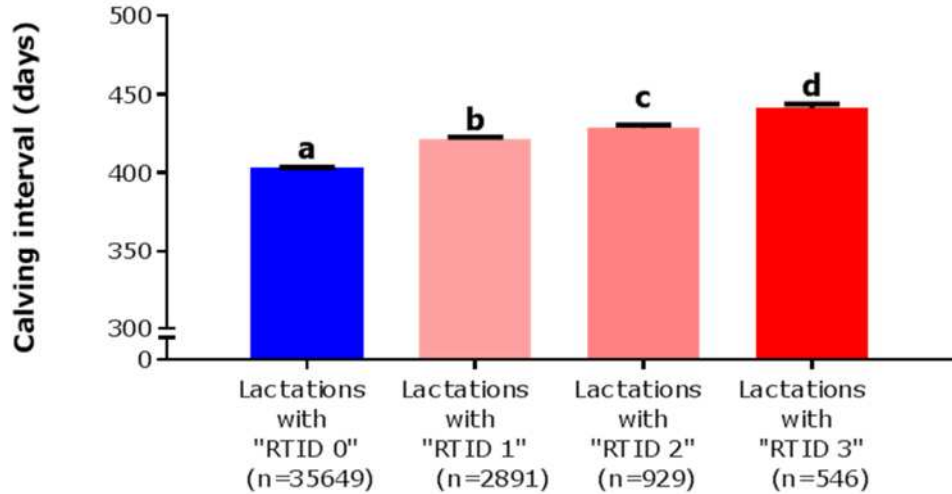
Figure 2.13: The association between calving interval and incidence of RTID. Mean calving interval was significantly different between control (RTID 0) and those with different number of RTID observations (*P*<0.05). Differences between lactations are indicated by different letters a<b<c<d. Data are mean±SEM.

2.3.3.6 Milk yield [305 day]

Lactations that had RTID during their lactation produced slightly more milk over 305 days (173L) than control lactations (P<0.01, Fig 2.14A). There was minimal difference in the distribution of milk yield in control lactations and those that experienced RTID during the lactation (Fig 2.14B). When the RTID lactations were split into their number of RTID cases, significance between the groups was detected (P<0.01, Fig 2.15). This showed no difference between the RTID groups, but that these were all greater than the control.
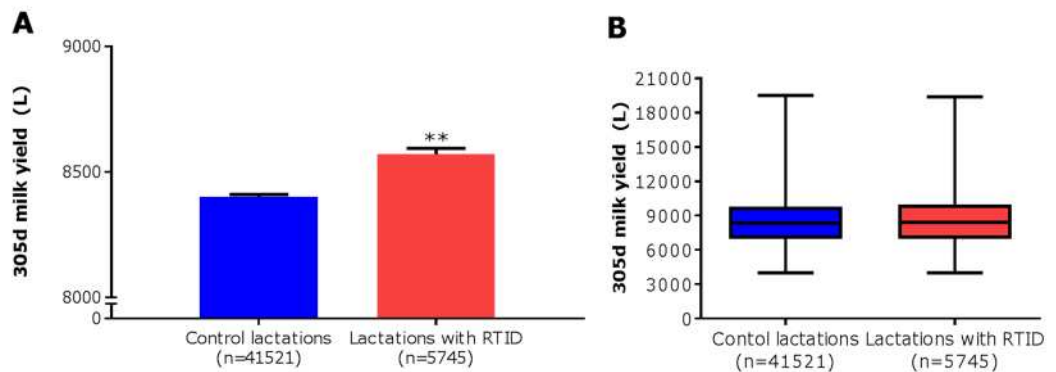
Figure 2.14: The association between lactations experiencing RTID on 305d milk yield. (A) 305d milk yield in control lactations was lower than those that experienced RTID (**;*P*<0.01). Data are mean±SEM (B) Box and whisker plots comparing the variation in 305d milk yield for control and RTID groups. The mid line within the box represents the median while the whiskers represent minimum and maximum.
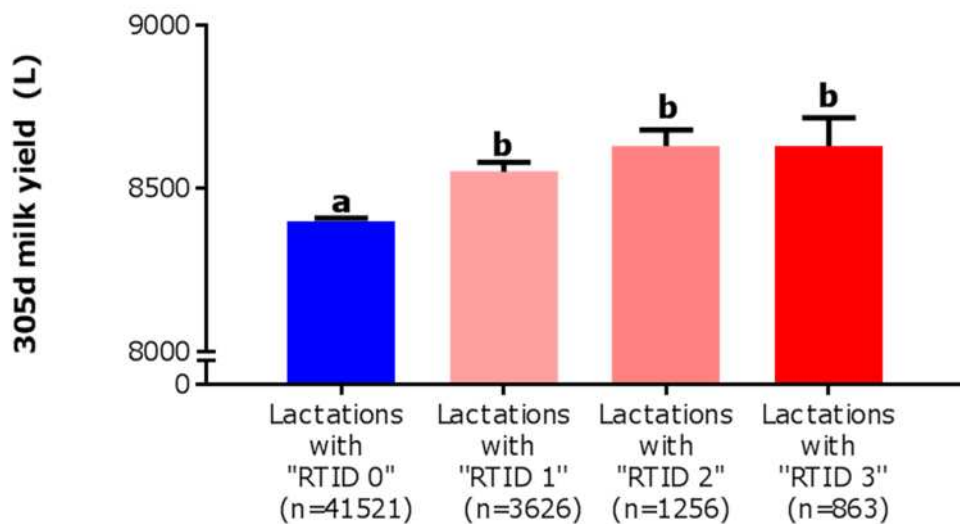
Figure 2.15: The association between 305d milk yield and number of RTID incidences. Mean 305d milk yield was lower in control lactations compared with 1, 2 and 3 observations of RTID, (a<b; *P*<0.01). Differences between lactations are indicated by different letters a<b. Data are mean ± SEM.

2.3.3.7 Exit rate from the herd

The overall herd exit rate was 21.7% per year (Table 2.7) and did not vary much between years from 2000 to 2007. The exit rate in the 80 different herds ranged from 5 to 35% (Fig 2.16). Lactations that experienced RTID exhibited a higher exit rate compared to control lactations by about 4 percentage points ($P<0.01$, Fig 2.17).

Table 2.7: The herd exit rate of lactations in 80 UK commercial dairy herds across England and Wales from 2000-7.

Year of calving	% of exiting rate	Total number of records
2000	20.4	2352
2001	21.9	2841
2002	17.8	4159
2003	21.1	6502
2004	20.2	7275
2005	19.6	11322
2006	24.4	11976
2007	21.9	12691
Total	21.7	59118
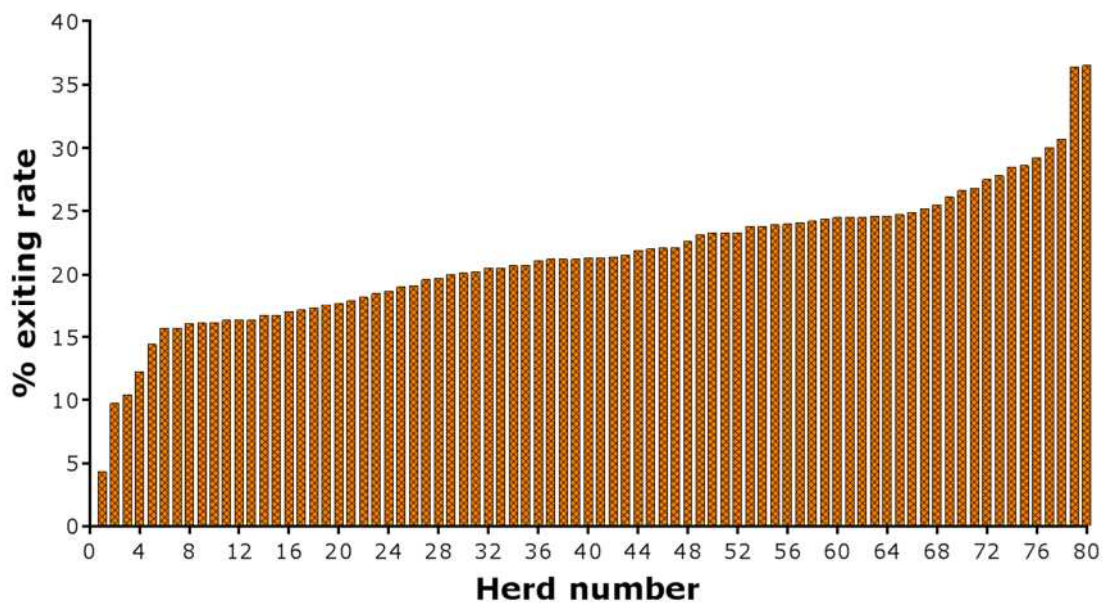
Figure 2.16: The variation in the exit rate between the 80 commercial dairy herds.
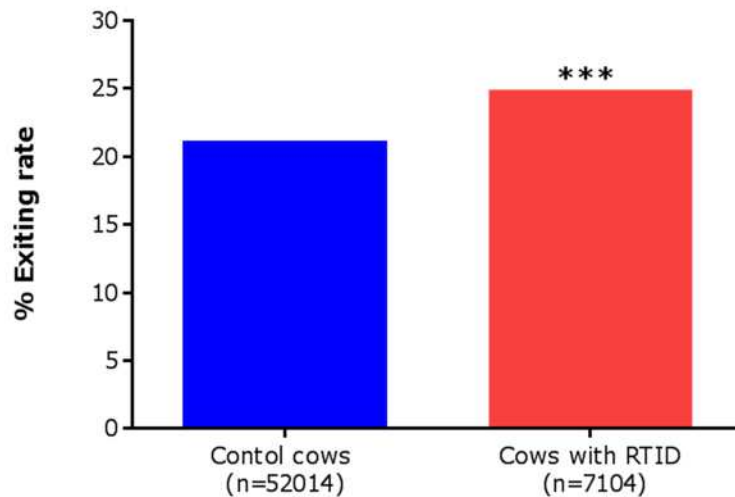
Figure 2.17: The herd exit rate in lactations that were positive or negative for RTID during that lactation. Cows with RTID had a higher cow exit rate (24.9±0.4) compared to control cows (21.2±0.2) (***;*P*<0.001). Data are mean.

When the RTID group was split into the number of reported RTID observations, the data showed that an increased number of RTID cases was associated with a higher exit rate (*P*<0.001, Fig 2.18). In those lactations with 3 RTID observations, the mean exit rate was over 30%, which is approximately 10 percentage points higher than control lactations.
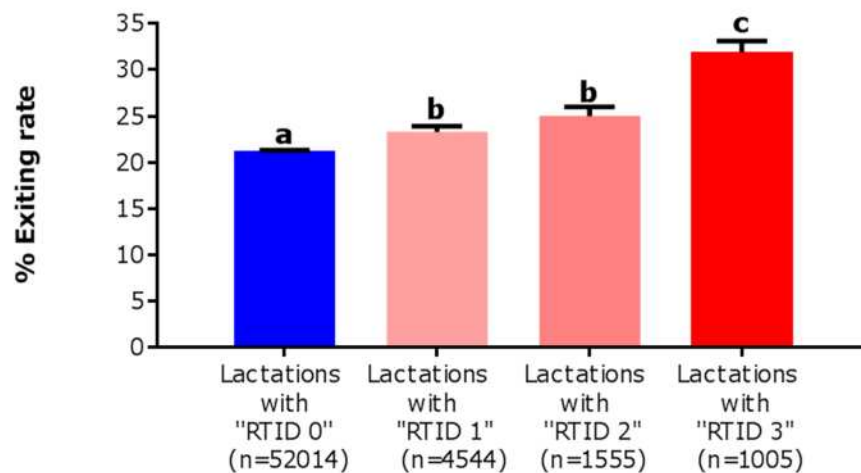
Figure 2.18: The association between exiting rate and number of RTID incidences. Mean exiting rate was different between lactations with (RTID 0) and lactations with RTID 1, 2 and 3 (P<0.001). Differences between lactations are indicated by different letters a<b<c. Data are mean ± SEM.

2.4 Discussion

2.4.1 Prevalence of RTID

The present study characterised the prevalence of RTID and their effects on reproduction of Holstein Fresian dairy cows in commercial dairy herds across England and Wales. This large-scale study is one of the few reports investigating RTID in Holstein dairy cows in multiple commercial herds across England and Wales. The mean prevalence of RTID in this study was 12%. Moreover, cows diagnosed with RTID had increased DFS (5 days), calving-conception interval (22 days), services per conception (about 1 service) and exit rate (4%). This was associated with reduced conception rate at first service. These results highlight the importance of having a sufficient health program throughout the transition and post-partum period in high yielding dairy cows.

The present study reported that the prevalence of RTID varied between herds ranging from 5-33%. The large range probably reflects differences in management between the herds analysed and the clinician's exact diagnosis definition. The overall prevalence of RTID in the current study was 12% across 80 commercial dairy herds in England and Wales. This was similar to Gautam et al. (2009), who reported 14.3% incidence of RTID during 29-60DIM in Japan but slightly lower than the rate (15%) previously reported in UK (Esslemont and Kossaibati 1996). It was 5 percentage points lower than that reported by (LeBlanc et al. 2002) in North America who diagnosed by vaginoscopy and cervical diameter from 20-33 DIM. Much higher prevalences were reported in the UK dairy herds (Esslemont and Peeler 1993) and in two Argentinean studies where the prevalence was 20-21% between 15-62DIM based on manual examination (Madoz et al. 2008, De la Sota et al. 2008). There are though a few studies reporting much higher rates of 29.4% in Ireland (5751 cows; diagnosed by ultrasound, Mee et al. 2009) and 37.5% in North America (975 cows, Ribeiro et al. 2013).

The difference in the prevalence of RTID in these studies are likely to depend on several factors. The most significant reasons are likely to be the different diagnostic methods employed and different times of RTID detection. Additionally, different characteristics of the herds and their different management practices will also have an influence (Kaneene and Miller 1995, Lewis 1997, Heuwieser et al. 2000, LeBlanc et al. 2002). The present study found that prevalence of RTID was significantly associated with retained fetal

membranes. Cows with retained fetal membrane had 38% greater prevalence of RTID than those without retained fetal membrane. The association between the retention of fetal membrane and uterine disease has been previously established (Kim and Kang 2003, Paisley et al. 1986, Potter et al. 2010). The current hypothesis is that the retained fetal membranes provide favourable medium and environment for bacterial growth.

2.4.2 Reproductive performance across all cows

To understand more about the management across the cows, the current study investigated the reproductive performance of cows as a whole. Cows with days to first service <50 DIM had an increased number of services per conception; but shorter calving to conception interval, and shorter calving interval compared to cows with days to first service 50-60 and>60 DIM. If fertility is measured by the average number of services per conception or conception rate at first service then fertility is clearly lower in cows bred prior to day 60. This is consistent with several other previous studies which have reported a lower conception rate at 1st service and a higher number of services per conception in cows first bred before 60 days postpartum (Williamson et al. 1980, Shannon et al. 1952, Dohoo 1983). However, the same studies demonstrated that there was no justification to delay breeding after 60 days postpartum, because cows served after 60 days had signifcantly longer CCI. It is interesting to note that cows with a calving interval <400 DIM had shorter days to first service, shorter calving to conception interval, higher conception rate at 1st service, lower number of services per conception and more milk production compared to cows with CI >425 days.

Calving to conception interval is regarded as a function of many factors, but of these, the management decision on when to start breeding following parturition may be the easiest to influence. However, calving to conception interval may not be a good indicator to measure reproductive status of the herd. It is difficult to calculate calving interval for cows that have exited the herd because there is no later calving; therefore, cows exiting the herd for reasons such as failure to conceive, or delayed rebreeding do not contribute to the herd's average CI value.

It is important to view the mean of the reproductive performance of animals. The mean of the three intervals included DFS, CCI and CI in the present study were 76.4; 124.1 and 405.6 days, respectively. Similar intervals were observed in the previous study by (Biffani et al. 2003). Generally, some factors have been attributed to extend these intervals such as, poor oestrus

detection, inadequate nutrition, poor housing and pathological problems such as uterine inflammation. In addition, the average number of services per conception in this study was 2.14 services/conception. The present thesis found that the conception rate at 1st service was 36.3%. A similar large-scale UK study by Royal et al. (2000), found that conception rates were 39.7% from 1995-98. While, Darwash et al. (1999) found that conception rate to any particular service were 40% from 1994-1997. This would indicate that there was a further decline in conception rates from the 1990s through to the 2000s. Though, the rate of this decline was less than previously predicted.

Not surprisingly, this study observed that cows with CI <400 days had better reproductive performance than cows with >400 days in terms of DFS, CCI, SPC and conception rate at 1st service. However, cows with calving interval of more than 400 days had higher milk production. This is in agreement with Smith and Becker (1996) who reported that cows with a calving interval >425 days produced more milk compared to cows with a calving interval <400 days. In contrast, Ratnayake et al. (1997) reported that extending the calving interval from 15 to 18 months may have a positive influence on the reproductive performance in terms of conception rate. It can be concluded for these studies that cows with shorter CI exhibited better fertility over a variety of indicators.

2.4.3 Impact of clinical RTID on reproductive performance

To achieve a high pregnancy rate in cows within an adequate period of time, there needs to be a successful and economically efficient reproductive management (Dijkhuizen et al. 1985, Plaizier et al. 1997). For that reason, a voluntary waiting period enables time for the ovarian cyclicity to be resumed and the uterus fully involuted, devoid from inflammation. This should increase the capability for conception, implantation and embryo survival. Generally, it has previously been agreed that clinical RTID impairs subsequent reproductive performance of dairy cows (LeBlanc et al. 2002, Sheldon et al. 2006).

An added level of complexity is that it is not only clinical RTID but also subclinical RTID that could impair fertility (Kasimanickam et al. 2004). Previously, it has been reported that RTID has a negative impact on fertility, in spite of appropriate treatments with PGF2α or locally administered broad-spectrum antibiotics (Sheldon et al. 2006). This large-scale study involving

over 59,118 lactations from commercial herds also showed the negative effects of RTID on subsequent reproductive performance.

In the current study, the mean days to first service was 5 days longer in cows that experienced RTID compared to control cows. Similar extensions to DFS were observed by LeBlanc et al. (2002) . However, other smaller scale studies also found longer DFS in RTID-affected cows by 23.3, 3, and 9 days respectively (Kim and Kang 2003, Williams et al. 2005, Gilbert et al. 2005). However, in some other studies reported that days to first service were not different (Dini et al. 2015). Moreover, this thesis found that there was a further increase in the days to first service with increased observations of RTID. The increased RTID observations are most likely the result of cows having persistent infection and/or failing to respond to the treatments.

The underlying mechanism which results in extended DFS remains to be fully elucidated. Cows with endometritis have lower peripheral plasma oestradiol concentrations due to slower growth of the first (and subsequent) postpartum dominant follicles which has been associated with lower peripheral plasma progesterone concentration after ovulation (Sheldon et al. 2002a, Williams et al. 2007, Battaglia et al. 1999, Peters et al. 1989). Cows which experienced RTID had a greater degree of NEB (Galvão et al. 2010a) and cows with NEB have lower circulating concentration of IGF-1 during the early postpartum period (Lucy 2000, Wathes et al. 2009).

The bovine endometrium expresses the IGF system (Llewellyn et al. 2008), which plays an important role in tissue repair, promoting proliferation and healing during uterine involution. In addition, cows with severe NEB during post-partum period had suppressed pulsatile LH secretion, suppressed ovarian responsiveness to LH stimulation and also decreases in the functional competence of the follicle characterized by low oestradiol production that subsquently leads to delayed ovulation (Zaleha et al. 2013). Another explanation for this, is that bacterial derived endotoxin lipopolysaccharide and/or various mediator cytokines stimulated following bacterial infection (e.g. tumour necrosis factor TNFA and interleukin (IL)-1) suppress the secretion of GnRH from the hypothalamus (Rivest et al. 1995, Battaglia et al. 2000, Williams et al. 2007). It is worth noting that DFS is influenced by management and VWP, thus the DFS does not necessarily reflect days to onset of cyclicity.

 In the current study, cows that experienced RTID took 22 days longer to become pregnant than RTID-negative cows. This difference is much greater

than observed for the DFS but is consistent with previous smaller scale studies (Toni et al. 2015, Giuliodori et al. 2013, Fourichon et al. 2000, Lincke et al. 2006, De la Sota et al. 2008, Mosquedq et al. 2011). However, it was much lower compared than (Gilbert et al. 2005) who showed that cows with RTID became pregnant 88 days later than cows without RTID. Increased freguency of RTID within the lactation had further detrimental effects on calving to conception interval. One explanation for this, is that RTID delays uterine involution resulting in delayed breeding time.

The likely effects of RTID on fertility are decreased oestrus behaviour which then may be missed by the farmer or an affect on the CL such that it does not produce enough progesterone to maintain pregnancy. Furthermore, animals with RTID need to be detected and treated efficiently as soon as possible to prevent problems later on. On dairy farms however, RTID diagnosis is performed by the veterinarian, often only during routine herd health checks. This means that in several cases, early warning signs of RTID go unnoticed until such time that the disease is in its full clinical stage. At this time, it has become chronic and much more difficult to treat.

The major contributor to impaired reproduction in RTID positive cows was a severe reduction in conception rate at 1st service. This study demonstrated that the 1st service conception rate was significantly higher in RTID-negative cows (38%) than RTID-positive cows (24%). These results are in agreement with a previous report by Gilbert et al. (2006) which demonstrated that cows with RTID had a lower conception rate (26%) compared to cows without RTID (36%). The meta-analysis by Fourichon et al. (2000) summarized the impact of RTID on reproductive performance from 24 studies in intensively housed conditions. The effect of RTID on reproductive performance was quantified as a 20 percentage point decrease in conception at 1st service. Moreover, the increased number of RTID recordings was associated with further detrimental effects on 1st service conception rates.

The reduction in 1st service conception rate might be associated with prolonged uterine tissue repair, attenuated uterine re-epithelisation and delayed uterine involution (Sheldon et al. 2003, Shrestha et al. 2004a) and/or decreased steroid production by the ovary (Green et al. 2011), and disturbance of ovarian cycles (Opsomer et al. 2000, Sheldon et al. 2002b, Herath et al. 2009). Alternatively, persistent bacterial infections could impair conception by changing the uterine environment resulting in impairment of sperm transport, the death of sperm and/or creatation of a hostile

environment to the subsequent embryonic development (Rahman et al. 1996).

In a similar manner, the number of services per conception increased to 2.9 for cows which experienced RTID (vs. 2.1 for control cows). In accordance to previous findings (Gilbert et al. 2005, Fourichon et al. 2000, LeBlanc et al. 2002) which reported that cows with endometritis had a greater number of inseminations per conception than cows without endometritis. The present study reported that RTID has a negative impact on calving interval. Cows experiencing RTID had a longer (426.4 days) calving interval than control cows (403.2 days) with a difference of 22.3 days. These results were different from the findings of Mosquedq et al. (2011) who reported that cows with RTID had longer calving interval by 38 days. Abnormally lengthened calving intervals due to RTID have been shown to be related to low productivity and lead to serious economical implications for the herds as productivity of the livestock farm is decreased severely (Lee and Kim 2007, Tayebwa et al. 2015).

The present study demonstrated that cows with RTID produced slightly more milk in 305d compared to cows without RTID. Thus is in accordance with a study by Gröhn et al. (1990) who reported that milk yield increased the risk of metritis, retained placenta, days to calving interval and ovarian cysts. The reasons beyond this increase are unknown, but it might be due to cows with RTID having prolonged calving to conception interval, and thus have a longer lactation curve. However, cows with uterine infection had lower milk production for the first 100 days in milk (Bell and Roberts 2007). They suggested this was due to reduced daily dry matter intake. There is a clear association between high milk yield and increased risk of negative energy balance that begins prior to calving and extends into early lactation (Knop and Cernescu 2009, Butler 2012).

Negative energy balance (NEB) has been linked to impaired polymorphonuclear neutrophil (PMN) function (Hammon et al. 2006). It has been reported that severe negative energy balance leads to reduced circulating white blood cells (WBCs) especially lymphocytes, and increased gene expression for inflammatory mediators at 14DIM (Wathes et al. 2009). It should be noted that other reports have shown that cytological endometritis and purulent vagina discharge were not associated with milk yield (Dubuc et al. 2011, Fourichon et al. 2000).

When to remove a cow from the herd is regarded as one of the most complex decisions dairy farmers make. The most likely reason for exiting the herd was culling with both biological and managerial factors influencing the dairy farmer's decisions. There are different aspects considered in culling decisions such as animal's health, fertility (or lack of), milk yield, and stage of lactation as well as the value of the replacement animal and it's cost (Barkema et al. 1992, Beaudeau et al. 1994). The present study demonstrated that the average exit rate was 21.7%. The rate of exited animals in this study was unaffected by calving year. This was similar to the previous UK studies which reported culling rates of 22.1% (Whitaker et al. 2000) and 23.8% (Esslemont and Kossaibati 1997). This suggests that the standard culling rate in the UK therefore, seems to be fairly steady about 22%. However, these are much lower than that reported by Lee and Kim (2007) with a culling rate of 46.4%, which was twofold higher compared to current study.

The observed exiting rate was significantly different between control cows and those that experienced RTID during the lactation. Furthermore, cows with RTID were 1.2 more as likely to exited in herds as cows without RTID. Similarly, Opsomer and de Kruif (2009) reported cows with clinical endometritis to be 1.2 times more likely to be culled for reproductive failure. However, the difference was lower than expected and did not agree with Beaudeau et al. (1994) who demonstrated that more than half of all culling were related to health disorders. Furthermore, the effect of RTID on exit rate was less compared to previous reports by Plöntzke et al. (2011) and LeBlanc et al. (2002) who reported that cows with clinical endometritis were 1.6 and 1.7 times, respectively as likely to be removed from herd as cows without clinical endometritis.

2.5 Conclusion

Overall, this study quantified the reproductive performance in 80 commercial dairy herds across the England and Wales. The incidence of RTID and its impact in extensive dairy production system in England and Wales was similar to previously published data from dairy farms with intense production systems. In this study, postpartum RTID had a significant influence on the subsequent reproductive performances in dairy cows. Cows which experienced RTID were less likely to become pregnant, required more services per conception and were more likely to be removed from the herd. This study has quantified the negative associations between cows experiencing uterine disease and subsequent reproductive performance. The biggest relationship appearing to occur on the ability of cows to re-conceive. To our knowledge this was the first study determine the association between RTID and reproductive performance in multi-commercial dairy herds England and Wales. Therefore, the next chapter will study on the effect of RTID on the reproductive performance of dairy cows in the University of Nottingham dairy centre.

3 The association between uterine disease and subsequent reproductive performance at the University of Nottingham and postpartum ovarian cyclicity

3.1 General introduction

In dairy cows, one calving each year is generally accepted as the optimum calving interval to maximise economic profit (Dijkhuizen et al. 1985). To achieve this, cows should resume their ovarian cycles within 5 weeks post calving and conceive within 85 days post-partum. The previous chapter showed that in the immediate postpartum period approximately 12% of dairy cows developed RTID and that this inhibited reproductive performance and impaired fertility. Endometritis is regarded as the principle infectious disease of the uterus and is caused by persistent bacterial infection after calving (Runciman et al. 2008, Sheldon et al. 2006, Barlund et al. 2008, Sheldon et al. 2009a, Gilbert et al. 2005). Chapter 2 indicated that RTID was associated with an increase in days open (22 days), increase numbers of services per conception (0.9 services) and an increased risk of culling (4%). Often, the real impact of endometritis is only measured by subsequent statistical analysis, while the economic impact of this disease remains to be quantified, but it is speculated to exceed millions of pounds annually for the UK dairy industry alone (McNaughton and Murray 2009).

The mechanism by which uterine infection with bacterial endotoxin during the post-partum period adversely affects fertility is likely to be multi-factorial influencing fertility both locally and systematically. For example, there is a disruption in the production of $PGF_{2\alpha}$ and PGE_2 by the endometrium (Herath et al. 2009). At the same time, there is an alteration to the immune system mediators and production of cytokines, which can affect the function of hypothalamus and pituitary gland (Sheldon et al. 2009a). An important observation was that uterine infection affected follicular growth and function (Sheldon et al. 2002b). Indeed, the bacterial endotoxin, LPS has been detected in the follicular fluid. There has been an association between clinical signs of uterine inflammation and post-partum ovarian cyclicity (Opsomer et al. 2000, Mateus et al. 2003, Shrestha et al. 2004a, Taylor et al. 2003, Williams et al. 2007, McCoy et al. 2006).

Over the last few decades, milk progesterone monitoring has been used widely for assessment of ovarian function, reproductive patterns and abnormalities in dairy cows (Bulman and Lamming 1978, Bulman and Lamming 1979, King et al. 1976). It has also investigated the impact of environmental factors on reproductive fertility. Previous studies have revealed that cows with an early onset of luteal activity (OLA) after calving had an increased chance of an earlier 1st service, shorter interval to conception, and higher conception rates (Darwash et al. 1997). However, early OLA has also been regarded as a risk factor for irregular luteal cyclicity in a form of a persistent corpus luteum (Opsomer et al. 2000, Petersson et al. 2006).

Cows with abnormal ovarian cycles have a significantly longer calving to first service interval, lower conception rates to 1st service, longer calving to conception interval and increased number of services per conception. Reduced conception rates have been related to lower post-ovulatory progesterone concentration or reduced number of cycles before insemination (Shrestha et al. 2004a, McCoy et al. 2006). In the United Kingdom, the reduction in pregnancy rate from 1975 to 1998 was related to an increased the incidence of atypical progesterone profiles (Royal et al. 2000). Prolonged luteal phases (high progesterone levels for >20 days in absence of pregnancy) were considered to be the most common type of irregular ovarian cycles. The incidence of prolonged luteal phase has increased from the 1970s to 1980 in Belgium and Japan (Lamming and Darwash 1998, Opsomer et al. 1998, Shrestha et al. 2004b).

It has been reported that prolonged luteal phases were associated with an abnormal uterine environment which perturbed the production of prostaglandins (Opsomer et al. 1998). The risk factors for prolonged luteal phase in cows were metritis (odd ratio (OR) =11.0), abnormal vaginal discharge (OR=4.4), retained fetal membrane (OR=3.5) and parity (increased in multiparous) (Opsomer et al. 1998, 2000). This supported the concept that prolonged luteal phases are primarily associated with uterine problems rather than follicular dysfunctions. In this chapter, two experiments were performed to investigate these associations further.

Hypothesis

RTID is associated with a negative impact on post-partum reproductive performance and increased abnormal post-partum ovarian activity. This hypothesis was tested by conducting detailed investigations (herd performance, clinical data and endocrinological) on a high-yielding dairy herd

in the UK. The herd chosen was University of Nottingham dairy centre whose 256-cow herd averaged a 305d milk yield of 10306L

3.2 Experiment 1: The association between RTID and reproductive performance at the University of Nottingham dairy centre

3.2.1 Objectives

The detailed investigations were broken down into the following objectives:

1. To quantify the incidence of RTID in the herd form cows calving between 2008 and 2014.

2. To assess the current status of fertility performance at the Nottingham University dairy centre

3. To assess the impact of cows experiencing RTID on the following fertility and production traits:

   - Day to first service (DFS)
   - Calving to conception interval (CCI)
   - Conception rate at first service
   - Number of services per conception (SPC)
   - Calving interval (CI)
   - Milk yield 305d

3.2.2 Materials and methods

3.2.2.1 Experimental animals

The present study involved 256 cows (n=708 lactations) British Holstein dairy cows calving from 2008 to 2014, at the University of Nottingham dairy centre. The parity of the cows ranged from 1-7. Animals were classified into two groups according to their veterinarian reported uterine health status: (1) control cows (n=603 lactations, these were not recorded RTID during lactation) and (2) cows with RTID (n=105 lactations). The following information was collected from Uniform Agriculture computer software programme for each animal:

- Current animal number
- Ear tag number
- Calving date
- Inseminated date
- Reproductive diagnosis interventions

Cows with RTID were treated by a veterinarian deemed appropriate for that particular case no experimental interventional introduced. Treatments were used included: Antibiotics such as (Naxcel® injection (S/C) and Metricure syringe (intrauterine) and hormonal treatments such as PGF2α. Cows (n=58) left the herd due to multifactorial causes.

3.2.2.2 Feeding and management

All animals were managed under the herd's standard procedures. The cows were housed continuously indoors, calved all year round and were kept in a loose house with individual rubber-matted cubicles. The cows were fed *ad lib* on a total mixed ration based on maize and grass silage and a variety of additional ingredients such as rolled wheat, sugar beet pulp nuts, molasses and mineral supplements. Additional concentrates were fed in robotic milking machines according to milk yield. Cows were milked by robot on average 3 times per day. Volume of milk yield at each milking was recorded and stored in the database and then transferred each month. All breeding was performed by artificial insemination at natural oestrus (n=1868) and all inseminations were recorded by the herdsperson. Pregnancy diagnoses were based on routine methods such as transrectal uterine ultrasound and rectal palpation by a veterinarian according to normal herd practice. This was routinely done from 30–60 days after the cows have been inseminated. Any post-partum problems or abnormalities such as RTID were recorded by the veterinarian or herdsmen. Once abnormalities were investigated, a therapeutic management plan (including antibiotics or hormones) was initiated.

3.2.2.3 Definition of fertility parameters

Fertility traits for both primiparous and multiparous Holstein dairy cows used for comparison of reproductive performance between control cows and cows that experienced RTID from 2008-2014 are explained in Table 3.1.

Table 3.1: The definition of fertility traits.

Fertility trait	Definition
Day to first service (DFS)	Number of days from parturition to the first insemination after calving
Conception rate at first insemination	The percentage of the number of cows that became pregnant at the first insemination.
Conception rate at second insemination	The percentage of the number of cows that became pregnant at the second insemination.
Conception rate at third insemination	The percentage of the number of cows that became pregnant at the third insemination.
Calving to conception interval (CCI)	Number of days from calving to conception
Number of services per conception (SPC)	Number of inseminations required for a cow needs to become pregnant.
Calving interval (CI)	Number of days between two subsequent calvings.

3.2.2.4 Data processing

All animal data included (calving date, any calving difficulties, insemination dates, pregnancy diagnoses outcome, milk production, the occurrence of diseases during post-partum period and date of treatments) were recorded by the herdsmen into the Uniform Agriculture computer software programme. Before statistical analysis was performed, these data were imported into Microsoft Excel 2010 (Microsoft Corporation, 2010) individually. The presence/absence of RTID and pregnancy diagnosis outcome were coded into dichotomous values with "1" or "0" as appropriate. In order to compare groups of cows with different fertility traits the following categories were created based on: (1) Days to first service and (2) calving interval (Table 3.2).

Comparisons between "control" cows with different reproductive performance

Within control group only, three sub-groups were created depending: 1) on the cows' day to first service interval (DFS) as follows: <42, 43-70 or >70 days; 2) calving interval as follows: <365, 365-400 and ≥401 DIM. For these sub-groups, reproductive parameters analysed were calving to conception interval (CCI), conception rate at 1st service, number of services per conception and milk yield 305d.

Table 3.2: The number of lactations in different reproductive performance categories.

Reproductive performance category	Number of lactations
Number of services	
1-10 services	708
Days to first service	
19-160	708
Days open	
20-353	664
No data	44
Calving interval	
<365 days	257
365-400 days	144
≥401 days	250
No data	57*
Milk yield 305 d	
5000-20000	268
No data	440**

*Includes cows that were removed and thus no calving interval

**Includes cows that were removed and thus no complete 305d milk yield, and data that was not recorded into dairy record.

RTID and its association with subsequent reproductive performance

Data from each lactation were entered and stored in Microsoft Excel 2010 (Microsoft Corporation, 2010). Dichotomous values were used for pregnancy outcome at 1st insemination, RTID status where "1" indicated they were pregnant/had RTID were coded into "0" when animals did not conceived at 1st service and/or free from disease.

3.2.2.5 Statistical analysis

The normality of the data was checked using residual plots and appropriately transformed where required.

The prevalence of RTID

The prevalence of RTID between calving years, and lactation number were analysed using binomial logistic regression in GenStat 16th Edition (VSN International Ltd, Hemel Hempstead, UK).

Comparisons between control cows with different reproductive performance

In order to assess the reproductive performance of cows with different DFS values (<42 (early), 43-70 (normal), >70 (late) DIM), one-way ANOVA was performed with the DFS categories as the factor. Similarly, one-way ANOVA was conducted to compare reproductive performance of cows with different CI values (<365, 365-400 and >401 DIM). One-way ANOVA was used to compare SPC between the different DFS and CI categories. Binomial logistic regression was performed to compare conception rate at 1st, 2nd and 3rd service between DFS groups and CI groups.

The association between RTID and subsequent reproductive performance

In addition, univariate, unbalanced ANOVA was performed to determine the association between RTID status (included as the factor) and reproductive performance (DFS, CCI, SPC and CI), as a response variable, blocked by the cow identity. Kaplan-Meier survival analysis was performed to compare calving to conception interval between cows that experienced RTID and those that did not. The proportion of non-pregnant cows represented the survival probability, which was plotted against the time (calving to conception interval). Logistic regression was performed to compare conception rate at 1st, 2nd and 3rd service between control cows and cows experienced with RTID. All candidate variables were kept in the model with significant attributes at $P<0.05$.

3.2.3 Results

3.2.3.1 Prevalence of RTID

The results demonstrated that the prevalence of RTID was significantly different between calving years (P<0.01; Fig.3.1A). The mean prevalence of RTID across the whole study was 14.8%. The highest incidence was observed in 2008 and 2009 at 25, 55.6%, respectively. Thereafter, the prevalence has been lower between 6-18%. It should be noted that there were fewer cows in 2008, 2009 and 2010 database. However, this study did not find any difference in the prevalence of RTID between the different lactation numbers (P>0.05; Fig. 3.1B).
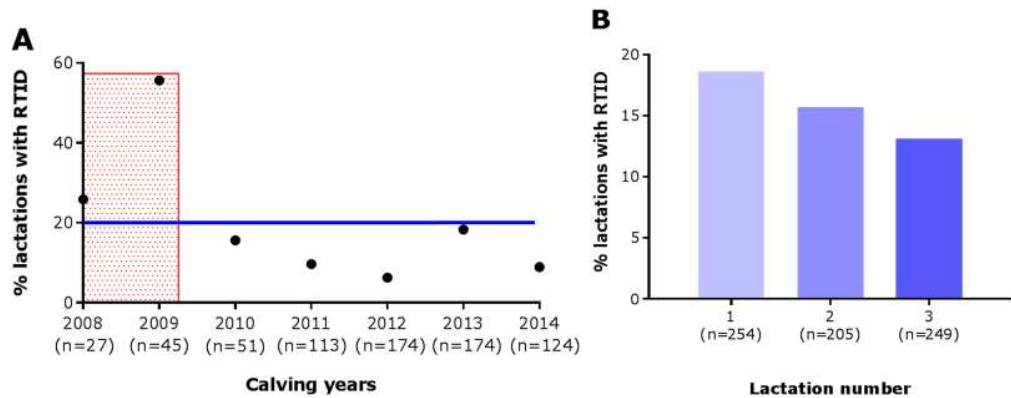
Figure 3.1: The prevalence of RTID in lactations at Nottingham dairy centre. (A) Effect of calving years on the prevalence of RTID. The highest percentage was seen in 2008 and 2009 (highlighted red column). (B) Incidence of RTID across different parities. There was no difference in the RTID incidence between parity (P>0.05).

3.2.3.2 Reproductive performance of the herd at the University of Nottingham dairy centre

This study performed descriptive statistics to measure reproductive performance across the herd. The mean values, range and standard deviation for the different endpoints are shown in Table 3.3.

Table 3.3: Descriptive statistics for production variables for all 708 lactations at the University of Nottingham dairy centre from 2008-2014.

Variable	Minimum	Maximum	Mean	SEM	Number
Days to first service	19	160	58.4	0.87	708
Calving to conception interval (days)	27	353	119.6	2.7	664
Service per conception (n)	1	10	2.98	0.08	708
Calving interval (days)	283	583	397.1	2.6	651
Milk yield 305 (L)	5000	20000	10214	157.0	271*
Conception rate at 1st AI	29.5%				
Conception rate at 2nd AI	35.3%				
Conception rate at 3rd AI	39.6%				

*= low number of data due to 58 lactations were removed before completed 305d, others lactations had no data.

Lactations with the early DFS (<42 days) required nearly one more service in order for them to conceive versus DFS >70 days ($P<0.05$; Table 3.4). Significant differences in conception rate between the different DFS categories were detected ($P<0.05$; Table 3.4). Lactations served after 70 days postpartum had 15% higher conception rate at 1st AI compared to cows served earlier ($P<0.05$, Table 3.4). However, there was no difference in 305d milk yield between lactations with different DFS range ($P>0.05$, Table 3.4).

Table 3.4: Mean±SEM for days to first service, services per conception, calving to conception interval, calving interval, conception rate at 1st AI, and 305 day milk yield with different days to first service: <42; 43-70 and >70days.

Reproductive performance	Days to first service category			
	≤42 days	43-70 days	>70 days	*P* value
Days to first service	36.1±1.1 n=165	54.3±0.6 n=384	92.3±1.0 n=159	<0.001
Number of services per conception	3.2±0.1 n=165	2.9±0.1 n=384	2.5±0.1 n=159	0.028
Calving to conception interval (days)	107.7±5.6 n=152	114.2±3.5 n=360	142.7±5.4 n=152	0.001
Conception rate at 1st AI	24.3%	28.3%	39.6%	0.03
Calving interval (days)	385.7±5.4 n=149	382.7±3.4 n=354	418.7±5.3 n=148	0.002
Milk yield 305 days (L)	10240±418.8 n=142	10665±260.6 n=79	10178±384.6 n=114	n.s.

A similar study was performed to compare the herds fertility performance by categorising lactations according to their calving interval. Lactations with the

shortest CI (<400 days) had an approximate 11 day shorter DFS compared to lactations with the longest CI (P<0.001; Table 3.5). The striking observation was that cows with the shortest CI had the least number of services to conceive (P<0.001; 2-3.7 services less) and had a significantly higher conception rate at first service (P<0.001) by over 40 percentage points. However, intriguingly, there was no difference in 305d milk yield between lactations with different CI (P>0.05; Table, 3.5).

Table 3.5: Mean±SEM days to first service,service per conception, calving to conception interval, calving interval, conception rate at 1st AI, and milk yield 305d of lactations with <365; 365-4200 and >401d CI.

Reproductive performance	Calving interval (CI) category			
	<365 days	**365–400 days**	**>401 days**	**P value**
Calving interval (days)	354.8±1.7 n=398	412.2±3.9 n=79	486.3±2.7 n=174	<0.001
Days to first service	55.3±1.1 n=398	64.8±2.6 n=79	66.3±1.8 n=174	<0.001
Number of services per conception	1.7±0.1 n=398	3.1±0.2 n=79	5.1±0.1 n=174	<0.001
Calving to conception interval (days)	76.4±1.8 n=398	132.1±4.1 n=79	210.3±2.8 n=174	<0.001
Conception rate at 1st AI	46.6%	10.8%	2.2%	<0.001
305 day milk yield (L)	10203±263.9 n=143	9981±552.6 n=34	10852±362.5 n=79	n.s.

3.2.3.3 Reproductive performance between control cows and cows positive with RTID

Days to first service, calving to conception interval, number of service per-conception and calving interval

Lactations that experienced RTID had a longer day to first service compared to control lactations by about 11 days. The mean for the control lactations was 57.4±0.9 days to first service in comparison with lactations positive with RTID where it was 68.3±2.1 days (P<0.01; Fig. 3.2A). However, no difference was seen between control lactations and lactations in which RTID was experienced in terms of CCI, SPC and CI (P>0.05; Fig. 3.2B-D).
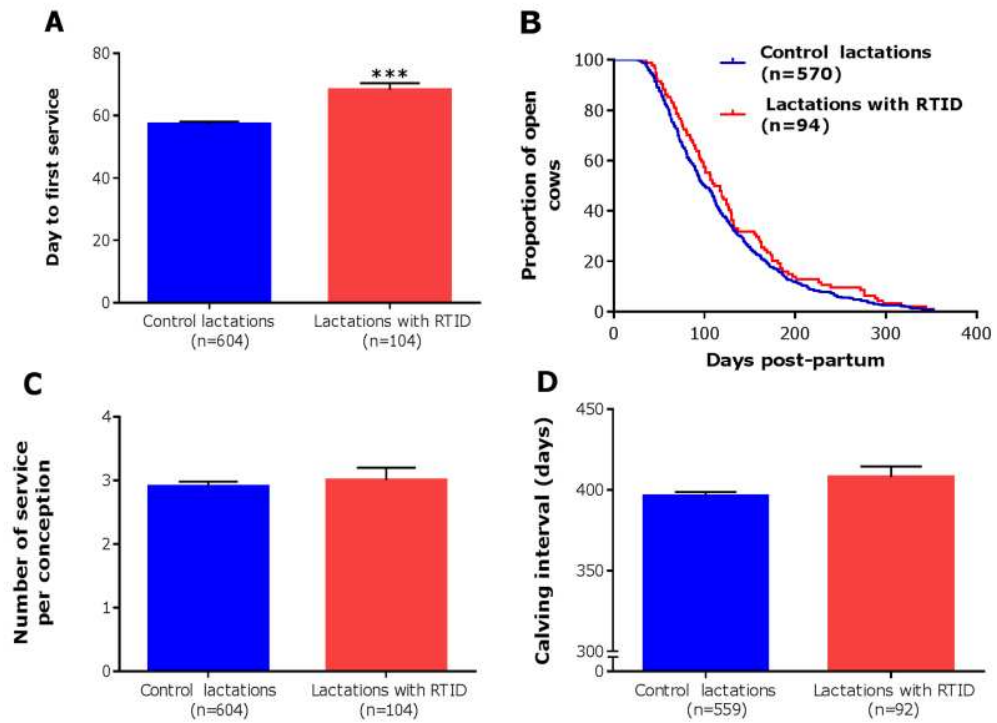
Figure 3.2: Impact of lactations having RTID post-partum on reproductive indices. (A) Days to first service (DFS) was greater in lactation that had RTID and those that did not (*P*<0.001). (B) Kaplan-Meier survival analysis of days open between RTID and control lactations. Median day for lactations with RTID (114.0 days), vs control lactations (100.5 days) (*P*>0.05). (C) Number of services per conception (SPC) was not different between lactations that had RTID (3.0±0.2) and those that did not (2.9±0.1, *P*>0.05). (D) Calving interval was not different between control lactations (396.1±2.6) vs lactations positive with RTID (407.9±67, *P*>0.05).

Conception rate at first service

There was an increase in the conception rate with increasing service number (P<0.05). This study demonstrated that RTID had no significant effect on conception rate at 1st, 2nd and even at 3rd service (*P*>0.05; Table 3.6).

Table 3.6: The conception rates at 1st, 2nd and 3rd service between cows that experienced RTID and those that did not.

Conception rate	Control cows	RTID cows	P value
At 1st service	28.9%	34.1%	0.525
At 2nd service	34.8%	31.9%	0.964
At 3rd service	41.1%	29.2%	0.321

Milk yield 305d

The results showed that control lactations produced higher 305d milk yield compared to the lactations experienced with RTID (P<0.01; Fig.3.3).
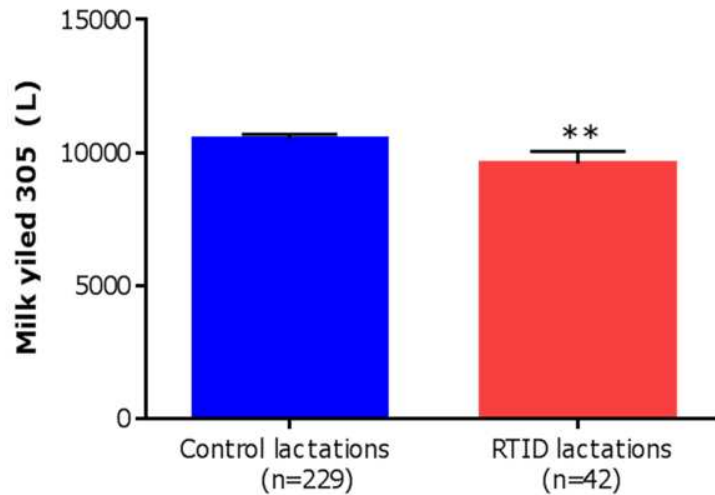
Figure 3.3: Effect of lactation having RTID on 305 d milk yield. The average of 305d milk yield was higher in the lactations with RTID vs lactations experienced with RTID (P<0.01).

3.2.4 Discussion

The study examined the prevalence of RTID in the University of Nottingham dairy centre from 2008-2014, and the effect of RTID on the post-partum reproductive performance of dairy cows.

3.2.4.1 Prevalence of RTID

The prevalence of RTID in the present study was 14.7%, which was close to the findings in the Chapter two. This was not too far from the findings of previous other studies (Chunjie et al. 2013, De la Sota et al. 2008, LeBlanc et al. 2002), they reported prevalence of endometritis were 17.4%, 20-21% and 17%, respectively.

However, the result of this study was lower compared to study of Ribeiro et al. (2013) who observed prevalence of clinical endometritis 37.5% and 59.0% of subclinical endometritis in North-America (from 957 lactating multiparous dairy cows). Furthermore, Plöntzke et al. (2011) reported higher prevalence of

endometritis 35% than in the present study. The variation in the incidence of endometritis between studies makes it challenging to define what the current situation is. This is hindered by different management systems under which the animals where kept and probably more importantly the different diagnostic methods used that are used at different times postpartum period (Heuwieser et al. 2000, Kaneene and Miller 1995, Lewis 1997).

There are equivocal reports about the effect of parity on the prevalence of RTID. The present study reported that parity did not influence prevalence of RTID. This is supported by several other studies that also demonstrated that parity had no effect in prevalence of endometritis (Matiko et al. 2008, Noakes et al. 2001, Nakao et al. 1997, Hare et al. 2006). In contrast, LeBlanc et al. (2002) observed that parity had an effect on the prevalence of endometritis, and demonstrated that a higher risk has been described for cows in the third or higher lactation in intensive high-yielding dairy systems. Conversely, McDougall (2006) reported that primiparous cows are more at risk than multiparous cows in more extensive farming conditions. It can be speculated that while in some conditions, parity has an impact on the prevalence of endometritis it is likely to be a relatively minor player.

The present study found that the prevalence of RTID was greater in 2008 and 2009 compared to other years. Many previous studies have been published about different risk factors for prevalence of endometritis such as calving difficulties (normal vs abnormal); metabolic disorders; calving season; retained placenta; negative energy balance and day in milk. There was no specific reason reported which accounts for the higher levels, but it might be related to the different environment conditions between years, or the quality of the feed.

Current fertility parameter in The University of Nottingham dairy centre

Prior to investigating the association between RTID and reproductive performance in dairy cows, this study described the fertility parameters at the herd level. The present study used a range of indices to describe reproductive performance of cows within a herd. The mean interval from calving to first service on herd level was about 58.4 days. This interval falls at the lower end of the recommended range of 50 to 70 days after calving (Noakes et al. 2009) and was lower than that reported in Chapter 2 across multiple herds in the UK. This

could reflect the herd's management strategy to serve earlier than most herds or that the cows in this herd returned to cyclicity relatively early. Interestingly, though cows with mean DFS >70 days had fewer services per conception and higher conception rates at 1st service compared to cows with <42 days. This agrees with Chapter 2 and is supported by previous reports (Williamson et al. 1980, Shannon et al. 1952, Dohoo 1983, Tenhagen et al. 2003, Řehák et al. 2009, Buckley et al. 2003). This might be due to that uterine tract was fully recovered from previous calving and conception results were normally high.

The mean calving to conception interval and calving interval in the present study were 120 and 397 days, respectively. This was lower than 405.6 days in Chapter 2. Cows with <400 days had lower number of services, and higher conception rate at first service compared to cows with longer CI >400. Similar to the previous chapter which reported that cows with <400 days CI, had better performance than cows with longer than >425 days CI. However, this parameter had no effect on reproductive performance of dairy cows (Ratnayake et al. 1997).

The overall mean number of services per conception and conception rate at 1st service was 2.98 services and 29.5%, respectively. These observations were lower than findings in the Chapter 2 (36.3%). This study supports the previous report by Royal et al. (2000) who demonstrate that pregnancy rate at first service decreased from 56% in 1975–1982 to about 40% in 1995–1998 in the UK, a decrease of about 1% per year. It has been reported that days to first service has a significant effect on conception rate at first service in dairy cows (Tenhagen et al. 2003, Řehák et al. 2009, Buckley et al. 2003). In the current study, this parameter did affect conception rate at first insemination, and it found that cows inseminated after 70 days had significantly higher first service conception rate than inseminated before 42 days. The reason is likely to be due to that uterine tract in cows inseminated before 2 months was not fully restored from previous calving. In the present study, mean 305d milk yield was 10306 litres, which was higher than the mean of 305d milk yield of 8425 liters shown in Chapter 2. This indicated that this herd was a very high-yielding herd.

3.2.4.2 Impact of RTID on reproductive performance of dairy cows

It should be noted that this retrospective study did not check cows for subclinical endometritis, thus the reference control cows may have included some animals having subclinical endometritis which may have also affected reproductive performance and, for that reason, this study could have underestimated the effect of RTID on reproductive performance. Those cows that experienced RTID had longer days to first service than control cows about 11 days. This is in strong agreement with reports that cows with RTID had longer DFS compare to cows without RTID (Lincke et al. 2006, Borsberry and Dobson 1989, LeBlanc et al. 2002). This was relatively longer than Gilbert et al. (2005) who reported that cows with clinical endometritis had 5 days longer compared to cow that did not have clinical endometritis. This was similar to that observed in Chapter 2. The causes of extended days to first service in cows with RTID in this chapter is likely to be due to cows having a shorter voluntary waiting period thus cows less likely to have fully recovered before 1st service.

It is interesting to note that, this study did find any significant effect of RTID on calving to conception interval; service per conception, conception rate at 1st service and calving interval. All these were adversely affected numerically in similar way to Chapter 2. In accordance to the previous studies by Kinsel and Etherington (1998); Galvão et al. (2009) and (Marques et al. 2015) they did not find an effect of endometritis on calving to conception interval. Moreover, in agreement with Kasimanickam et al. (2006) who found that the presence of endometritis has no effect conception rate at first insemination. The results of the present study were contrast to the findings of LeBlanc et al. (2002); Gilbert et al. (2005); Lincke et al. (2006) and the results of Chapter 2. Alternatively, the reasons in the present study did not find an association between RTID on reproductive performance might relate to: 1) good management factors, highly efficient and early diagnosis of uterine disease, and efficient treatments; or 2) due to limited numbers to fully determine significance.

The present study found that there was a significant difference in milk production between cows which experienced RTID and control cows. This was similar to the findings of Bell and Roberts (2007) who concluded that post-partum uterine infection was associated with reduced feed intake, which would explain the

decreased milk yield and lower milk protein composition. Dubuc et al. (2011) and Mee et al. (2009) showed that metritis was associated with decreased milk yield in multiparous cows, but not in primiparous cows. Another study by Ribeiro et al. (2013) using daily milk data from 500 cows in one herd demonstrated a negative impact of metritis on milk production in all cows, and found that cows with metritis produced less milk. Control cows produced more 305d milk than cows with endometritis. In contrast to chapter 2 which reported that cows with endometritis produced more milk in 305d compared to control cows.

3.2.5 Conclusion

Prevalence of RTID at the University of Nottingham Dairy Centre was 14.8%. In this herd, cows with RTID had extended days to first service by 11 days. However, RTID had no adverse effect on the subsequent reproductive performance in dairy cows such as calving to conception interval, service per conception, conception rate at 1st service and calving interval. Additionally, cows that had RTID had lower 305d milk yield. The next experiment focused on the impact of endometritis on pattern of ovarian activity and cyclicity in the same UK dairy herd.

3.3 Experiment 2: Effect of RTID on post-partum ovarian cyclicity

3.3.1 Objectives

1. To quantify the incidence of typical and atypical progesterone profiles in a high yielding dairy herd.
2. To determine the effect of endometritis and abnormal vaginal discharge score on the postpartum ovarian cyclcity.
3. To quantify the postpartum reproductive performance between cows with typical and atypical progesterone profiles.

3.3.2 Materials and methods

3.3.2.1 Animals and herd

Three sets of animals were used in this experiments: 1) The first group involved 46 multiparous Holstein dairy cows calving from August 1999 to February 2000

selected from the Nottingham University commercial dairy herd (Manor Farm). This group involve: 33 control cows and 13 cows with endometritis; 2) The second group 38 animals from another local dairy herd (Rangemoor Farm), and classified into 24 control and 14 endometritic cows; 3) The last group of animals involved 86 cows from the University of Nottingham dairy centre, which calved from July 2013 to April 2014: 61 control cows and 25 cows which experienced endometritis. For analysis, this study combined all these cows together, and classified into: 118 control cows and 52 cows that experienced endometritis. In the later group, the lactating cows were kept indoors in groups of approximately 40. Housing consisted of a purpose-built shed with four pens, which was well ventilated, with rubber matting, cubicles and shavings for comfort whilst lying. All cows were fed the same silage based total mixed ration diet, with concentrated feed at milking. Cows were milked by 4 robotic milkers (Lely Astronaut A3 AMS units) voluntarily; visiting from 2 to 6 times per day. Milking was done on average 3 times per days. In the other 2 groups, cows were milked in a traditional parlour. In all three groups, cows were checked for uterine disease through inspection of the vaginal discharges and categorised into two groups: endometritis (n=52) and control group (n=118). Endometritis defined by the presence of abnormal vaginal discharge within 3 weeks after calving. All cows in all 3 groups were managed according to normal management practices. Generally, control cows selected when there was no abnormal vaginal discharge seen from 3 weeks after calving until cows getting pregnant.

3.3.2.2 Reproductive management

All cows with reproductive disorders were treated as appropriate by the inspecting clinician.

3.3.2.3 Milk sampling

In the first two groups of animals, milk samples were collected manually, from the milking jar and after milk had mixed thoroughly. A sample was taken twice weekly (p.m., Monday and Friday) from each cow, starting two weeks after calving and continued until 15 weeks post-partum. However, at the University of Nottingham dairy centre, milk samples were collected via a Lely Shuttle connected to the robotic milking machine for progesterone analysis. The milk sampling procedure was started from 10-14 days postpartum period and

continued until confirmation of a new pregnancy either by rectal palpation or ultrasonography. Milk samples were collected thrice weekly (Monday, Wednesday, and Friday). As cows entered the robot for milking, they were identified to be sampled and approximate 20ml milk was collected. Samples were collected in plastic tubes contain broad spectrum Microtabs®II (containing Bronopol and Natamycin; D&F Control Systems, Inc., USA), which preserved and prevented growth of bacteria, yeasts and molds. Milk samples were preserved with one tablet per sample and refrigerated at 4°C until measurement. If the cow was confirmed as pregnant, milk sampling of these cows was stopped. Occasionally, milk samples were missed either due to the automated nature of sampling or failure of robotic system due to power-cut communication which lead to an error in robotic system. However, this had no impact on the ability to interpret the progesterone profiles.

3.3.2.4 Assay for milk progesterone

The concentration of progesterone in milk was determined using a 96-well microtiter plate-based enzyme-linked immunosorbent assay (ELISA) supplied as a commercially available kit (Ridgeway Science Ltd., Alvingdon, UK). Microtiter plates pre-coated with progesterone-antibody were stored at 4°C and were warmed to room temperature prior to use. The foil seal was removed, wells emptied and the plate blotted dry onto tissue paper. Prior to each assay the following reagents: whole milk standards: 0, 1, 2, 5, 10, 20 and 50ng/ml; quality controls: 2ng/ml and 8ng/ml in milk from ovariectomized cows and samples were brought to room temperature and thoroughly vortexed to ensure homogeneity of the samples and to ensure the fat was thoroughly mixed with milk. Standards, quality controls and samples (10µl each) were added to the wells in duplicate. Progesterone enzyme label (200µl) was added to each well and the plate incubated at room temperature for 1 hour 30 minutes. After incubation, the wells were emptied, washed with cold tap water and tapped dry on paper. This was repeated a total of 3 times. The supplied substrate solution (200µl) was then added to each well and incubated in the dark at 25°C for 20-30 minutes to allow the colour to develop. The immunosorbance of each well was recorded at 570nm using an automated plate reader (Labsystems Multiskan Ascent 354) and processed using specific software to read the absorbance and transform into progesterone concentration (Ascent Software Version 2.6, Thermo Labsystems).

The software used a 4-parameter logistic regression line to fit the standards. The ELISA's reliable reading range was from 1.0 to 10ng/ml. All samples were reading below or above this range were taken to be 1.0 to 10ng/ml, respectively. All samples with a coefficient of variation >15% were repeated. The intra and inter-assay coefficient of variation were 6.3 and 7.7%, respectively.

3.3.2.5 Onset of luteal activity (OLA)

The interval from calving to OLA was defined as the first day that milk progesterone concentrations were ≥3 ng/ml for two successive measurements more than 10 days after calving (Mann et al. 2005, Garmo et al. 2009). The average value was calculated for OLA and used to classify cows according to early (on or before average day of OLA) or late OLA (greater than the average OLA).

3.3.2.6 Ovarian cycle patterns analysis

This study described the nature of typical and atypical postpartum ovarian patterns (as observed within the herd). The obtained ovarian activity profiles were defined as previously reported. (Mann et al. 2005) Namely, the ovarian activity profiles were split into five categories:

1. ***Normal profile (normal ovarian activity):*** Ovulation occurred ≤45 days post-partum (detected rise in progesterone) with progesterone concentration >3ng/ml for 14 days, followed by regular ovarian cycles with about 2 weeks of luteal phase (high progesterone) and 1 week of the follicular phase (low progesterone). A short luteal phase <10 days after the first ovulation was considered as normal.
2. ***Anovulation or delayed first ovulation***: The first detected ovulation occurred more than 45 days after calving.
3. ***Cessation of cyclicity***: Ovulation occurred within the normal period i.e ≤45 days after parturition, but it was followed by progesterone concentrations <3ng/ml for >12 days.
4. ***Prolonged luteal activity***. It was characterised by progesterone concentration >3ng/ml for >21 days without mating and thus not pregnant.

5. ***Short luteal phase.*** Progesterone concentration >3ng/ml for less than 10 days (excluding the first oestrus cycle).

3.3.2.7 Reproductive examination

Vaginal discharge scoring

The vaginal discharge scoring during post-partum was assessed by a veterinarian, starting three weeks after calving. The cow's vulva was wiped thoroughly with a dry paper towel. Thereafter, a clean gloved hand lubricated with gel was inserted through the vulva to the vagina. The dorsal, ventral and lateral aspects of the vagina and the posterior part of the cervix of each cow were palpated, and the vaginal contents withdrawn manually for inspection. Intravaginal fluid was collected for examination prior to scoring it. Any discharge during each vaginal examination was noted. According to Sheldon (2004), the character of vaginal discharge was scored as follows:

(0) Clear or translucent mucus (healthy cows)

(1) Mucus containing flecks of white or off white pus

(2) Exudates containing ≥50% purulent material, usually white or yellow (Fig. 3.4).
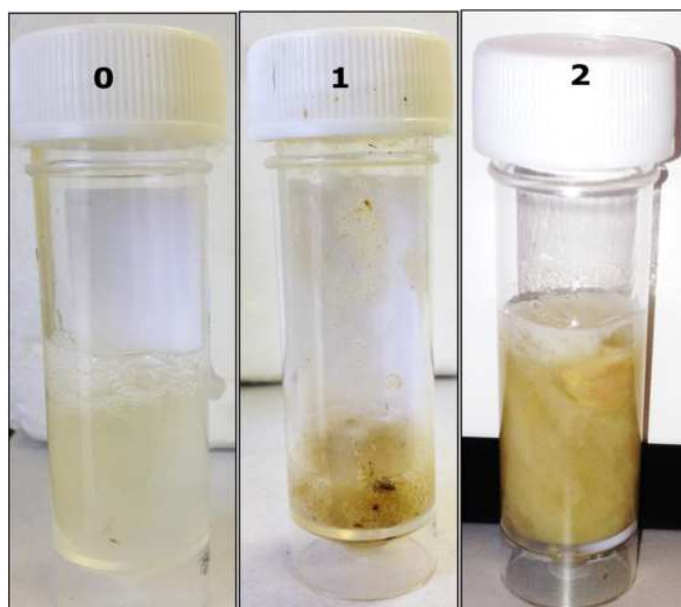
Figure 3.4: Typical samples of vaginal mucus character: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; and score 2 = discharge containing >50% purulent material, usually white or yellow (Sheldon 2004).

Rectal palpation

Reproductive tracts of cows were examined by the vet and rectally palpated three weeks after calving. Palpation of the uterus during the post-partum period was carried out to assess the involution of the uterus and to detect any uterine abnormalities. Both ovaries were palpated for identification of normal ovarian structures, the examination of any possible abnormal pathological condition and pregnancy diagnosed after 35 days post insemination. At the same time, ovaries were checked for appropriate structures. By rectal palpation, ovarian cysts were identified and distinguished mainly by size and consistency. A follicular cyst was diagnosed as soft, thin walled large follicle. In contrast, a luteal cyst was characterised as a firm, thick-walled structure.

Ultrasonography

A portable transrectal B-mode ultrasound scan was carried out with a 7.5-MHz linear-array transducer used to monitor the ovarian activity, examine the reproductive tract during post-partum period and for pregnancy diagnosis by an experienced veterinarian. Cows with a negative pregnancy test and with persistent corpus luteum or luteal cyst were treated with 2ml of Estrumate®

(PGF2α). However, cows with a follicular cyst were treated with 5ml of receptal (GnRH agonist; buserelin).

3.3.2.8 Statistical analysis

All data were stored in Microsoft Excel 2010 (Microsoft Corporation). All statistical analyses were performed in GenStat version 16th Edition. This study combined three different sets of data for analysis. Various types of progesterone profiles during the pre-service period were converted to a percentage of the total. One-way ANOVA used to compare the OLA and vaginal discharge score between normal and abnormal ovarian cycles. Kaplan-Meier survival curves were used to compare days to first insemination between cows with typical and atypical progesterone profiles. The χ^2 test was used to compare conception rates at first insemination between progesterone profiles. One-way ANOVA was performed to compare the onset of luteal activity between the endometritis and control groups, and the same test was used to compare vaginal discharge score (VDS), DFS and conception rate at 1st service between early and late OLA. Binominal logistic regression was used to determine odds ratio and the effect of risk factors (endometritis and vaginal discharge score 0, 1 and 2) on the incidence of typical and a typical progesterone profiles. All candidate variables were kept in the model with significant attributes at P<0.05.

3.3.3 Results

3.3.3.1 Types of typical and atypical progesterone profile

The various types of ovarian cyclicity detected during the postpartum period based on milk progesterone are shown in Fig.3.5.
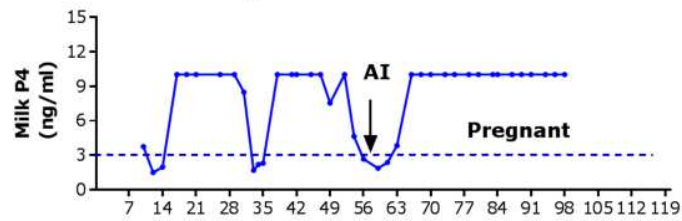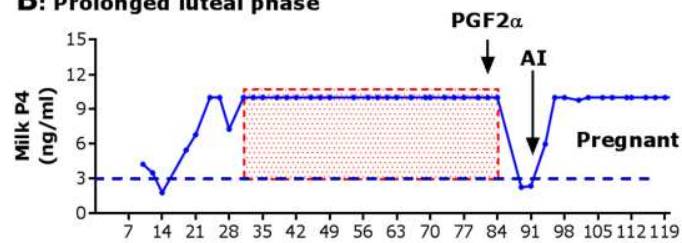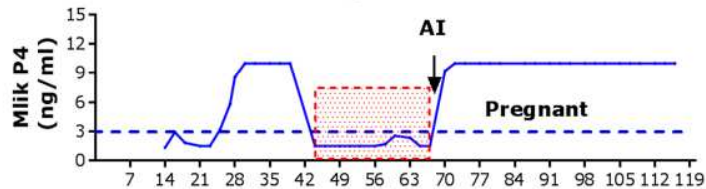
Figure 3.5: Schematic illustration of the types of reproductive cycles identified in Holstein dairy cows by analysis of progesterone in milk: These represent: a) normal cyclicity, b) prolonged luteal activity, c) cessation of cyclicity, d) short luteal activity, e) delayed cyclicity. Red boxes represent the period during which specified problem occurred. The blue dotted lines represent the limit of progesterone for luteal activity. AI: time of artificial insemination; PGF$_2\alpha$: prostaglandin F$_2\alpha$.

3.3.3.2 The incidence of typical and a typical progesterone profiles

A total of 82 animals had normal resumption of ovarian activity and typical ovarian cycles. The other 88 cows experienced abnormal ovarian cycles (Table 3.7).

Table 3.7: Incidence of typical and atypical ovarian patterns. Out of total of 170 animals (51.8%) had at least one atypical ovarian profile

Class	Number	
Normal ovarian cycle	82	
Abnormal ovarian cycle	88	
Abnormal ovarian category		*percentage*
Prolonged luteal phase	41	46.6
Cessation of cyclicity	16	18.2
Short luteal phase	17	19.3
Delayed to first ovulation	14	15.9

3.3.3.3 Onset of luteal activity (OLA)

Overall, the mean interval of OLA (1st milk progesterone rise >3ng/ml) was 28.4±0.8 days and ranged from (12-77 days). The onset of luteal activity was significantly different between cows with normal or abnormal ovarian cycles ($P<0.05$). Cows with normal ovarian cycle had a lower mean discharge score compared to cows with abnormal ovarian cycle ($P<0.05$; Table 3.8).

Table 3.8: Mean±SEM time to onset of luteal activity, days to first insemination and vaginal discharge score at first insemination at Nottingham dairy centre exhibiting normal or abnormal ovarian cycles.

Characteristics	Normal ovarian cycles	Abnormal ovarian cycles	P value
Time to onset of luteal activity (days)	26.0±1.1 (n=82)	30.4±1.1 (n=88)	0.009
Vaginal discharge score	0.2±0.1 (n=82)	0.7±0.1 (n=88)	<0.001

3.3.3.4 The association between atypical progesterone profiles and the reproductive performance in dairy cows

Days to first service

A total of 6 normal and 8 abnormal cows were not included analysis because they were not inseminated. In addition, for the purpose of analysis, the delay to first ovulation and cessation groups were merged together into one group to enable full-satisfied analysis. Kaplan-Meier survival analysis revealed that days to first service (DFS) was different between ovarian cyclicity groups in dairy cows (Fig. 3.6A). Survival analysis demonstrated that the rate at which the cows with normal ovarian cycles were inseminated was higher than those cows with abnormal progesterone profiles (P<0.05, Fig.3.6A).
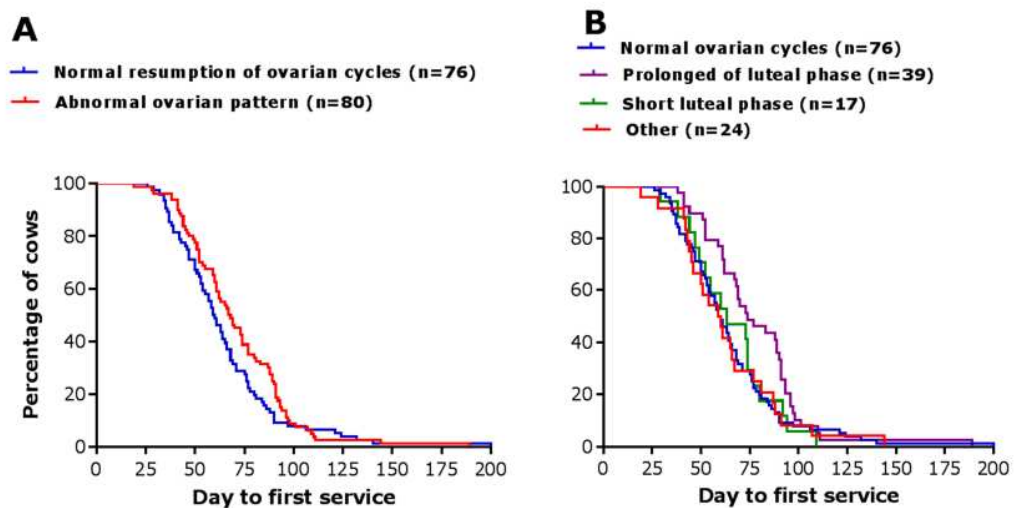
Figure 3.6: The comparison of normal and abnormal progesterone profiles on days to first insemination in cows that were served A) Kaplan-Meier survival curves for time to first insemination in cows with normal resumption of luteal activity (blue line, median survival=59.5) or abnormal ovarian activity (red, median survival=67.5) (*P*<0.05). B) Kaplan-Meier survival curves for time to first insemination in cows with normal resumption of ovarian cyclicity (n=76; blue line, median survival 59.5 days) or a prolonged luteal phase (n=39; purple line, median survival 74 days, *P*<0.01), or a short luteal phase (n=18; green line, median survival 63 days) or other abnormal cyclicities (n=24; red line, median survival 59.5 days) (*P*=0.88).

Median DFS for cows with normal and abnormal ovarian cyclicity was 59.9 and 67.5 days, respectively. In addition, this test was used to determine this interval between the different atypical progesterone profiles (short luteal phase and

others) and normal ovarian cycles (P=0.88). The prolonged luteal phase (median 74 days) had longer days to first insemination compared to control cows (median 59.5 days, P<0.01; Fig 3.6B). However, days to first insemination was not different between other abnormal cyclicity groups compared to control cows (median 59.5 days, P=0.88; Fig 3.6B).

Conception rate at 1ˢᵗ service

Over all cows, conception rate at first service in this study was 38.0%. The association between conception rate at 1ˢᵗ service and pattern of ovarian cyclicity are shown in Table 3.9. Chi square test, showed that cows with normal ovarian resumption of ovarian cycles had significantly higher conception rate at 1ˢᵗ service compared to cows with abnormal ovarian cycles (P<0.05; Table 3.9). Further analysis showed that among abnormal progesterone profiles, cows with delay to first ovulation had the highest conception rate at first service (Table 3.9).

Table 3.9: Conception rate at first insemination of cows that were served at Nottingham dairy centre exhibiting normal and abnormal ovarian activity.

Category	Total number of AI	Conceived at 1ˢᵗ AI	% Conception rate
Normal luteal activity	76	38	50.0[a]
Abnormal progesterone profiles	80	24	30.0[b]
Within abnormal progesterone profile			
Prolonged luteal activity	39	10	25.6
Delay to first ovulation	12	5	41.0
Cessation of luteal activity	12	3	25.0
Short luteal phase	17	6	35.3

Values in the same column with superscripts,[ab] differed from normal resumption of ovarian cyclicity: Chi-square ([a,b]=P<0.05).

3.3.3.5 Comparison of progesterone profiles between control cows and cows with endometritis

These results related reproductive data of cows which suffered from uterine disease with occurrence of normal and abnormal ovarian cycles post-partum.

Onset of luteal activity (OLA)

The interval from calving to onset of luteal activity was affected by endometritis. Control cows resumed cyclicity post-partum approximately 4 days earlier compared to cows with endometritis ($P<0.05$; Fig. 3.7). The reproductive performance of cows with early OLA (≤ 28 days) and those with late OLA (≥ 29 days) are shown in Table 3.10. Cows with early OLA had lower mean discharge score compared to late OLA cows ($P<0.05$). However, there was no difference in DFS and conception rate at 1st service between early and late OLA ($P>0.05$).
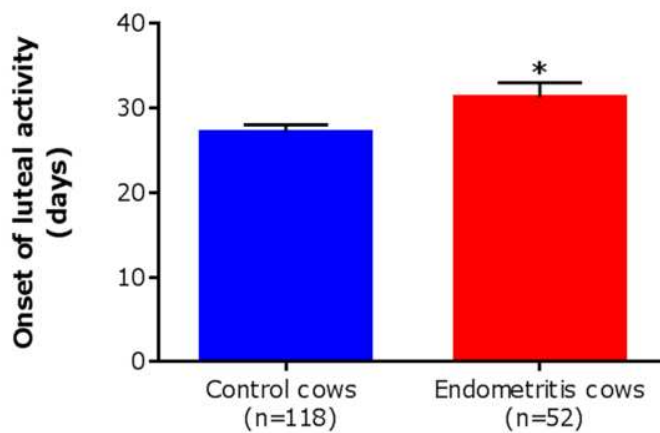
Figure 3.7: The comparison of onset of luteal activity between control cows and cows that experienced endometritis. Cows that experienced endometritis had significantly later onset of luteal activity compared to control cows about 4 days, (*; $P<0.05$).

Table 3.10: Mean±SEM vaginal discharge score, day to first service, and conception rate at 1st service in cows with early or late onse of luteal activity (OLA).

Class	Early OLA (n=101)	Late OLA (n=69)	P value
Vaginal discharge score	0.3±0.1	0.7±0.1	**0.01**
Days to first AI	66.5±2.9	69.9±3.5	0.37
Conception rate at 1st AI	44.5	32.8%	0.14

The incidence of typical and atypical progesterone profiles

Cows that had endometritis had a greater incidence of atypical ovarian patterns ($P<0.001$, Table 3.11). Cows that exhibited endometritis had significantly more prolonged luteal phases compared to control cows ($P<0.05$). However, incidence of cessation of cyclicity, short luteal phase, and delay to 1st ovulation were not different between cows with endometritis and control cows. While cessation of cyclicity was significantly higher in control cows than in cows experienced endometritis (Table 3.11).

Table 3.11: Incidence of typical and different types of atypical progesterone profiles in control and endometritic cows.

Class	Control n	Control %	Endometritis n	Endometritis %	*P*-value
Normal ovarian cycle	68	57.6[a]	14	27.0[b]	<0.001
Abnormal ovarian cycle	50	42.4[a]	38	73.0[b]	<0.001
Prolonged luteal phase	16	32.0[a]	25	65.8[b]	0.034
Cessation of cyclicity	13	26.0[a]	3	7.9[b]	0.02
Short luteal phase	12	24.0[a]	5	13.1[a]	0.20
Delayed 1st ovulation	9	18.0[a]	5	13.1[a]	0.54

Values in the same row with superscripts,[ab] differed from normal luteal phase ([ab] P<0.001; [aa]P >0.05).

Risk factors associated with normal and abnormal ovarian progesterone profiles

Logistic regression revealed that cows with endometritis had a reduced chance of having a normal ovarian cycle (P<0.01, Fig.3.8). Similarly, abnormal vaginal discharge also reduced the chance of a normal ovarian cycle (P<0.01) and this was further reduced in cows with the highest vaginal discharge score of 2. Endometritis significantly increased the proportion of prolonged luteal phase as discharge score increased, there was further increase proportion of prolonged luteal phase (p<0.05). However, endometritis had no effect on the risk of having delayed ovulation, cessation of luteal phase and short luteal phase as well (P>0.05, Fig.3.8).
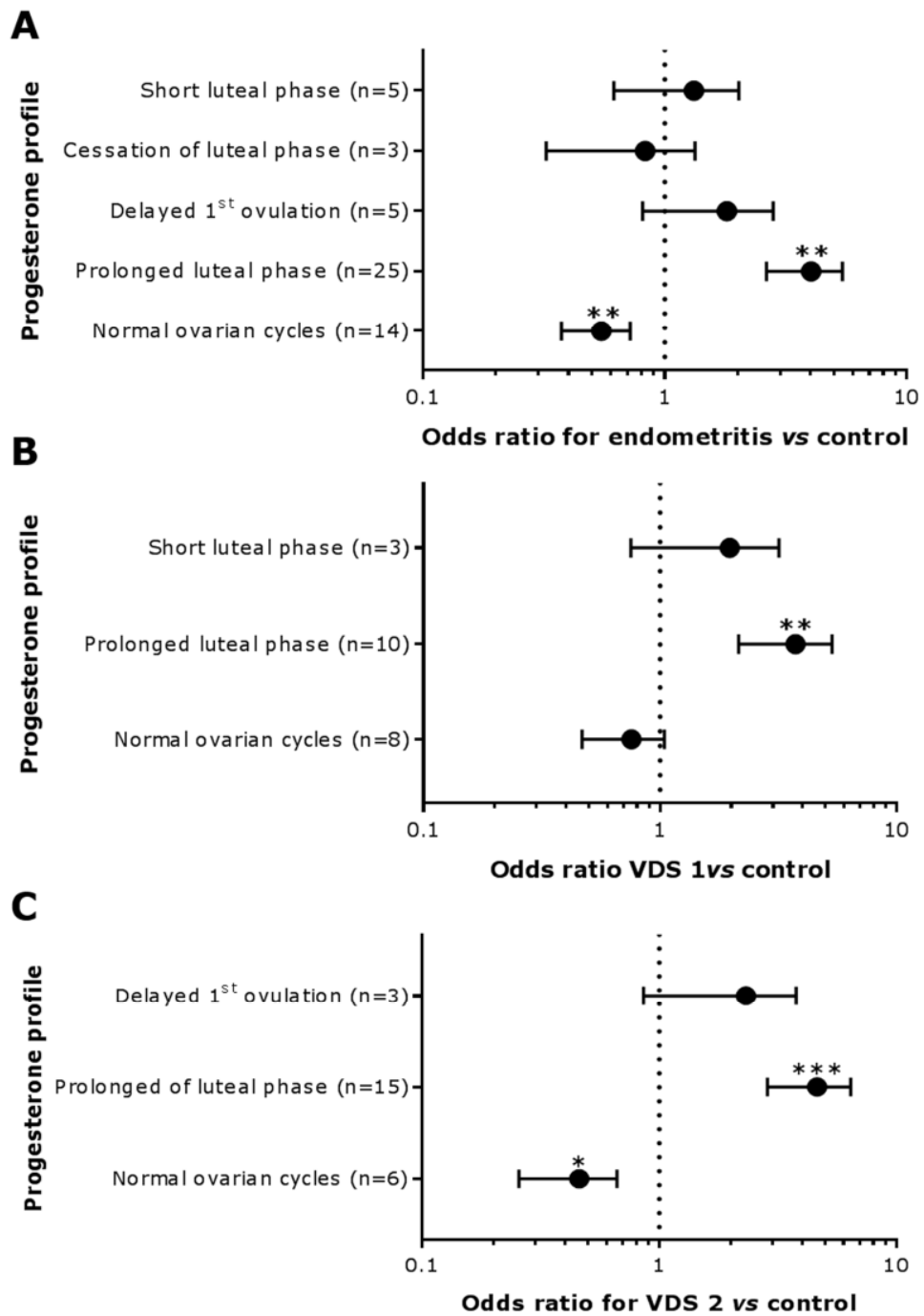
Figure 3.8: Odds ratio for the association between vaginal discharge and endometritis on the chance of having normal or abnormal progesterone profiles. A) Cows with endometritis had signifcantly reduced risk of having normal ovarian cycles (**$P<0.01$) and increased prolonged luteal phase (***$P<0.001$), however, endometritis had no effect on the risk having another abnormal progetertone profiles ($P>0.05$). B) Odds ratios of vaginal discharge score 1 *vs* control; cows with VDS1 had significantly increased prolonged luteal phase (** $P<0.01$). C) cows with VDS2 had signifcantly reduced proportion of normal ovarian cycles (*$P<0.05$) and increased prolonged luteal phase (***$P<0.001$). Note: Other types of progesterone profiles are not shown in A) or B) due to low numbers.

Conception rate at first insemination

Conception rate at first service between cows with or without endometritis having normal and abnormal progesterone profiles are shown in Table 3.12. While the control cows with normal and abnormal ovarian cycles had numerically higher conception rate at 1st AI but this was not significantly different. This might be due to the small of animals in the endometritis group.

Table 3.12: Conception rate at 1st service of control and RTID cows at Nottingham dairy centre exhibiting normal and abnormal ovarian activity.

Classes	Control cows	Uterine disease	*P* value
Normal ovarian cycles	50.8 (n=32/63)	46.2 (n= 6/13)	0.59
Abnormal progesterone profiles	32.6 (n=15/46)	26.5 (n= 9/34)	0.55
Prolonged luteal phase	35.7 (n= 5/14)	20 (n= 5/25)	0.28
Others	31.3 (n=10/32)	44.4 (n= 4/9)	0.46

3.3.3 Discussion

The objectives of this study were to determine the effect of post-partum bacterial uterine infection (endometritis) on the post-partum ovarian cyclicity and their effect on post-partum reproductive performance in Holstein dairy cows at Manor, Rangemoor and Nottingham University dairy centre. The results of the study supported the hypothesis that endometritis has a detrimental effect on postpartum ovarian cyclicity in Holstein dairy cows

The combined results of Manor, Rangemoor farms and the University of Nottingham dairy Centre showed that there was no association between the interval from calving to onset of luteal activity and subfertility in dairy cows during the postpartum period. The findings of the present study did not find any differences in day to first service between an early and late onset of luteal activity. In contrast to Darwash et al. (1997) who found early postpartum onset of luteal activity was positively linked with fertility. They recorded that cows with early onset of luteal activity had a reduced calving to first insemination interval

compared to cows with late onset of luteal activity. They observed that for each extra day of onset of luteal activity lengthened the period of calving to 1st service and calving to conception interval by 0.24 and 0.41 days, respectively. Other studies reported that calving to 1st service was longer in cows with abnormal ovarian cycles compared to cows with normal ovarian cycles (Petersson et al. 2006, Hommeida et al. 2005). Furthermore, this study did not find a relationship between onset of luteal activity interval and conception rate at 1st service. This is consistent with Royal et al. (2000) who failed to observe a relationship between conception rates and early onset of ovulation in Holstein Frisian cows.

The combined results of Manor, Rangemoor farms and the University of Nottingham dairy showed that postpartum reproductive cycle abnormalities were present in 51.8% of animals. These abnormal progesterone profiles were associated with reduced conception rate at 1st service, increased time to OLA and abnormal vaginal discharge. After reproductive cycles have re-commenced, many cows continue to cycle at regular intervals until they are successfully served. These results corresponded to the results of a previous Belgium study which reported 49.0% incidence of abnormal ovarian cyclicity (Opsomer et al. 2000). However, the results of the present study were relatively higher compared to previous study carried out at the University of Nottingham where about 32% of an abnormal pattern of the total (n=2503) cows (Lamming and Darwash 1998). Previous studies in 1980 carried out at the University of Nottingham found 22.5% of cows had an abnormal ovarian cycle (n=533) in the late 1970s (Claire and Wood 1980). Another study in the late 1990s reported 32-42% of cows had an abnormal cycle which was associated with reduced pregnancy rate at first insemination from 56 to 40%. (Royal et al. 2000).

Compared with earlier studies, this study demonstrated that modern high producing dairy cows are more susceptible to ovarian abnormal cycles in the immediate postpartum period, especially prolonged luteal phase type (positive progesterone level for >20 days without insemination). It should be noted that it is difficult to completely compare the results of this study with previous studies, because the definition of abnormal progesterone profiles used in current study are slightly different from other studies. The particular variation is due to the different number of days used in the definition. This study classified postpartum ovarian dysfunction into four types: Of the cows with different types of

progesterone profiles (14%, 16%, 17% and 41%) showed delayed to first ovulation, cessation of ovarian cyclcity, short luteal phase and prolonged luteal phase, respectively.

Findings of the present study showed that endometritis significantly impaired postpartum ovarian cyclicity in Holstein dairy cows. Cows exhibiting endometritis had the highest rate of atypical progesterone profiles particularly prolonged luteal phase, (65.8%) compared to control cows (32.0%). In agreement with Taylor et al. (2003) who found that cows with abnormal vaginal discharge had a 47.6% incidence of prolonged luteal phase. The results of the current study were higher than recently published by Ghanem et al. (2015) who observed that cows which experienced endometritis had 60% prolonged luteal phase. In addition, Ranasinghe et al. (2011) recorded 32% of prolonged luteal phase in cows with postpartum complications (retained fetal membrane, endometritis, metritis and pyometra), which was lower compared to current study.

The precise mechanism through which postpartum endometritis increases the incidence of prolonged luteal phase in dairy cows is unknown. It is feasible that it is through the disturbance of the luteolytic process that results in the extended CL lifespan (Williams et al. 2008a, Sheldon et al. 2008, Lamming and Darwash 1998). In addition, endometritis caused an increase in PGE_2 concentration in uterine fluid of cows (Mateus et al. 2003), with epithelial cell of endometrium exposed to bacterial endotoxin undergo an endocrine switch from luteolytic $PGF_{2\alpha}$ to luteotrophic PGE_2 production (Herath et al. 2009). Increased PGE_2 concentrations in uterine fluid were shown to cause luteal persistence in cows (Thibodeaux et al. 1992). This suggests that in cows with endometritis this prostaglandin plays a local role in the pathogenesis of prolonged luteal function.

It is interesting to note that, there was no relationship between endometritis and delayed first ovulation, cessation of ovarian activity or short luteal phase. This study showed that the incidence of delay to first ovulation was 13.0% in cows with endometritis. This is consistent with study of Ghanem et al. (2015) which observed there was no difference between cows with uterine bacterial infection and cows without uterine infection in terms of delay to 1st ovulation. Here they reported that cows positive with uterine infection are less likely to ovulate because they have a lower growth rate of the dominant follicle and lower plasma oestrogen concentrations. However, the incidence of delay to ovulation was

comparatively lower compared to other studies where they observed a delay to first ovulation in about 20–25% (Sheldon et al. 2008, Nakao et al. 1992)

The present study observed that abnormal vaginal discharge had a significant effect on subsequent progesterone profiles which was consistent with the hypothesis that clinical endometritis adversely affects post-partum resumption of ovarian cyclicity. As the abnormal vaginal discharge score increased, the frequency of atypical ovarian cycles was increased as well. This is consistent with the previous study which used a regression analysis for confirmation and quantification of the relation between vaginal discharge index and progesterone profiles which was significant (Gorzecka et al. 2011). However, others found that there was no significant relationship between vaginal discharge and abnormal progesterone profiles (McCoy et al. 2006).

The present study found that endometritis and abnormal purulent vaginal discharge were important risk factors for the development of abnormal ovarian cycles, particularly prolonged luteal phase. This study found that cows with endometritis and vaginal discharge score >1 were 4 times more likely to show a prolonged luteal phase than other abnormal progesterone profiles. In agreement to the findings of the previous study which observed that cows with postpartum complications (endometritis, metritis and pyometra) were 5 time more likely to show prolonged luteal phase (Ranasinghe et al. 2011). Four major risk factors for prolonged luteal phase have been previously identified; metritis, abnormal vaginal discharge, retained placenta and parity (Opsomer et al. 2000). The same study observed that metritis is the most important factor and cows with metritis were 11 times more at risk of developing prolonged luteal phase. In agreement with these data support the concept that prolonged luteal phases were associated with endometritis.

The present study attempted to quantify the severity of inflammation symptoms by examination of vaginal discharge based on visual inspection of vaginal discharge (Williams et al. 2005, Gorzecka et al. 2011), which have been shown to accurately reflect uterine bacterial infection and immune system. In addition to metritis, abnormal vaginal discharge and difficult calving have a negative impact on the postpartum ovarian cyclicity by day 50 PP (Opsomer et al. 2000) and on time to resume ovarian cyclicity (El-Din Zain et al. 1995). The impact of metritis on cyclicity may be partly explained by the relation between metritis and

negative energy balance (Galvão et al. 2010b, Huzzey et al. 2007, Hammon et al. 2006) and by the release of bacterial endotoxin (LPS) into the uterine lumen, and then enters into the blood stream (Mateus et al. 2003, Herath et al. 2009). As reported previously LPS can affect GnRH and LH release, follicle development, oestradiol production and ovulation.

In this study, reproductive cycle abnormalities were associated with reduced conception rate at 1st AI, increased OLA time and abnormal vaginal discharge. Once reproductive cycles have re-commenced, after a time of post-partum anoestrus, many cows continue to cycle at regular intervals until they are successfully inseminated. It should be noted that cows with endometritis had longer interval of onset of luteal activity compared to control cows. In agreement with (Borsberry and Dobson 1989, Holt et al. 1989) reported that cows with endometritis delayed onset of ovarian activity compared to healthy cows.

3.3.5 Conclusion

This study demonstrated that endometritis has a negative impact on the post-partum ovarian cyclicity in Holstein dairy cows in the University Nottingham dairy centre and elsewhere. Cows which experienced endometritis had longer onset of luteal activity, higher rate of abnormal ovarian cycles, particularly prolonged luteal phase. However, the occurrence of other types of abnormal ovarian cycles including delayed to first ovulation, short luteal phase and cessation of luteal activity was not influenced by endometritis. As vaginal discharge score increased, the occurrence of prolonged luteal phase significantly increased. Furthermore, this study found that abnormal ovarian cycle had negatively associated with fertility of cows during post-partum period. Cows with abnormal ovarian cycles had longer onset of luteal activity, longer calving to 1st AI interval, lower conception rate at 1st service versus cows with normal ovarian cycles.

4 The association between endometritis and vascularisation, progesterone production and steroidogenic enzyme expression in the bovine corpus luteum *ex vivo*.

4.1 Introduction

In post-partum dairy cows, infections in the bovine uterus with Gram-negative bacteria commonly cause endometritis (Sheldon et al. 2009a). Endometritis subsequently influences ovarian function through various mechanisms including delayed follicular growth, reduced circulating oestradiol concentrations, altered luteal phase duration, and disruption of ovarian cyclic activity (Sheldon et al. 2002b, Herath et al. 2007, Williams et al. 2008a; Chapter 3).

Uterine infection with Gram-negative bacteria leads to release of endotoxin such as LPS into the circulation that can cause ovarian dysfunction, in part, through its accumulation in follicular fluid (Herath et al. 2007). The extremely rapid growth and development of the CL that is associated with a period of intense angiogenesis might also be sensitive to bacterial endotoxin exposure. Inadequate angiogenesis is likely to result in reduction of progesterone production (Robinson et al. 2008).

Often angiogenesis and inflammation are co-dependent processes (Voronov et al. 2007), but their precise mechanisms and cross-talk in the regulation of luteal growth, differentiation, and regression are poorly understood. Endothelial cells are considered major targets of cytokine actions during the immune process, particularly in inflammation (Sprague and Khalil 2009). Because endothelial cells/pericytes are the most abundant cells in the developing CL, microvascular endothelial cells (O'Shea et al. 1989, Gaytan et al. 1999) and their responsiveness to cytokines are likely to influence luteal function (Cherry et al. 2008). The role of cytokines in regulation of luteal function has been investigated in humans (Yan et al. 1993), and cattle (Sakumoto et al. 2000, Nishimura et al. 2004, Skarzynski et al. 2005). For example, tumour necrosis factor alpha (TNFA) and interferon gamma (IFNG), have been shown to regulate bovine luteal endothelial cell function (Okuda et al. 1999, Fenyves et al. 1993, Pru et al. 2003). The innate immune system

depends on pattern recognition receptors on host mammalian cells to detect microbe- or pathogen-associated molecular patterns, such as LPS (Hoshino et al. 1999). LPS is recognised by Toll-like receptor 4 (TLR4) by binding with other co-receptors including CD14 and MD-2. This, in turn, stimulates cellular responses characterised by the production of inflammatory mediators, including interleukin-1β (IL-1β), IL6, and IL8 (Moresco et al. 2011). Indeed, LPS induced a TLR4-dependent inflammatory response in bovine granulosa cells collected from antral follicles and perturbed oocyte development (Bromfield and Sheldon 2011). It is less clear, however whether LPS affects ovarian endothelial cells and/or pericytes directly.

An *in vivo* study demonstrated that infusion of LPS (0.5mg/kg body weight) decreased luteal blood flow, and luteal size using power-flow Doppler ultrasound (Herzog et al. 2012). The level of *caspase-3* mRNA expression was increased and at the same time luteal expression of *STAR* mRNA was decreased. These were all associated with decreased plasma progesterone concentrations (Herzog et al. 2012).

Pericytes, myofibroblast and vascular smooth muscle cells are present in the CL, and have the same lineage but they differ in their location relative to endothelium. Pericytes are in close contact blood capillaries, pre-capillaries arterioles and are embedded within the endothelial cell basement membrane (Gerhardt and Betsholtz 2003, Armulik et al. 2005). Vascular smooth cells are principally involved in the regulation of blood vessel contraction and tone (Armulik et al. 2005). However, there is increasing evidence that pericytes are active players in the initiation of angiogenesis (Verbeek et al. 1994, Nehls and Drenckhahn 1993).

Hypothesis

Endometritis will reduce luteal vascularisation, size, and progesterone content and steroidogenic enzyme expression in dairy cows.

Aims

1. To determine the association between uterine infection and vascularisation of the bovine CL and the degree of pericyte coverage.
2. To evaluate the association between uterine infection, luteal progesterone content and expression of progesterone synthesis proteins.

3. To detect the expression of TLR4 and its co-receptors (CD-14 and MD-2) in bovine CLs at different stages of the oestrus cycle.

4.2 Materials and methods

4.2.1 Animals

In all experiments, bovine ovaries with corpora lutea were obtained from a local abattoir (Horton wood 60, Telford, Shropshire, TF1 7FA, UK) and transported back to the laboratory in 1x phosphate buffered saline (PBS) at room temperature. However, samples for mRNA analysis were transferred and placed into RNA Later within 20 min of slaughter. The same CL samples were used for the different components of this experiment.

4.2.2 Tissue collection and section preparation

Corpora lutea from the mid-luteal phase (day 8-12) were isolated from bovine ovaries of uterine disease-free cows (n=3) and cows with reproductive tract inflammatory disease (n=3). Beyond this, we had no further information about how long this disease had been on going or what treatments if any had been administered. The CL were classified according to the criteria that has been previously reported (Fig. 4.1, Ireland et al. (1980)), and their weights recorded. CLs were cut into three segments: (1) fixed in Bouins' solution for 6h for IHC analysis, and (2) snap frozen and stored at -80°C for Western blot and mRNA purposes (2 separate segments).

The uterus was collected from the same animals, to confirm the health status of the uterus. At the slaughterhouse, uterine horn cross-sections (ipsilateral to CL) were collected 5 cm from the oviduct-uterus junction these were fixed with Bouins' solution for 6 h, followed by a 1x PBS wash for 5 min to remove excess Bouins' solution. The remainder of the reproductive tract was checked for the presence of endometritis by examination of vaginal discharge and an incision made from the uterine tip to the cervix.

The fixed luteal and uterine tissues were dehydrated in a series of alcohols: 30% (v/v) ethanol for 5 min, 70% (v/v) ethanol 3 x 5 min. Sections were put into cassettes and dehydrated and then embedded as follows: 70% (v/v) ethanol for 2h; 80% (v/v) ethanol for 2h; 95% (v/v) ethanol for 2h; and 100% ethanol 2 x 2h, 95% (v/v) ethanol plus histoclear 1 x 2h, histoclear for

2 x 4h each time and molten paraffin-wax for 2x 2h in an automatic tissue processor (TP1020, Leica, Milton Keynes, UK).
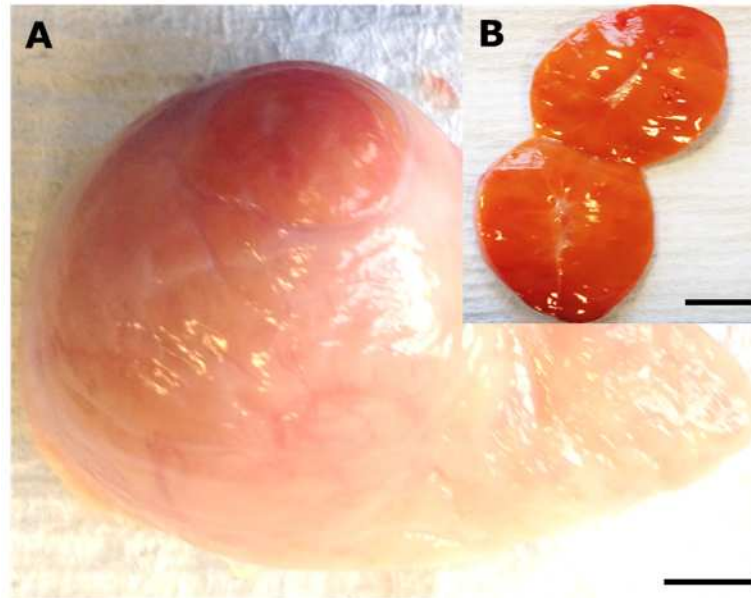
Figure 4.1: Image of a mid-luteal phase (day 5-12) bovine corpus luteum (CL). (A) Shows the CL within the ovary, while (B) shows it dissected out of the ovary. Scale bar: 10mm.

Embedded tissues were sectioned at 5µm using a microtome (HM355, Micron, Walldrof, Germany) and sections were floated onto water (40°C) and placed onto Superfrost Plus, positively charged slides. Sections then were heat fixed to slides to dry overnight at 50°C. Uterine sections were stained with haematoxylin and eosin to check histological appearance. This was done by placing slides in: Histoclear for 3x 5 min to remove excess wax followed by rehydration using decreasing concentration of alcohol, 100% (v/v) ethanol for 2 min, 95% (v/v) ethanol for 2 min, 70% (v/v) ethanol for 2 min. Rehydrated sections were stained with Harris's haematoxylin for 5 min and then washed with running tap water for 5 min to remove excess stain. The sections were differentiated with 1% (v/v) acid/ethanol for 2-4 second and washed with running tap water. Sections were dipped 8 times in 1% (v/v) ammoniated water to turn the stain blue in colour, and then slides were transferred to 1% (w/v) eosin for 5 min. Sections were washed with running tap water to remove excess eosin stain and were dehydrated with increasing concentration of alcohol: 70% (v/v) ethanol for 2 min, 95% (v/v) ethanol for 2 min, 100% ethanol for 2 min, histoclear 2 x 5 min. Sections were finally mounted using DPX (Raymond A Lamb, East Sussex, UK) mounting media.

4.2.3 Luteal content analysis

4.2.3.1 Luteal progesterone extraction and ELISA

The analysis of progesterone content in luteal tissue was performed as previously described (Tsang et al. 1990). Luteal tissue was thawed on ice and 200 mg was transferred to a 10 ml glass extraction tube to which 5 volume of ice-cold PBS was added and then vortexed for 1 min. Luteal tissue was homogenised using a tissue homogeniser (Polyton tissue grinder) for 3x 30 sec each time, with samples were kept on ice in between homogenisation. Then, in duplicate, 100 µl aliquot of homogenised samples was added to a fresh 10 ml glass extraction tube (previously cleaned with petroleum ether). Petroleum ether (2 ml; Acros, New Jersey, USA), was added and vortexed vigorously for 5 min. The homogenised tissue was then rapidly frozen by placing on methanol in dry ice. The ether liquid was transferred to a new glass tube (Borosilicate test tube, 12x75mm) and dried in a fume hood around 36 hours. The residue was reconstituted by adding 500µl progesterone media buffer (0.1% (w/v) bovine serum albumin (BSA) in 1X PBS) and vortexed for further 5 min. Immediately prior to analysis, the reconstituted solution was further diluted 1:800 in progesterone media buffer for cows with metritis and1:1000 for control cows. The progesterone concentration was then determined by a competitive progesterone ELISA (Ridgeway Science) as used for plasma samples and described in Chapter 3.2.3. This assay was designed for plasma/buffer progesterone samples and standards were; 0, 0.5, 1, 2, 5, 10 and 20 ng/ml in buffer. The lower limit of sensitivity was 0.2ng/ml and the intra-assay coefficient of variation was 5.6%. All samples were analysed in a single assay.

4.2.3.2 Protein extraction from luteal tissue

Corpus luteum tissue (100 mg) from both healthy cows (n=3) and endometritic cows (n=3) were transferred into 2 ml of ice-cold lysis buffer [0.1 M Tris pH7.4, 0.1% (w/v) dithiothreitol (DTT), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.18 mg/ml sodium orthovanadate and protease inhibitors [Complex protease inhibitor cocktail; Roche Diagnostic Ltd, East Sussex, UK]. The tissue was homogenised as described for progesterone extraction. The homogenised tissue was additionally sonicated for 3 × 20 sec on ice with >1 min on ice in between sonications. The luteal samples were then centrifuged at 13000**g** for 15 min. The supernatant was collected and

transferred to a new labelled tube on ice. The supernatant volume was recorded for the purposes of calculating total protein. Samples were aliquoted and stored at -80 °C until use.

4.2.3.3 Quantification of protein concentration

The protein concentration of the supernatant was quantified against a protein standard curve (0-0.6mg/ml bovine serum albumin (BSA); Sigma) by the method of Bradford assay using Bio-Rad dye reagent. Samples were diluted 1:100 with PBS buffer prior to analysis. Standards or samples (10µl) were added to the appropriate wells in triplicate. To this, 250µl of Bio-Rad reagent (Bradford Reagent, Sigma) was added to each well, mixed thoroughly using plate shaker and incubated at room temperature for 15 min. Absorbance was read using a microplate reader (Bio-Rad model 680) at 595 nm wavelength and protein concentration determined from the standard curve by linear regression (Microsoft Excel).

4.2.3.4 SDS-polyacrylamide gel electrophoresis

All reagents for Western blotting were purchased from Bio-Rad unless stated. Plates (1mm thick) were cleaned with water and ethanol. The gel apparatus was assembled with 1mm spacers and tested with distilled water for leaks. Then, 15ml of 12% (w/v) polyacrylamide solution required for 2 gels was carefully added. The polyacrylamide solution was prepared for four gels as follows:

- 4.9ml of dH$_2$O
- 3.8ml 1.5 M Tris HCl, pH8.8
- 150µl 10% (w/v) SDS
- 6.0ml 30% (v/v) acrylamide/bis-acrylamide solution
- 8µl 0.1% (v/v) N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)
- 150µl of ammonium persulphate (APS) (10% v/v) was added to start the cross-linking reaction.

The polyacrylamide was overlaid with water to avoid air interfering with the crosslinking reaction. Once set after approximately 40 min, water was removed by using blotting paper. The stacking gel (4% (w/v) polyacrylamide) 5.8ml was prepared for two gels and composed of:

- 4.0ml dH$_2$O

- 750µl 0.5M Tris HCl, pH6.8
- 60µl 10% (w/v) SDS
- 1.0ml 30% (v/v) acrylamide solution
- 5µl TEMED
- 60µl 10% APS.

This was immediately laid over the separating gel and the gel comb inserted making sure no bubbles were present. The staking gel was allowed to polymerise for 40 min and then the comb was removed. Luteal protein samples (20µg) were prepared by adding 10µl protein sample buffer (5% (v/v) β-mercaptoethanol in Laemmli buffer) and then were boiled at 100°C for 10 min.

The gel was removed from casting rig and placed into the electrophoresis chamber (gel tank). Running buffer (1X TGS [2.5 mM Tris HCl, 19.2 mM Glycine, 0.01 % SDS, pH 8.3]) was poured into the centre of the chamber until full. The first well of the gel was loaded with 10µl molecular weight marker (Bio-Rad Kaleidoscope). Samples were added to subsequent wells, wells between samples were loaded with loading buffer only. The tank was filled with 1x running buffer and gels were electrophoresed at 150V for 45-60 min until the proteins had through across the gels.

4.2.3.5 Protein transfer

Immediately prior to transfer, PVDF membrane was prepared by briefly soaking in methanol and then in transfer buffer (250 mM Tris HCl, 19.2 mM glycine, pH8.3 in 20% methanol) for 2 min. Sponges and blotting paper were soaked in cold transfer buffer. Gels were removed from the electrophoresis tank and gently removed from the glass plates and rinsed in dH₂O. Gels were equilibrated with transfer buffer and made in a blotting sandwich.

The sandwich was rolled with a glass pipette to remove air bubbles and wiped away excess buffer. Semi-dry electroblotting (Owl Panther Semi-dry Electroblotter) was used for protein transfer. Transfer was carried out at 150V for 40 min. Once transfer was complete, the membrane was carefully removed and protein transfer was checked with Ponceau S stain for less than 1 min. Subsequently, the membrane was de-stained in dH₂O and then 0.1% (v/v) Tween20 (Sigma) in PBS (PBST) until the solution became clear.

4.2.3.6 Western blotting

After protein transfer, membranes were blocked with 5% (w/v) skimmed dried milk (Marvel) in PBST for 1 h at room temperature. Following blocking, membranes were washed briefly with PBST, and then membranes were further incubated with primary antibody overnight at 4°C (Table 4.1).

Table 4.1: Primary antibodies used for Western blotting.

Antibody	Source Cat no	Size (kD)	Dilution	2nd Ab
Histone (H3)	Abcam, ab1791	15	1/1000	Rabbit
Smooth muscle actin (ACTA2)	Sigma, A2547	42	1/1000	Mouse
3beta-hydroxysteroid dehydrogenase (HSD3B)	Dr Richard Parker, University of Alabama	42	1/200	Rabbit
Steroidogenic acute regulatory enzyme (STAR)	Abcam, ab96637	32	1/3000	Rabbit
Cholesterol side-chain cleavage enzyme (P450scc/CYP11A)	Abcam, ab75497	61	1/1000	Rabbit

On the following day, membranes were washed 5 times in PBST for 10 min each. Subsequently, the membranes were further incubated with secondary antibody (Table 4.2) for 90 min diluted with blocking solution (5% (w/v) skimmed dried milk in PBST). The membranes were again washed 5 times in PBST for 10 min each and then rinsed in distilled water twice.

Table 4.2: Secondary antibodies used for Western blotting.

Antibody name	Company	Cat.No.	Dilution	Time
Goat anti-Rabbit IgG, HRP conjugate	Sigma	A9169	1/8000	1 h
Sheep anti-mouse IgG, HRP conjugate	GE Healthcare	NXA931	1/50000	1 h

4.2.3.7 ECL detection

Enhanced chemiluminescent (ECL) Plus detection kit (GE Healthcare, RPN2132) was used to detect the HRP conjugate. Membranes were covered with mixed ECL reagent (2 ml per gel) for 5 min. The blot was covered away

from direct sunlight at all times. The ECL was poured off the membranes which were transferred to a piece of cling film and wrapped.

4.2.3.8 Development of film

Under a safe light, one piece of film (Fuji Medical X-ray film, Fujifilm UK) was placed on the top of the membrane and the cassette was closed. The film was exposed to the membrane for 30 sec prior to being placed in developer (Sigma) for 30 sec and then washed with water. Subsequently, films were placed in fixer for 1 min, washed in water and hung to dry. The films were scanned to obtain a digital image and the quantification of protein in samples was assessed by using image analysis (Image J 1.47).

4.2.4 Immunolocalisation of endothelial cells, pericytes and steroidogenic cells in bovine CL

4.2.4.1 Dewaxing and rehydration

Slides were placed in histoclear (3 x 5 min) to remove paraffin wax then rehydrated through a graded series of decreasing concentration of ethanol (100%, 95% (v/v), 70% (v/v)), then slides were washed twice in PBS for 5 min each, prior to immunohistochemical analysis.

4.2.4.2 Antigen retrieval

This step was performed to expose epitopes and permit antibodies to bind. There are two different antigen retrieval methods which were performed in this study (1) trypsin digestion (2) boiling in 10mM citrate buffer pH6.0. This was dependent on the primary antibody, which is described in Table 4.3.

Trypsin retrieval

Antigen retrieval was performed by incubating sections in 0.4 mg/ml trypsin and 0.25 mg/ml $CaCl_2$ in PBS for 15 min at room temperature (RT). The sections were then washed twice in PBS for 5 min each.

Citrate retrieval

Antigen retrieval was performed by boiling sections in 10 mM citrate buffer, pH6.0 in an 800 W microwave for 10 min. Following this, sections were allowed to cool for 30 min at RT, and then the sections were washed twice in PBS for 5 min each.

4.2.4.3 Blocking

An endogenous peroxidase block step was performed by incubating sections in 3% (v/v) H_2O_2 in methanol for 10 min at RT, and then sections were washed with PBS buffer for 10 min. After this, sections were blocked with an appropriate normal serum for 30 min at RT (Table 4.3) to minimise non-specific primary antibody binding.

4.2.4.4 Primary antibody/isolectin-B4 step

von Willebrand factor, smooth muscle actin and HSD3B

For VWF, the primary antibody and control rabbit IgG was diluted in 2% (v/v) normal goat serum. While, PBS was used to dilute ACTA2 and HSD3B primary antibodies and their respective IgG controls. For control slides, the primary antibody was replaced with rabbit or mouse IgG at equivalent concentration. Then slides were incubated in a humidified chamber overnight at 4°C.

Table 4.3: Primary antibodies used to detect endothelial cells, pericytes and large luteal cells in the bovine corpus luteum.

Primary antibody	Target cell	Supplier	Retrieval	Blocking serum	Conc/ dilution	Species
VWF	Endothelial cell	Dako; A0082	Trypsin	20% NGS	20µg/ml	Rabbit
Isolectin B4	Endothelial cell	Vector; B1205	Citrate	4% NGS	10µg/ml	n/a
ACTA2	Pericyte	Sigma; A2547clone 1A4	Citrate	4% NHS	5µg/ml	Mouse
HSD3B	Steroidogenic luteal cells	Gift[1]	Citrate	4% NGS	1:400	Rabbit

[1]Gift from Richard Parker, University of Birmingham, Alabama; NGS= normal goat serum; NHS= normal horse serum

Biotinylated Griffonia (Bandeiraea) simplicifolia isolectin B4

Biotinylated isolectin was diluted with PBS buffer, and for control slides to this 500mM galactose was added. Then slides were incubated in a humidified chamber overnight at 4°C.

4.2.4.5 Secondary antibody

On the following day, the primary antibody was removed, and slides were washed twice with PBS for 5 min except for VWF IHC where slides were washed with 0.1% (v/v) Triton-x-100 in PBS as the first wash to decrease the

background staining. Then, the primary antibodies were detected using the Vector ABC Elite method, that used biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG as secondary antibodies.

The biotinylated secondary antibodies were diluted 1:250 in 2% (v/v) normal goat or horse serum, added onto the slides and incubated for 30 min at RT. Then slides were washed with PBS twice for 5 min each time. However, for isolectin-B4, sections, the biotinylated step was omitted and went straight to the ABC step. The ABC solution (prepared by adding 2 drops of solution A and 2 drops of solution B to 5 ml of PBS buffer 30 min prior to adding to sections. ABC solution was then added onto each slide and incubated for 30 min at RT. This step was followed by two washes with PBS for 5 min.

4.2.4.6 Detection of antigen

3,3'-diaminobenzidine tetrahydrochloride (DAB) was used for visualisation of antigen-antibody binding. The DAB solution (prepared as per instructions) was added to slides and incubated for 1-5 min, and then the reaction of development was stopped by rinsing slides into tap water for 10 min.

4.2.4.7 Counterstaining and mounting

Sections were counterstained with haematoxylin for 20 sec then washed with running tap water for 5 min, before being dipped (8 dips) in 1% (v/v) ammoniated water. Slides were washed once with tap water before being dehydrated in a series of increasing ethanol concentrations (70%, 95%) and 100% (v/v) for 2 min each time then placed in histoclear for 2 x 5 min. Slides were then mounted with DPX mounting medium. Pictures across each whole section were taken under brightfield with a Leica DM 4000 B microscope (Leica Microsystem (UK) Ltd, Milton Keynes, UK), fitted with a Qlmaging Micropublisher 5.0 RTV colour camera (Qlmaging (UK) Ltd, St. Helen, Merseyside, UK).

4.2.5 Quantification of immunohistochemistry staining

The quantification of VWF, ACTA2 or isolectin B4 was based on that previously described by Robinson et al, (2006). Slides were allocated a random code to ensure unbiased analysis. For each slide, 10 fields of view were randomly selected across the whole section under 20x objective and analysed to

determine % area of staining. For each section, the mean was then taken from these values. The brown colour was selected using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK), which converted this staining into a % area of staining. The estimated total endothelial cell (VWF or isolectin) or pericyte (ACTA2) weight was calculated by multiplying the CL weight by the mean percentage per-area of staining for each CL.

4.2.6 Expression of TLR4, CD14 and MD2 in bovine CLs at different stage of oestrus cycle.

4.2.6.1 Tissue collection

Ovaries were collected within 20 min of slaughter at a local abattoir (Telford abattoir). Corpus luteum were removed from bovine ovaries of disease-free uteri (n=9) and categorised by stages into: early, mid, regressed CL (n=3 per time point) as previously described (Ireland et al. 1980). Three CL from uterus with reproductive tract inflammatory disease explained in Section 4.3.1 and for confirmation see Fig.4.2. The CL segments were placed in RNALater until RNA extraction.
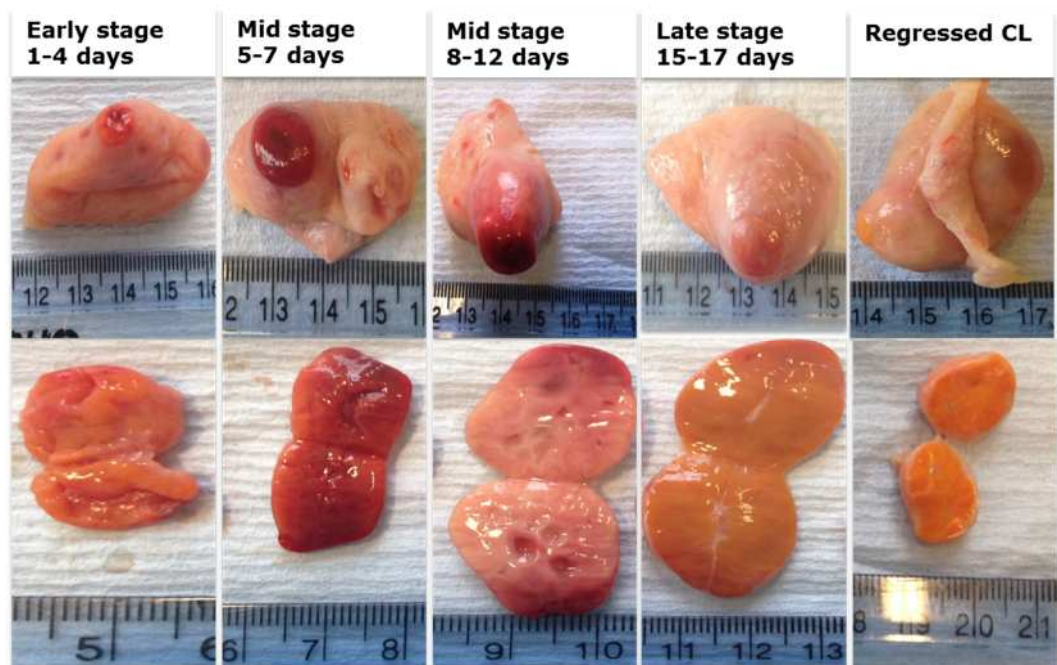
Figure 4.2: Images of bovine corpus luteum (CLs) from different stage of the oestrus cycle.

4.2.6.2 RNA extraction

Luteal tissue (15mg) was transferred into tube containing 350µl of TRK lysis buffer (MicroElute™ Total RNA Kit, OMEGA Bio-Tek) and 7µl β-mercaptoethanol added, and then luteal tissue was homogenised using a tissue homogenizer. The homogenised lysate has transferred into 1.5ml Eppendorf tubes and centrifuged at 13000**g** for 2 min. The clear supernatant was then transferred into a new 1.5ml Eppendorf tube. An equal volume of ethanol (70%) was added and vortexed thoroughly, and the entire lysate was transferred to a MicroElute™ RNA column. The lysate was then centrifuged at 13000**g** for 15 sec. The flow through was discarded and the column transferred to a new 2ml collection tube, then 500µl of RWF wash buffer I added to column, and then centrifuged at 13000**g** for 30 sec. Flow-through was discarded and then 500µl of RNA wash buffer II added to column and then centrifuged at 13000**g** for 30 sec, the last step was repeated once.

The micro-column was centrifuged at 13000**g** for up to 2 min to completely dry it. The column was to a new cooled 1.5 ml Eppendorf tube, and then 10µl of DEPC water was added to the column. Lastly, columns were centrifuged at 13000**g** for 1min and eluted RNA was stored at -70°C. RNA yield was, measured using ultraviolet spectrophotometer (Eppendorf UK Ltd, Cambridgeshire, UK) at absorbance of 260nm. Purity of RNA was assessed using the A260nm:A280nm ratio and only samples with a ratio between 1.8 and 2.0, were used for further analysis.

4.2.6.3 Primer design

Primers were designed using the DNA sequence obtained from the NCBI Genbank for *Bos taurus* TLR4, CD14, and MD-2 and SUZ12 (Table 4.4). This study used SUZ12 as a housekeeping gene as it showed a good stability across bovine luteal samples (Rekawiecki et al. 2012). Primers were positioned in different exons to avoid the amplification of genomic DNA.

Table 4.4: Primer sequences for multiplex PCR in bovine CL samples.

Gene	Sequences (5´-3´)	Size (bp)	Anne. temp	Accession No.
MD-2	5'GAATCGTTGGGTCTGCAACT'3	125	60	AB072456.1
	5'AGAGTATTCCACGGCTTCC'3			
TLR4	5'TCCCCGACAACATCCCCATA'3	224	60	NM_174198.6
	5'AAAGGCTCCCCAGGCTAAAC'3			
CD14	5'GAAGCCTGACTGGTCTAGCG'3	230	60	D84509.1
	5'CCTTGAGACGAGAGTACCCG'3			
SUZ12	5'AACGAAATCGTGAAGATGGG'3	286	60	XM_58260
	5'CTAGTGGCAAGAGGTTTGGC'3			

4.2.6.4 Multiplex PCR

Multiplex forward primers were prepared adding 5µl each primers (TLR4, CD14, MD2, and SUZ12; 0.5µM) into 25µl of nuclease free water. Multiplex reverse primers were prepared by adding 5µl each primer (CD14, MD-2, SUZ12; 0.5 µM) or 10µl of TLR4 into 1.5ml Eppendorf tube containing 25µl of nuclease free water. Multiplex PCR reactions were assembled as described in Table 4.5 and performed using the Eppendorf Master cycler (Eppendorf, Stevenage, UK). The reactions were started at 48°C for 30 min and for other 30 min at 42°C, this cycle generate single strand cDNA. Then followed with thermal cycles (denaturation) 95°C for 15 min, 95°C for 30 sec, 68°C for 30 sec, 72°C for 30 sec, 95°C for 30 sec, 55°C for 30 sec (annealing), 72°C for 30 sec. The last 72°C (extension) used to seal the production ends of newly synthesised PCR products (Table 4.6).

Table 4.5: The PCR reaction reagents.

Reagent	Volume (µl)
Multiplex TEMPase 2x master mix	5
Multiplex forward primer	0.5
Multiplex reverse primer	0.5
RNA	1 (1µg)
RNAase inhibitor (40U/µl)	0.3
Reverse transcriptase enzyme (200U/µl)	0.3
dH$_2$O	2.4
Total volume	10

Table 4.6: PCR amplification protocol.

Cycles	Steps	Temp.°C	Time (min)	Purpose
1 (1x)	1	48	0.30	
	2	42	0.30	
2 (1x)	1	95	15.00	Initial denaturation
3 (12x)	1	95	0.30	
	2	68	0.30	
	3	72	0.30	
4 (40x)	1	95	0.30	
	2	55	0.30	Annealing
	3	72	0.30	Extension
5(1x)	1	72	3.00	Elongation
6 (1x)	1	15	0.00	

4.2.6.5 Agarose gel electrophoresis

Aliquots (10µl) of the PCR reaction were mixed with loading dye and electrophoresed on a 1.8% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer with 50 ng/ml ethidium bromide at 82V until sufficient separation was directly visualised on an ultraviolet trans-illuminator (UVP Ltd, Cambridge, UK) and photographed using Polarised film. To estimate the size of the products a 100bp DNA molecular weight marker was used.

4.2.6.6 DNA Sequencing

In order to confirm the identity of the multiplex PCR product, DNA sequencing was performed, following gel electrophoresis. The DNA band was excised from the agarose gel using scalpel under UV light machine (Fig 4.3) and purified as per the Zymoclean™ Gel DNA Recovery kit protocol (Omega Bio-Tek). Briefly, the DNA band was dissolved at 50°C on a heat block (Techne DRI-BLOCK DB.3A) in a 3 volumes of agarose dissolving buffer to release the DNA for 5-10 min. When the gel had dissolved fully the samples were vortexed thoroughly. Then, samples were transferred to a Zymo-Spin Column with a 2ml collection tube and samples centrifuged (Mini Spin, Eppendorf, Stevenage, UK) at 10,000**g** for 1 min. Wash buffer (200µl) was added to Zymo-Spin Colum, centrifuged at 10,000**g** for 1 min, and the process repeated to wash the DNA. The empty column and collecting tube were then centrifuged at 10,000**g** for 1 min to remove the residual wash buffer (alcohol). The columns were transferred to a clean 1.5ml Eppendorf tube.

DNA Elution buffer (10µl; 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) was added directly to the Column matrix to elute the DNA, and centrifuged for 1 min to collect the purified PCR product. Samples were sent for sequencing (Beckman CEQ8000 Sequencer).
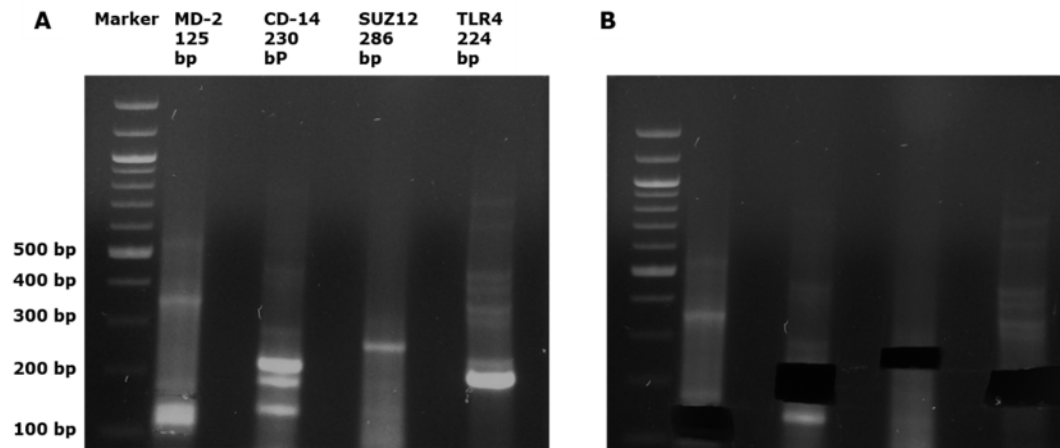
Figure 4.3: Agarose gel showing DNA bands for MD2, CD14, SUZ12 and TLR4 examined under UV light (A) before and (B) after excision for sequencing.

4.2.7 Statistical analysis

All data were checked for normality and homogeneity of variance by using residual plots and Bartlett's test, respectively. Data were log transformed where appropriate. The effect of endometritis on the percentage area of von Willebrand factor or ACTA2 staining as well as the expression of progesterone synthesis proteins were analysed using general analysis of variance ANOVA in GenStat 16th Edition. Disease (endometritis) was added as the factor to the fixed model to account it effect on the responses variance (area stained of endothelial cell, estimated total ECs weight, estimated total of pericytes weight, CL weight and luteal progesterone content). For all experiments $P<0.05$ was considered as significant and all data are presented as mean±SEM.

4.3 Results

4.3.1 Gross morphology of the uterus

Macroscopically, both uterine horns from diseased-free uteri cows were similarly sized and had completely involuted. However, in diseased cows the uteri were characterised by thickened walls, congested, and contained exudates (Fig.4.4). They were similar in overall size to the diseased-free uteri cows and looked like they were completely involuted as judged by gross examination and palpation of both uterine horns.

4.3.2 Histology of the uterus

Histologically, all uterine layers in the control cows appeared normal, with a complete luminal epithelium, and more extensive formation of defined glands compared to endometritis cows (Fig 4.5). However, in endometritis cows, there was a sloughing of epithelial surface and severe infiltration of inflammatory cells (especially neutrophils and macrophages under light microscope) in the sub-epithelial layer as determined by H&E staining (Fig. 4.6). In addition, uteri with endometritis were characterised by a dilatation of blood vessels; scattered of inflammatory cells in the basal layer of endometritis, around uterine glands, and even in the myometrium (Fig. 4.6C-F).
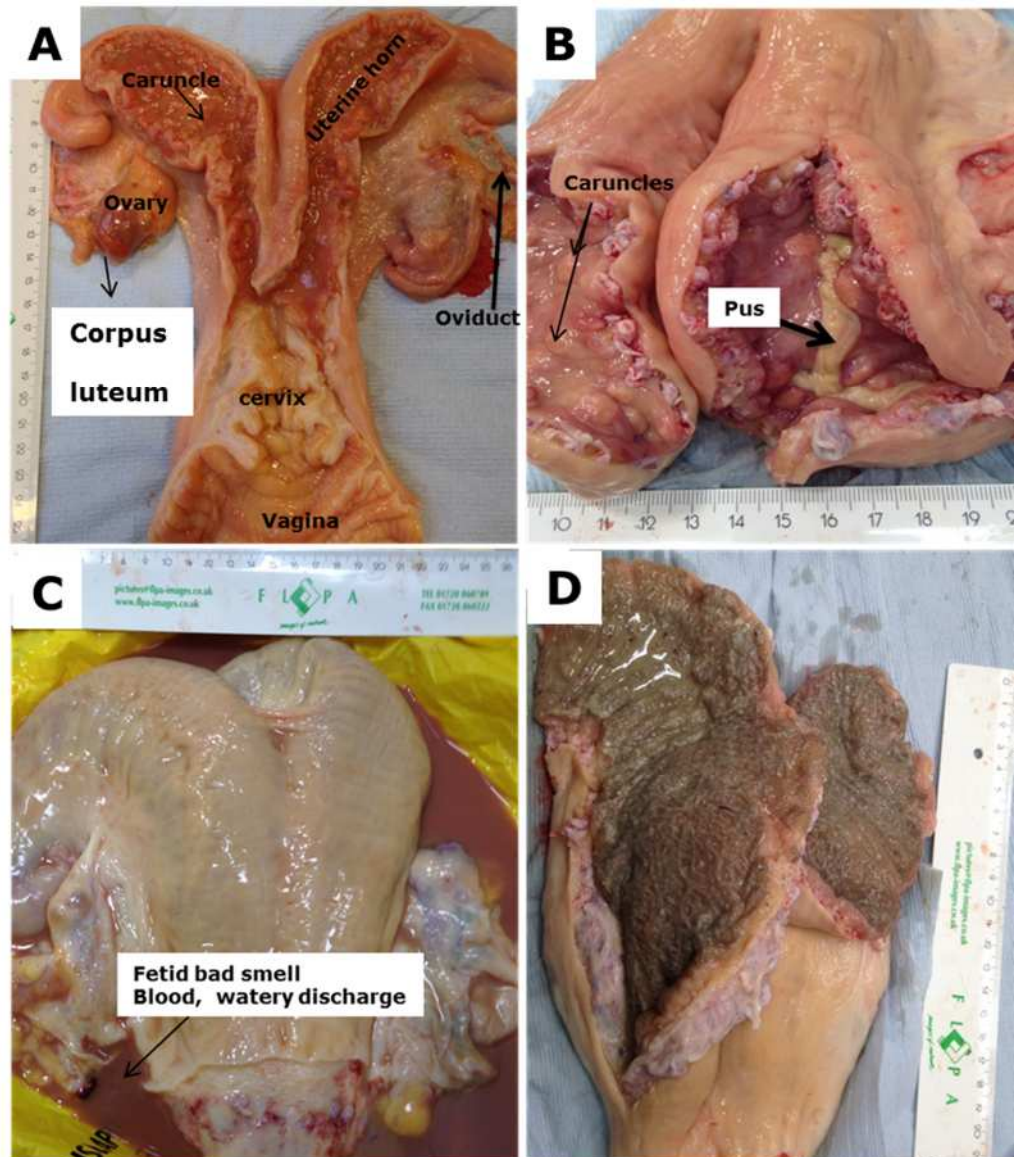
Figure 4.4: Reproductive tract of cows used in this Chapter: (A) diseased-free uterus, (B) Cow 1 with endometritis, purulent discharge presented within uterine horn. (C & D) Cows 2 and 3 with severe metritis and bad odour bloody watery discharge inside the uterus.
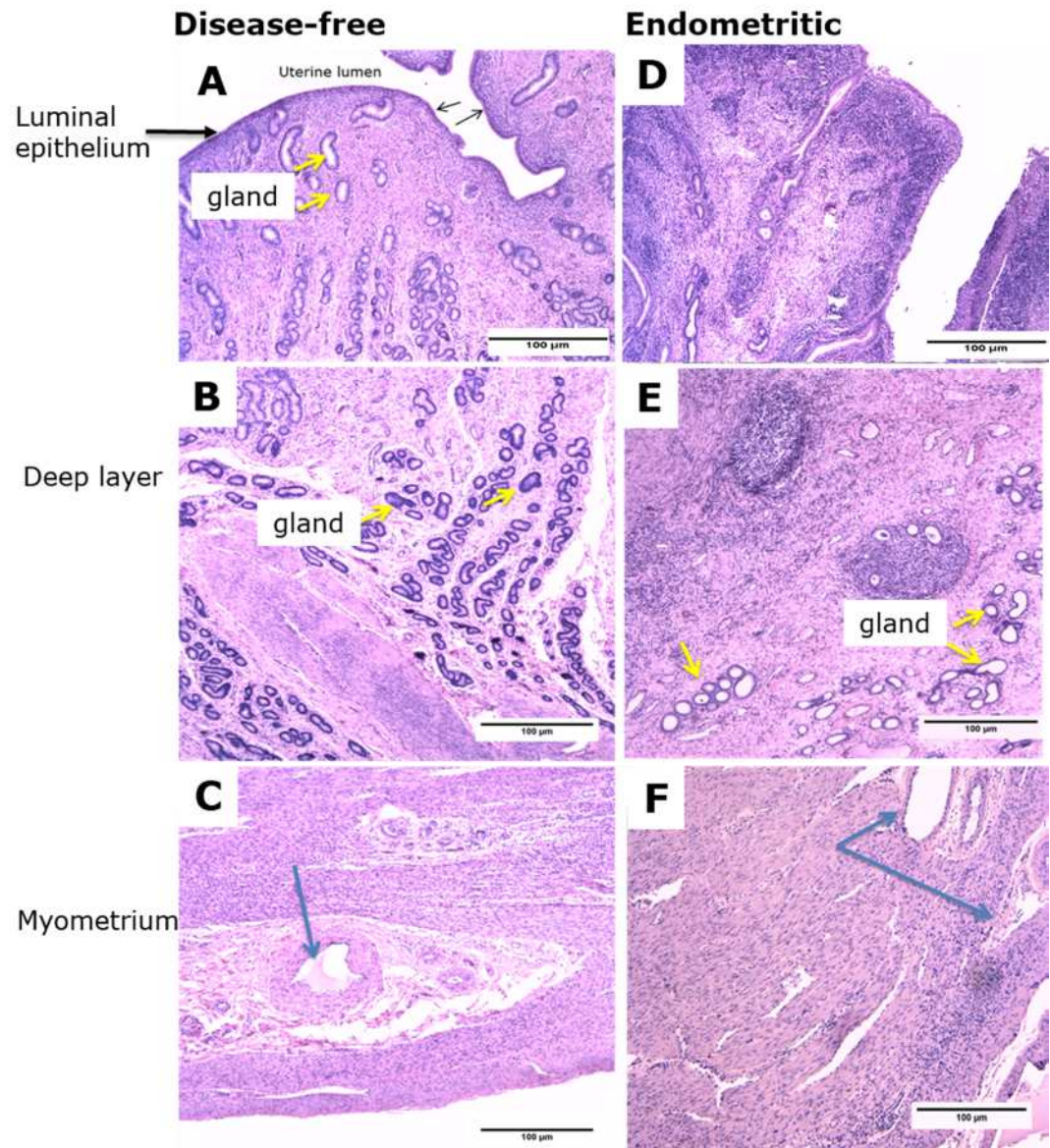
Figure 4.5: Histological analysis of the uterus from (A-C) disease-free uteri and (D-F) endometritic cows; (A&D) show the functional layer of endometrium with luminal epithelium (black arrows), uterine glands (yellow arrows). (B&E) show the deep layer of endometrium. (C&F) show myometrium and perimetrium with blood vessels (blue arrows); scale bar = 100µm.
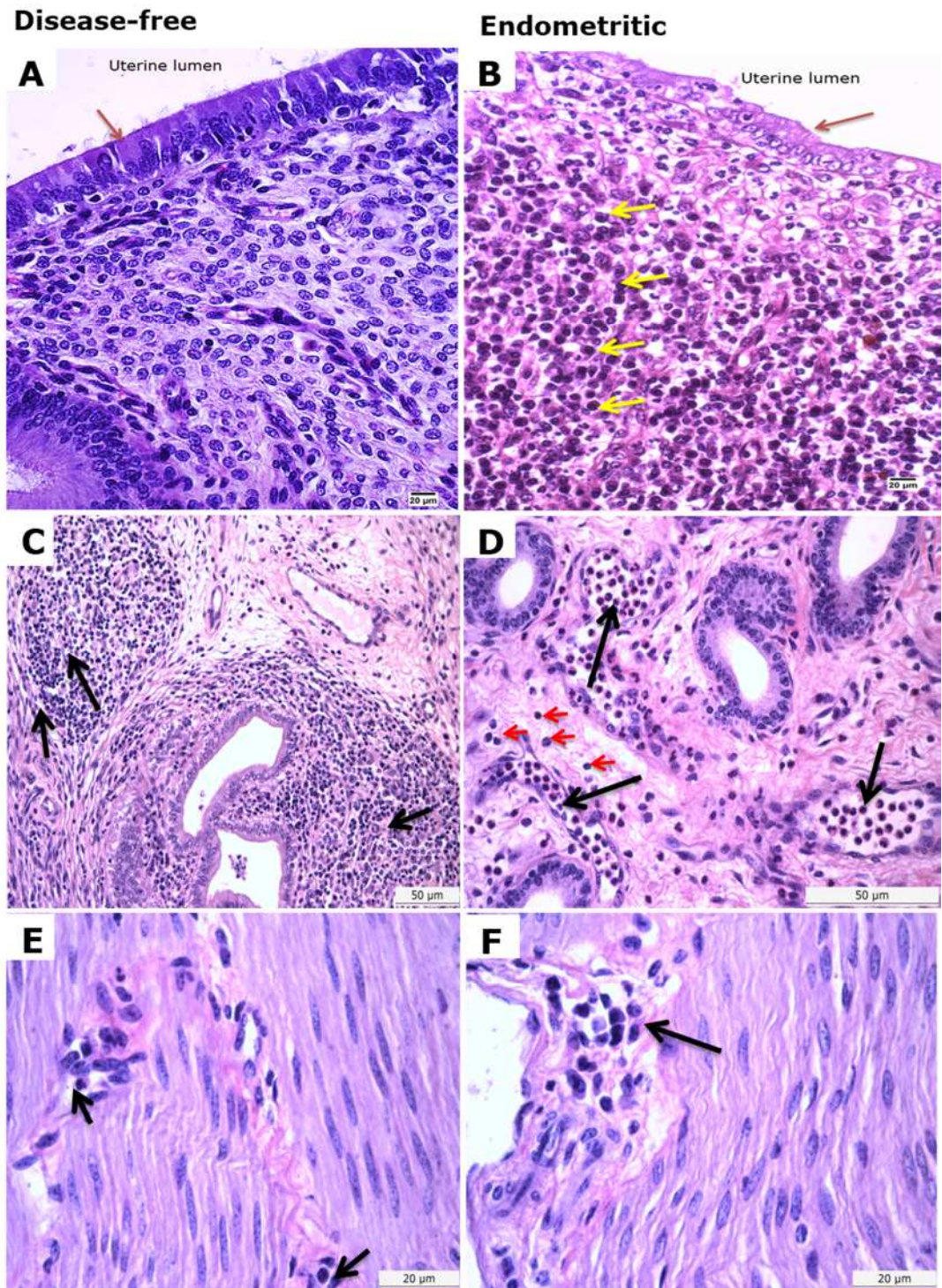
Figure 4.6: Photomicrograph showing epithelial and sub-epithelial layers of the endometrium in (A) disease-free uteri and (B-F) endometritic cows (A) Shows that both epithelial (red arrow) and sub-epithelial layers were normal. (B) Shows infiltration of polymorphonuclear cells (PMC) in sub-epithelial layer of endometrium (yellow arrows). Scale bar = 20 µm. (C) Aggregation of inflammatory cells around uterine glands in the sub-epithelial layer (black arrows), Scale bar = 50 µm, (D) Dilatation of blood vessels (black arrows) and scattered inflammatory cells in the basal layer of endometrium (red arrows), scale bar =50 µm. (E&F) High magnification micrographs showing infiltration of inflammatory cells in the myometrium (black arrows), which indicated that this cow had severe metritis. Scale bar =20 µm.

4.3.3 Corpus luteum weight, luteal progesterone content and expression of progesterone synthesis proteins in cows with endometritis

The results showed that endometritis affected CL weight and luteal P4 content. Cows with endometritis had a 1.2-fold smaller CL (P<0.05) and a 1.2-fold lower luteal progesterone content per unit mass (P<0.05) compared to control cows (Fig. 4.7). This resulted in the total progesterone content being nearly 1.5-fold less in the CL from endometritis cows.
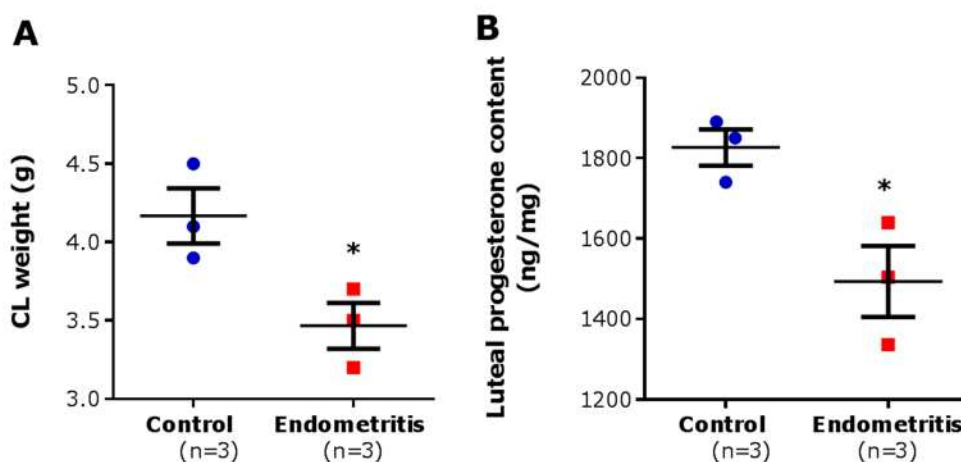
Figure 4.7: (A) CL weight and (B) luteal progesterone (P4) content in cows with endometritis. Cows with endometritis had decreased CL weight vs. control cows (*; *P*<0.05 vs. control); (B) shows that cows with endometritis had lower luteal progesterone content compared to control cows compared to control cows (*; *P*<0.05 vs. control).

Western blot showed that there was a single prominent band for STAR (25kD), HSD3B (42kD), CYP11A (61kD) and histone H3 (15 kD) at expected molecular weights (Fig. 4.8-4.10). Quantification clearly showed that the expression of these key proteins associated with progesterone synthesis were lower in the CL from cows that had endometritis. Namely, STAR (2-fold), HSD3B (8-fold) and CYP11A (3-fold) protein levels were lower in cows with endometritis versus control cows, (P<0.01; Fig. 4.8-4.10). Histone (H3) is housekeeping gene was being stable across the control samples.
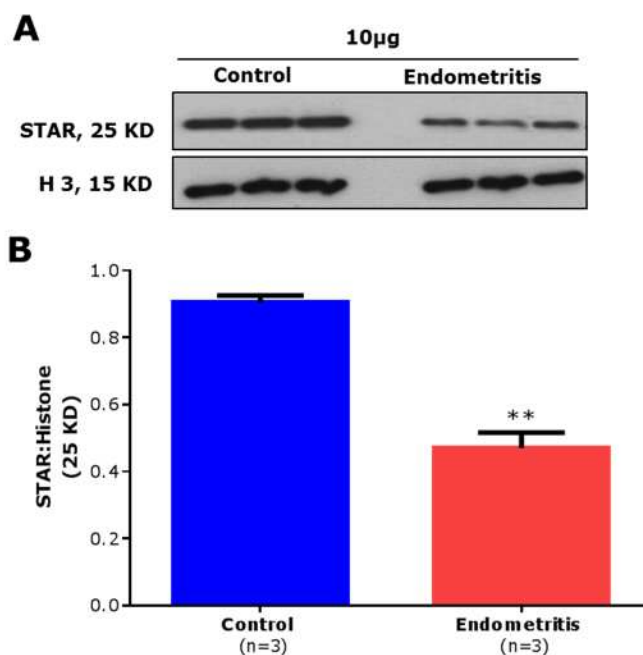
Figure 4.8: The expression of steroidogenic enzyme acute regulatory (STAR) protein in the CL of cows with and without endometritis. (A) Western blot of STAR and histone H3 showed single bands at 25 and 15kD respectively. The housekeeping protein histone H3 (H3) expression was consistent across the different samples. (B) The expression of STAR protein (normalised to histone H3) was different between control and endometritis cows ($P<0.01$; ** *vs.* control). Data are mean±SEM.
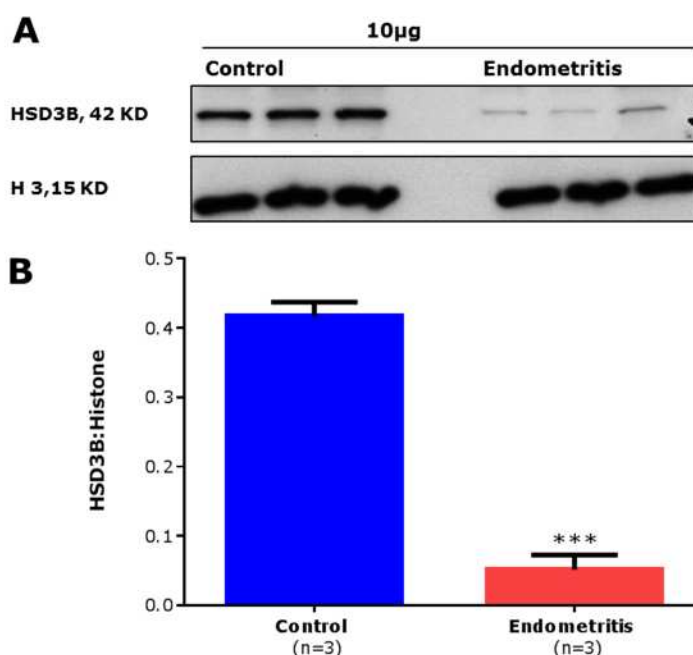
Figure 4.9: The expression of 3β-hydroxysteroid dehydrogenase (HSD3B) in the CL of cows with and without endometritis. (A) Western blot image of HSD3B protein showing a single band for HSD3B at 42kD and the housekeeping protein (Histone H3) was unchanged across the two groups. (B) The expression of HSD3B (normalised to histone H3) was different between control and endometritic cows $P<0.001$; ** *vs.* control). Data are mean±SEM.
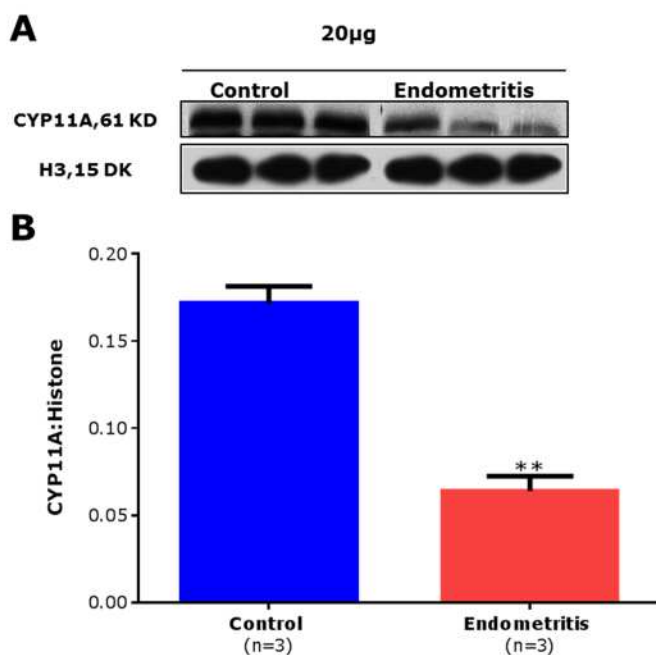
Figure 4.10: The expression of cholesterol side-chain cleavage enzyme (CYP11A) protein in the CL of cows with and without endometritis. (A) Western blot image of CYP11A protein revealed a single band for CYP11A at 61kD and the housekeeping protein (Histone H3) was unchanged across the two groups. (B) The expression of CYP11A (normalised to histone H3) was different between control and endometritis cows $P<0.01$; ** vs control). Data are mean±SEM.

4.3.4 Immunolocalisation of endothelial cells *in* ex vivo CL

There was a different pattern of endothelial cell staining in the luteal tissue between the control and endometritic cows (Fig. 4.11). There was an extensive microvasculature across the whole CL section in control cows with steroidogenic cells very close or adjacent to endothelial cells (Fig. 4.11A&B) this appeared much greater compared to CL of endometritis cows where there was limited staining of endothelial cells (Fig. 4.11C&D). The negative IgG controls were blank (Fig. 4.11E&F).
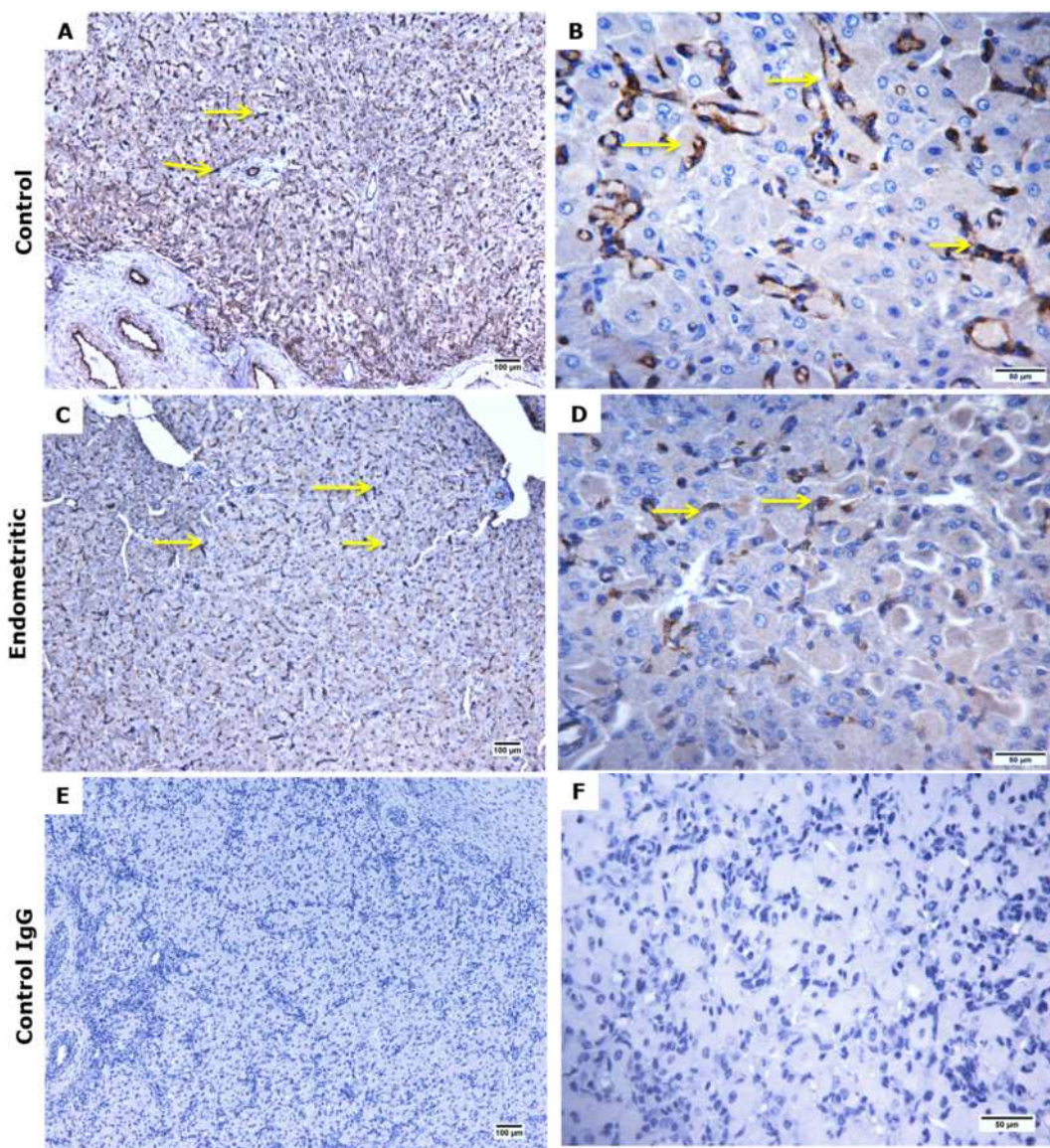
Figure 4.11: Immunohistochemical localisation of von Willebrand factor in the corpus luteum from (A&B) control cows, (C&D) cows with endometritis and (E&F) with the control rabbit IgG (primary antibody absent). In control cows, there was widespread staining (brown) for endothelial cells compared to corpus luteum of cows experienced with endometritis. Arrows indicates area positive with staining VWF. A, C & E; 10X; B, D & F; 40X magnification.

The area of VWF staining in the CL was affected by endometritis (Fig. 4.12; $P<0.001$) with a 3-fold greater percentage area of endothelial cells in control cows compared with cows that had endometritis. The estimated total endothelial cell weight was also affected by endometritis ($P<0.05$), and showed that this was also about 4-fold greater in control compared to cows with endometritis (Fig.4.12B).
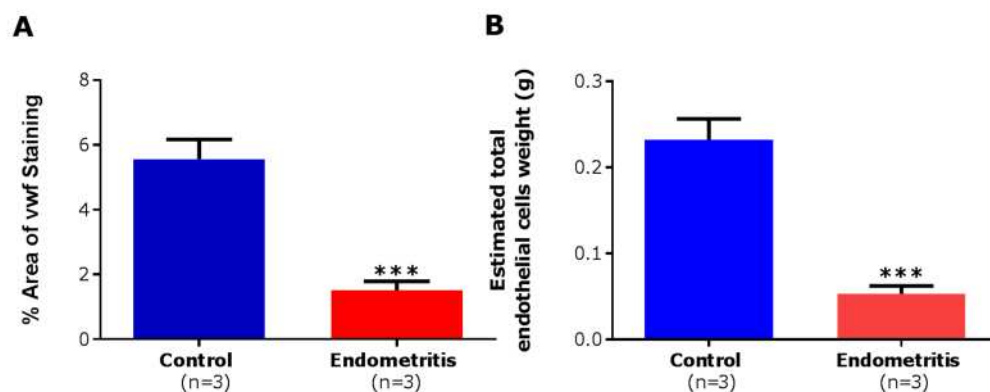
Figure 4.12: The quantification of (A) % area of VWF staining and (B) total estimated weight of endothelial cells in bovine corpus luteum of control cows and those with endometritis. There was a significant effect of endometritis on % area of VWF staining (***; *P*<0.001 vs. control) and estimated total endothelial cells weight (***; *P*<0.001 vs. control). The values are mean±SEM.

In the control cows, the pattern of isolectin B4 staining in the CL was similar to that for VWF immunohistochemistry (Fig 4.11). Additionally, the proportion of luteal EC positively stained with isolectin-B4 in the CL of diseased cows was lower than in control cows (Fig. 4.13A-D). The addition of galactose to the isolectin B4 revealed no positive staining thus confirming specificity of isolectin staining (Fig. 4.13 E&F).

Quantification of isolectin-B4 staining revealed that the percentage area of isolectin-positive endothelial cells was greater (4-fold) in control cows than cows that were experiencing endometritis (P<0.001). The estimated total weight occupied by isolectin B4 positive-endothelial cells in the CL was also 5-fold lower in cows with endometritis (P<0.001; Fig. 4.14B).
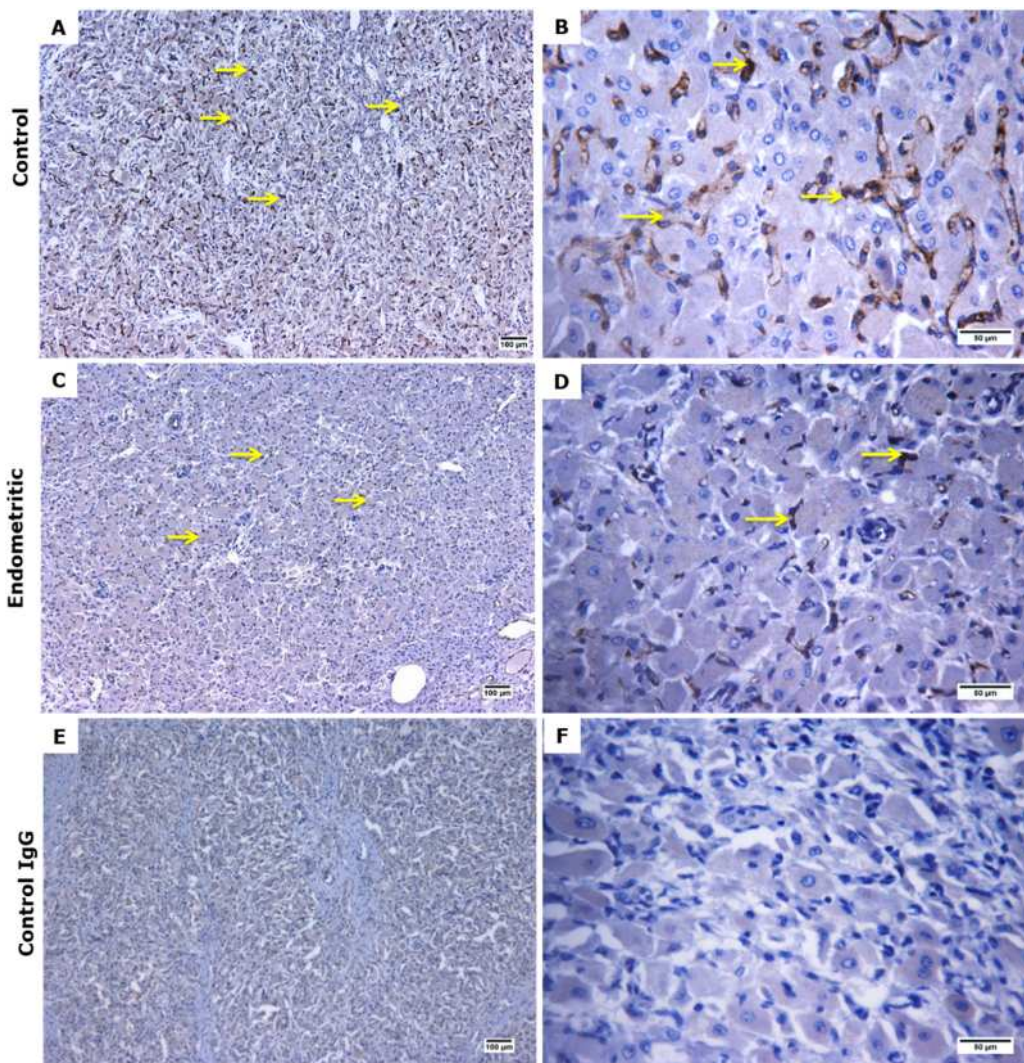
Figure 4.13: Immunohistochemical localisation of endothelial cells using isolectin B4 as a marker. (A, B) shows corpus luteum from a control cow. (C,D) shows corpus luteum from a cow with endometritis cows. (E,F) Represent the control section showing no positive staining. In control cows, there was widespread staining of endothelial cells compared to corpus luteum of cows with endometritis. Arrows indicates positive staining.
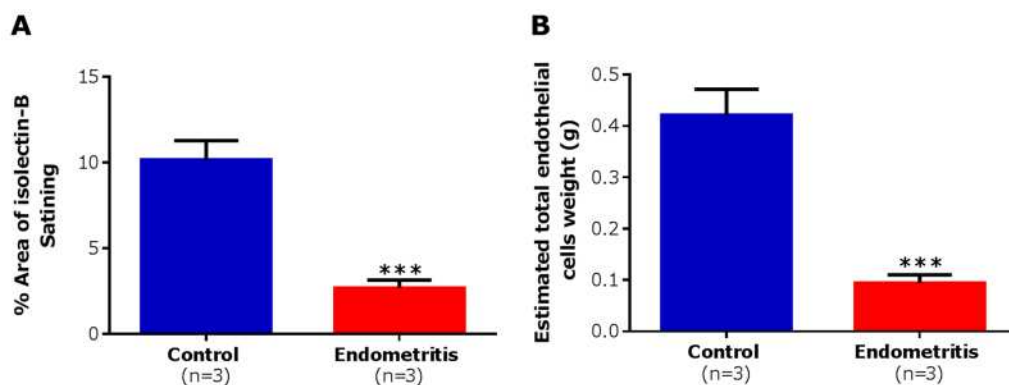
Figure 4.14: The quantification of (A) % area of isolectin B4 staining and (B) estimated total of endothelial cell weight in bovine corpus luteum of control cows and those with endometritis. The % area of isolectin staining (***;$P<0.001$ vs. control) and estimated total luteal endothelial cell weight (***;$P<0.001$ vs. control) were reduced in cows that had endometritis. The values are mean±SEM.

4.3.5 Immunolocalisation of α-smooth muscle actin (ACTA2) in ex vivo CL

Pericytes/vascular smooth muscle cells were stained with ACTA2 in the CL. Staining of pericytes with ACTA2 in the CL from cows with endometritis was visibly less extensive compared to control cows (Fig. 4.15). Furthermore, the staining that was present had a lower intensity appearance. The possible exception was in the larger blood vessels where staining was similar to that for control cows. The negative IgG control revealed no staining (Fig. 4.15E&F). Quantification of ACTA2 showed that the area of ACTA2 was greater in control cows by about 4-fold than cows with endometritis ($P<0.001$; Fig. 4.16A). In addition, estimated total weight of pericytes 4-fold greater in control cows compared with cows that had endometritis ($P<0.001$, Fig.4.16B).
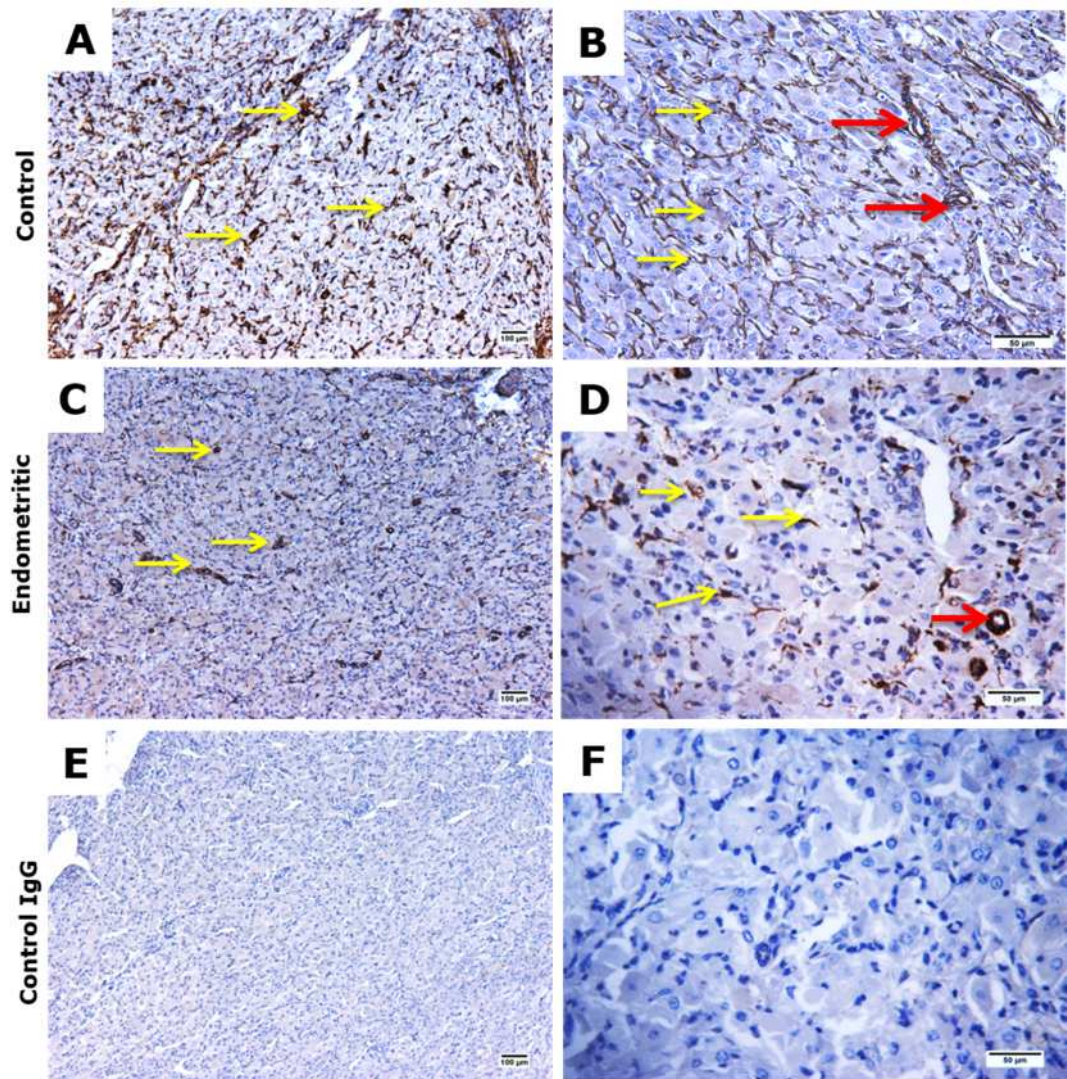
Figure 4.15: Immunohistochemical localisation of α-smooth muscle-actin (ACTA2) in bovine CL. (A,B) shows CL from control cows while (C,D) shows CL from cows with endometritis. (E,F) shows negative mouse IgG controls where no staining was evident. The ACTA2 staining was more extensive and covered a greater area in control cows compared to those from cows with endometritis. Arrows indicates positive staining of pericytes (yellow) and vascular smooth muscle cells (red).
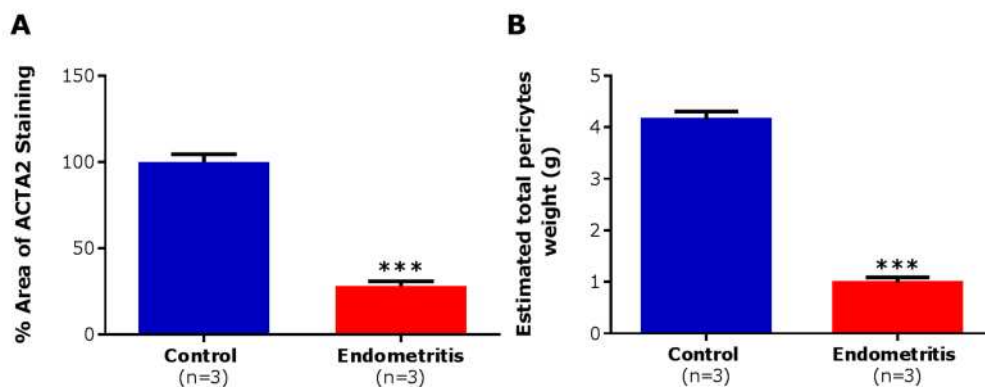
Figure 4.16: The quantification of (A) % area of ACTA2 staining and (B) total occupancy of pericytes in the bovine CL. There was a significant decrease in % area of ACTA2 staining (***;*P*<0.001 vs control) and total amount of pericytes (***;*P*<0.001 vs control) in cows With endometritis. The values are mean+SEM.

The Western blot for ACTA2 revealed a single band at the expected molecular weight of 42kD. Quantification of this band showed that ACTA2 protein expression was markedly lower (6-fold) in endometritic cows compared to control cows (P<0.001; Fig.4.17 A&B).
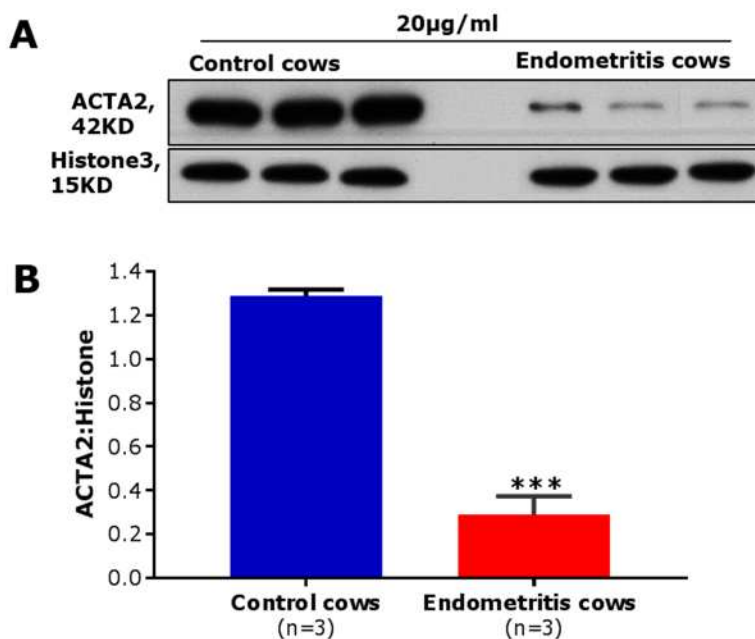
Figure 4.17: The expression of α-smooth muscle actin (ACTA2) protein in the bovine CL in cows with and without endometritis. (A) Shows a representative Western blot analysis for ACTA2 with a single band at 42kD and a single band for histone H3 at 15kD which was constant across the two groups. (B) The expression of ACTA2 (normalised to histone H3) was greater in the control cows than those that had endometritis (P<0.001). The values are mean±SEM

4.3.6 Expression of TLR4, CD14, MD-2 and SUZ12 mRNA in the bovine CL at different stages of oestrus cycle

Using TLR4, CD14, MD-2 and SUZ12 primers (Fig. 4.18), the DNA bands from the PCR were at the expected sizes of 224, 230, 125, and 286 bp, respectively. The appropriately-sized bands were observed in all disease-free CLs and diseased CLs, indicating the presence of mRNA encoding the LPS receptor and its co-receptors mRNA throughout the oestrus cycle. Multiplex PCR products were sent for sequence analysis, which confirmed that fragment sequences generated were 100% homologous in both directions to the bovine sequence already published (Genbank accession numbers, Table 4.4). No quantification or qPCR was performed, thus it was not possible to compare the expression levels between samples. Negative control samples were blank.
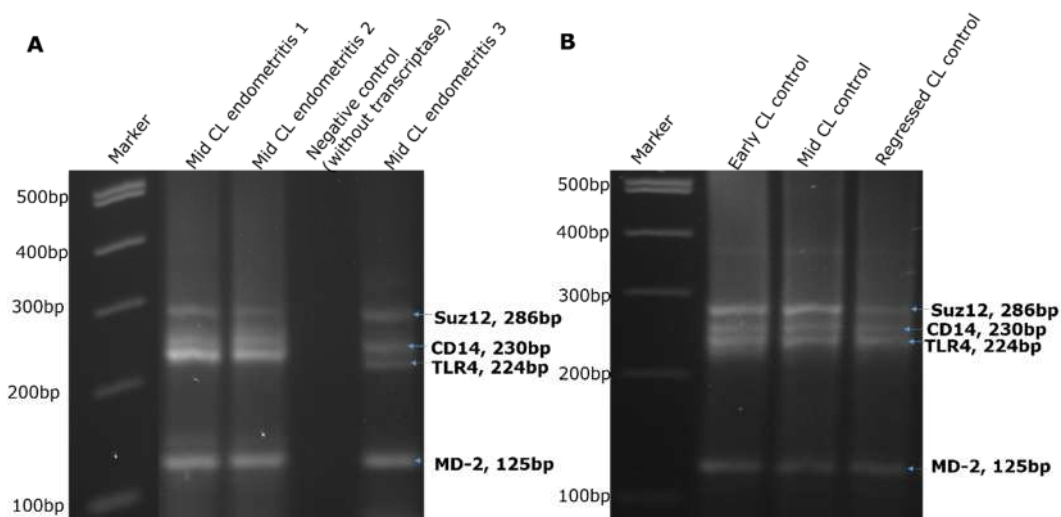
Figure 4.18: Representative multiplex PCR reactions showing the expression of TLR4 mRNA (LPS receptor) and its co-receptors in bovine CL at different stages of oestrus cycle. Far left lane shows the DNA molecular weight marker. A)1=Marker, 2= mid stage CL (endometritic cow), 3= mid stage CL (endometritic cow), 4= negative control [no reverse transcriptase enzyme], 5= mid stage CL (endometritic cow). B) 1=Marker, 2=early stage CL (control), 3= mid stage CL (control), 4= regressed CL (control). bp= base pairs.

4.4 Discussion

The present study determined the association between endometritis and luteal vascularisation and steroidogenic capacity in dairy cows. Endometritis was defined as an inflammation of the superficial layers of the uterus which was

further diagnosed by microscopic examination of pathological lesion of uterine sections with the presence of purulent discharge inside the uterine horns. Metritis is distinguished from endometritis by inflammation of all layers of the uterus, and presence of fetid blood, bad odour and discharge from the vagina and inside the uterus. Animals with endometritis, are microscopically characterised by infiltration of polymorphonuclear cells in the sub-epithelia layer and among the uterine glands, but this does not extend to the deeper layer of the uterus (Sheldon et al. 2006). However, cows with metritis are characterised by an erosion of the superficial layer with severe infiltration of leukocytes in the submucosal layer and among uterine glands. Furthermore, blood vessels appear dilated and blood cells scattered in the myometrium (myometrium degeneration) (Moss et al. 2002, Chapwanya et al. 2012, Sheldon et al. 2006, McEntee 2012).

It has been reported that uterine infection not only affects endometrial function, but also affects ovarian function. Cows with uterine infection particularly those infected with *E.coli* infection have decreased ovarian follicle growth and functions and are less likely to ovulate (Williams et al. 2007, Peters et al. 1989, Sheldon et al. 2002b). In this Chapter, cows with endometritis/metritis had smaller CLs and lower luteal progesterone content versus oestrus cycle-matched control cows. The logical extension of this would be that these endometritic/metritic cows, where ovulation had clearly occurred, would have lower circulatory progesterone concentrations, although this was not tested. This agrees with previous reports that demonstrated that cows with metritis that had small CL and produced less progesterone *in vivo* (Williams et al. 2007, Strüve et al. 2013). Furthermore, treatment of cows with an intra-uterine infusion of LPS reduced CL size and plasma progesterone transiently (Sheldon et al. 2009b, Herzog et al. 2012).

In addition, uterine disease directly perturbed steroidogenesis by ovarian granulosa cells (i.e. reduced *aromatase* expression and oestradiol production) (Sheldon et al. 2002b, Herath et al. 2007). It is likely that this could reduce the ability of the follicle to ovulate and/or form a fully functional CL. However, it is important to note in this study, the CL were collected had the appearance of those from the mid-luteal phase, thus any compromised follicular function in these cows was not sufficient to prevent ovulation.

The reduced luteal progesterone content was strongly supported by markedly reduced luteal protein expression of key proteins involved in progesterone synthesis in cows with endometritis. Namely, the expression of steroidogenic

acute regulatory protein (STAR) protein (Miller 2007) which transports cholesterol from the outer to the inner mitochondrial layer was decreased; cholesterol side-chain cleavage P-450 complex (CYP11A) protein, which converts cholesterol to pregnenolone (Tuckey and Stevenson 1984) was decreased, and expression of 3β-hydroxysteroid dehydrogenase (HSD3B) protein, which converts pregnenolone to progesterone (Rekawiecki et al. 2005) was reduced in cows with endometritis compared to control cows. Similarly, it has been reported that intra-uterine LPS infusion down-regulated luteal STAR mRNA expression which concurred with reduced plasma P4 concentrations (Herzog et al. 2012, Magata et al. 2014). The mechanism through which LPS inhibits steroidogenic enzyme expression in bovine luteal cells is unclear. But this could be as a result of bacterial endotoxins decreasing luteal blood flow which coincided with decreased plasma progesterone as well as reduced steroidogenic enzyme activity (Herzog et al. 2010).

There are some discrepancies about the effect of LPS on plasma P4 concentrations. Some studies have shown that LPS treatment actually increased P4 concentration at some earlier time points after administration (Herzog et al. 2012, Gilbert et al. 1990). However, several studies support the concept that LPS decreases plasma progesterone concentrations. It was reported that CL size decreased after treating animals with *E.coli* LPS (0.5µg/kg body weight) on day 10 of the oestrus cycle (Herzog et al. 2012) and between day 29 and 39 of pregnancy (Debertolis 2015). Also, they found reduced expression of STAR mRNA at 12h after infusion. The same observations were reported by Williams et al. (2008b) where systemic progesterone concentrations were decreased over several days in cows after intrauterine infusion of LPS. However, previously it has been reported that reduced plasma P4 concentrations occurred concurrent with decreased luteal blood flow (Herzog et al. 2012). In that regard, plasma progesterone concentrations decreased to minimal values between 9 and 24h after LPS treatment, consistent with the close correlation between luteal blood flow and P4 (Herzog et al. 2012).

The results of the current study demonstrated that CL from cows with endometritis had a significantly reduced degree of vascularisation and estimated total luteal vasculature. Additionally, there was reduced pericyte coverage in the CL. A particularly interesting aspect to this novel finding was the high amount (4-fold) by which these vasculature parameters were affected. The logical extension of this observation would be markedly reduced

luteal blood flow, reduced biochemical/metabolite exchange and decreased supply to steroidogenic cells of cholesterol/LDL for progesterone production.

It is important to understand the mechanisms through which uterine inflammation could affect luteal angiogenesis. This could be via altered cytokine production such as tumour necrosis factor alpha (TNFA). When leukocytes and macrophages infiltrate the endometrium *TNFA* expression is up-regulated (Haddad et al. 1995, Bondurant 1999, Janowski et al. 2013). TNFA could then act directly via the circulation on luteal cells and endothelial cells (Williams et al. 2008b, Crespo et al. 2010). Circulating TNFA is increased in cows with endometritis (Brodzki et al. 2015, Kasimanickam et al. 2013). It known that TNFA exerts actions on the CL though multiple signalling pathways involving two receptors: TNFR1 (responsible for induction of the cell death) and TNFRII (implicated in cell proliferation) (Korzekwa et al. 2008). Both luteal cells and endothelial cells possess TNF receptors (Okuda et al. 1999, Friedman et al. 2000). It has been reported that TNFA is cytotoxic for endothelial cells derived from the corpus luteum (Friedman et al. 2000). This was mediated through TNFRI and preceeded structural luteolysis, which is a prerequisite for the initiation of apoptosis in endothelial cells (Pru et al. 2003, Friedman et al. 2000). TNFA enhanced apoptosis which associated with both morphological and biochemical features of apoptosis, including caspase-3 activation, shrunken nuclei, and DNA fragmentation (Petroff et al. 2001, Jo et al. 1995). Thus, altered systemic TNFA as a consequence of an extensive uterine infection could have a role in the regression of the luteal microvasculature.

Several studies demonstrated that LPS affects cytoskeletal filaments in endothelial cells and in so doing makes gaps between adjacent cells, and disrupts intercellular gap formation that led to endothelial barrier dysfunction, (Chakravortty et al. 2000, Goldblum et al. 1993, Stasek et al. 1992). Vascular endothelial cadherin (VE-cadherin) is located exclusively at the endothelial intercellular junction, and plays an important role in the maintenance and regulation of the endothelial barrier via cell-cell adhesion (Dejana et al. 1999, Lampugnani et al. 1992). *In vitro* LPS (20µg/ml) reduced VE-cadherin expression in human umbilical vein endothelial cells (Hama et al. 2008). Furthermore, Bogatcheva et al. (2009) observed that lower doses of LPS (100ng/ml) caused disturbance in the endothelial cytoskeletal organisation. They speculated that this gap was due to decreased expression of VE-cadherin in human lung microvascular endothelial cells.

It has been reported that transforming growth factor B1 (TGFB1) has been implicated in the positive and negative regulation of angiogenesis *in vivo* by inhibiting the proliferation of endothelial cells *in vitro* (Iruela-Arispe and Sage 1993, Ferrari et al. 2009). Previously, it was reported that LPS induced upregulation of TGFB1 (Chen et al. 2008) and this could be a mechanism by which LPS could reduce the degree of vascularisation in the CL that requires intense angiogenesis for its formation.

The sharp decrease in luteal blood flow after LPS infusion could be associated with, significant systemic problems such as septicaemia, and blood flow is diverted from the periphery to the core (Richardson et al. 1996). This might have accounted for the precipitous decline after LPS perfusion. Furthermore, luteal size decreased from 5.2 to 3.8 cm within 24 h after treatment with LPS and remained smaller throughout the remainder of the cycle (Herzog et al. 2012). It has been shown that LPS can contribute to the initial damage of various types of endothelium (Frey and Finlay 1998, Choi et al. 1998, Koide et al. 1996). Microvascular injury happens during sepsis, such as in liver, gut and lung. Generally, this event has been considered important in the pathogenesis of the septic shock syndrome (Morrison and Ryan 1987). It has been reported that critical role of endothelial cell apoptosis in the pathogenesis of endotoxin shock. However, prolonged exposure to lower LPS (0.01-100µg/mL) can lead to stimulation of angiogenesis. For example, Kim et al. (2008) reported that LPS increased *VEGFA* mRNA expression pericytes from rat lungs.

The results of this study established that TLR4 and its LPS co-receptors are expressed in bovine CLs at different stages of the oestrus cycle, using multiplex PCR. The results showed that *TLR4*, *CD14* and *MD2* mRNA were all present in the bovine CL throughout the oestrus cycle from both control cows and cows with endometritis. While no quantification of this expression was performed, the next step would be to perform quantitative PCR in order to precisely quantify their expression. It has recently been shown that TLR2 and TLR4 mRNA is expressed in mid-cycle CL of isolated perfused bovine ovaries *in vivo* (Lüttgenau et al. 2016). The identification of receptors in the bovine CL that recognise LPS indicate that LPS could potentially directly influence the function and development of CL. It has been reported that TLRs are important mediators of the immune system and the TLR-mediated production of inflammatory cytokines could affect the other luteal cells populations (e.g. steroidogenic cell, endothelial cells, fibroblasts and pericytes). Previously, there was no differences in mRNA expression of TLR4 between healthy and diseased CL (Lüttgenau et al. 2016).

LPS receptors including *TLR4, MD2 and CD14* were expressed in bovine granulosa cells, which have an important role in initiating the innate immune response to Gram-negative pathogens. It has been demonstrated that granulosa cells exhibited functional TLR1, TLR2 and TLR4 responsiveness to PAMPs with increased accumulation of inflammatory cytokines and chemokines leading to activation of MAPK pathway (Price et al. 2013). The high level of *TLR2*, *TLR3*, *and TLR4* expression in luteolytic CL tissue could indicate a role in the initiation of luteolysis (Tyson 2014). Previously, reported that the degradation of luteal tissue may serve as a source of endogenous ligands for LPS receptors and would potentially activate inflammatory mediators like TNFA, IFNG, IL6, IL12, and TGFB (Tyson 2014).

4.5 Conclusion

There is no doubt that luteal angiogenesis is essential for the proper function of the CL and thus pregnancy. The % area of both endothelial cells and pericytes were markedly reduced in cows with endometritis. Furthermore, luteal progesterone content and expression of progesterone synthesis proteins were significantly reduced in cows with endometritis. The present findings suggest a novel mechanism by which endometritis could affect ovarian and in particular CL function *in vivo.* LPS receptor and its co-receptors TLR4, MD-2 and CD-14 were expressed in luteal tissue throughout the oestrus cycle of control cows and cows with endometritis. It seems possible that luteal and endothelial cell TLR4, MD-2 and CD14 are involved in the immune response of luteal tissue to LPS *in vivo*, which could be related to the production of pro-inflammatory cytokines and decreased ovarian steroidogenesis in cows. Future studies are required to measure the production of pro-angiogenic (e.g. FGF2, VEGFA, fibronectin), anti-angiogenic factors (e.g. TGFB, TNFA) as well as pro-inflammatory cytokines (e.g. IL-1, IL-6 and IL-8), in the CL and endometrium at different time points in cows with endometritis. To gain a deeper understanding of how endometritis could affect luteal angiogenesis, the next chapter focussed on dose-dependent effects of LPS on luteal endothelial cells and progesterone production in culture.

5 The effect of *E.coli* LPS on bovine luteal endothelial cell network formation and steroidogenesis *in vitro*

5.1 Introduction

In dairy cows, post-partum uterine inflammation caused by Gram-negative bacteria (e.g. *E.coli*) suppresses follicular function and reduces fertility. LPS is an endotoxin that is present on the outer membrane of Gram-negative bacteria. Furthermore, LPS is detected in follicular fluid (0.004-0.88μg/ml) of cows with endometritis. The mechanism by which LPS enters the follicular fluid is controversial. However, it has been suggested that LPS is absorbed by the uterus and then into the blood stream (Mateus et al. 2003, Dohmen et al. 2000). The corpus luteum (CL) is a site of high vascular formation, characterised by the formation of a dense capillary network. This plays a key role in suppling hormone-producing cells with oxygen, nutrients and hormone precursor required to synthesise and release of large amount of progesterone, that is necessary for establishment and maintenance of early pregnancy (Redmer and Reynolds 1996, Reynolds and Redmer 1998, Acosta et al. 2003). Angiogenesis in the CL involves a complex series of cellular processes and molecular changes. An establishment of vascular system in the CL depends on different cell types including: The inner lining endothelial cells, related to mural cells such as pericytes and vascular smooth muscle cells (vSMC). Such vessels remain quiescent until there is an angiogenic stimulus such as hypoxia, injury, or ovulation.

The degree of vascularisation can be determined by immunostaining for von Willebrand Factor (VWF) as a marker for endothelial cells (Ratcliffe et al. 1999, Antczak and Van Blerkom 2000). Angiogenesis in the CL is principally controlled by several factors, including vascular endothelial growth factor A (VEGFA) (Ferrara et al. 2003, Gerhardt and Betsholtz 2003, Woad et al. 2009), and fibroblast growth factor 2 (FGF2) (Woad et al. 2009). For example, immunoneutralisation of VEGFA in the early luteal phase decreased endothelial cell number and reduced progesterone production in marmoset monkeys (Fraser et al. 2000) and cows (Yamashita et al. 2008). Many previous reports have demonstrated the angiogenic potential of LPS (Mattsby-Baltzer et al. 1994, Harmey et al. 2002). However, there is controversy as to whether lipopolysaccharide (LPS) directly stimulates endothelial sprouting and angiogenesis, or whether it is mediated through cytokine production (Koolwijk

et al. 1996, Pidgeon et al. 1999). In addition, it has been suggested that the angiogenic effect of LPS may be secondary to the release of angiogenic factors from adjacent non-endothelial cells (Pidgeon et al. 1999, Leibovich et al. 2002). Conversely, LPS has induced apoptosis in various endothelial cell subtypes such as human umbilical vein endothelial cells and lung-derived normal human microvascular endothelial cells (Koide et al. 1996, Bannerman et al. 1998, Munshi et al. 2002). The present study determined whether LPS would adversely affect the proliferation of luteal endothelial cells and their ability to form networks. *In vitro* effects of LPS on luteal function and endothelial cells are poorly understood. In this chapter, two experiments were preformed, to investigate this further.

Hypothesis

E.coli LPS will reduce luteal endothelial network formation, through increased apoptosis and decreased proliferation of luteal endothelial cell. At the same time, LPS will reduce luteal progesterone production and steroidogenic enzyme expression *in vitro*.

Aims

In order to address this hypothesis, this chapter had two aims: (1) to determine the effect of LPS on luteal endothelial cell (EC) network formation and progesterone production and (2) to elucidate the effect of LPS on luteal endothelial cell proliferation and apoptosis as well as the expression of ACTA2 (smooth muscle actin).

5.2 Experiment 1: Effect of LPS on luteal endothelial cell networks and progesterone production in culture

5.2.1 Materials and methods

5.2.1.1 Luteal angiogenesis culture system

Coating of coverslips

Coverslips (circular, 19 mm diameter, 0.25 mm thick, SLS, Nottingham, UK) were placed in 70% (v/v) IMS for at least 1 hour. These coverslips were taken out individually and briefly dipped in 70% (v/v) IMS, then placed on a petri dish to dry at least 1 hour before being transferred to 12-well plates. Fibronectin (500µl; 10µg/ml) was added to each well, and then incubated in a

humidified chamber at 39°C. After 4 hours, the fibronectin was removed and the wells allowed to dry at 39°C overnight. On the next day, coverslips were washed once with 1ml of sterile distilled water, and then 1ml endothelial cell media (EBM-2, Lonza, Wokingham, UK) was added to each well. Plates were incubated at 39°C until cell plating, as required.

Preparation of specialised endothelial cell media

The endothelial cell media (ECM) is a proprietary specialised endothelial cell medium (EBM-2; Lonza, Wokingham, UK), which was supplemented as per manufacturer's instructions (Table 5.1). In addition, the media was supplemented with 5ng/ml LH (AFP11743B, biopotency 1.06, LH NIDDK-I-2; NIDDK,CA, USA), 100 units/ml penicillin, 10 μg/ml streptomycin and 5 ml of 100x of ITS (1.0mg/ml recombinant human insulin, 0.55mg/ml human transferrin (substantially iron-free), and 0.5μg/ml sodium selenite).

Table 5.1: Composition of endothelial cell media-2, Lonza [500ml bottle].

Supplement	Volume
Human epidermal growth factor	0.5 ml
Ascorbic acid	0.5 ml
Long™ R3 insulin like growth factor-1	0.5 ml
Fetal bovine serum	10 ml
Heparin	0.5 ml
Hydrocortisone	0.2 ml
VEGFA	1ng/ml[#]
FGF2	1ng/ml[#]
Gentamycin sulphate, amphotericin (GA 1000)	0.5 ml

[#] As appropriate

Tissue collection and luteal angiogenesis culture system

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warmed 1×PBS (PBS). Corpora lutea chosen were from an early stage (day 1-4) and mid stage (day 5-7) of the oestrus cycle. These were selected according to the standard classification system (Ireland et al. 1980, Fig. 5.1).
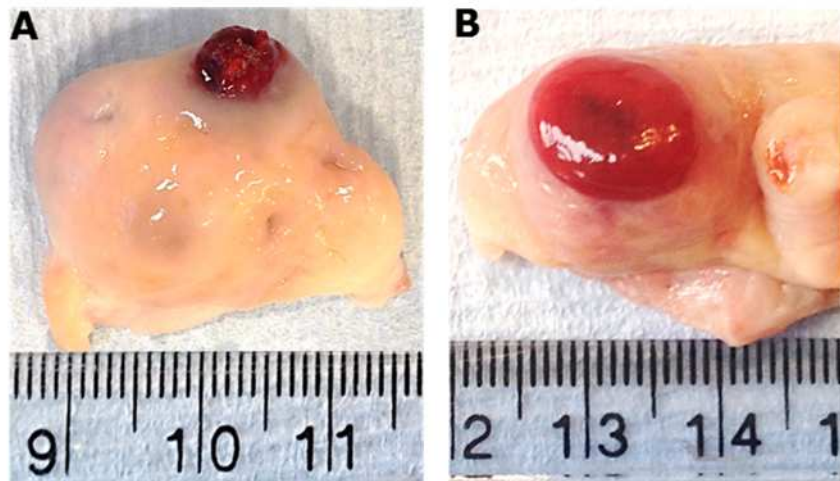
Figure 5.1: Bovine ovaries at different stages of oestrus cycle: (A) Ovary from very early luteal phase (day 1-4). (B) Ovary from mid-luteal phase (day 5-7).

The ovaries were rinsed with 70% (v/v) IMS and then rinsed with sterile PBS and handled in an aseptic manner henceforth. CLs were dissected from connective tissue into a petri dish and then sliced up using scissors and chopped with surgical blades. Chopped luteal tissue was dissociated in 10ml DMEM/Hams medium containing 2 mg/ml collagenase I type 1A (Sigma, C2674) and 25 µg/ml DNase I (Sigma; D0525) which was put into a conical flask and incubated in a shaking water bath for 30-45 minutes at 37˚C. Following incubation, the solution was poured into 50 ml falcon tubes and allowed to settle before the solution was pipetted off and filtered through a 70µm cell strainer (BO Biosciences, Oxford, UK) into a new falcon tube which was then centrifuged at 150**g** for 5 min. The supernatant was removed and the cell pellet resuspended in ECM media. Meanwhile, fresh dissociation solution was added to the remaining tissue from the first digestion and incubated in a shaking water bath at 37°C for a further 30-45 minutes. The cells were prepared as for the first digestion and then combined together before being centrifuged at 150**g** for 5 minutes.

The supernatant was removed and the pellet washed with 10 ml of ECM three times to remove any visible RBC and clean-up the cell pellet. After the final centrifugation, the pellet was re-suspended with 5 ml of ECM. The number of viable cells were counted using Trypan Blue exclusion and hemocytometer after diluting the cell suspension 1 in 20.

Experimental design

The cells were diluted to 1×10^5 cells per ml and 2ml of this dilution was added to each well (12 well plates; Fig.5.2). The cells were treated with *E.coli* O111:B4.lipopolysaccharide (Alpha diagnostic international, LPS11-1, is highly

purified with <3% impurities) (0, 0.01, 0.1, 1, 10µg/ml) in the presence and absence of FGF2 and VEGFA (both 1ng/ml) (Fig. 5.2A and B). The plate was then incubated at 39°C in humidified incubator in 5% CO_2; 95% air. The culture media changed every other day and media was collected and stored at -20°C for progesterone measurement. Total number of cultures performed at early stage (n=5) and at mid stage CLs (n=5).
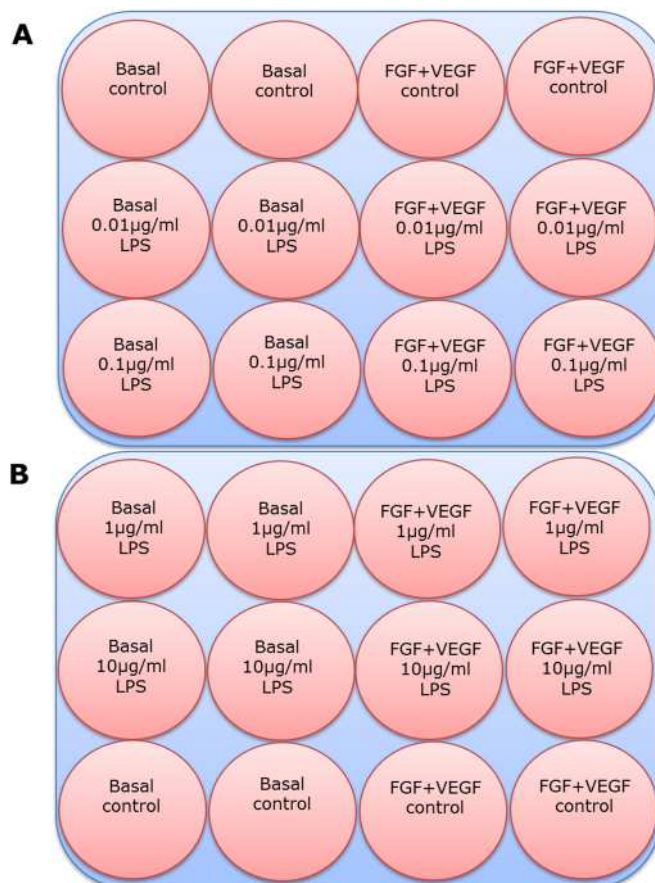
Figure 5.2: Cell plate layout for Exp 1. (A) Cells were treated with 0-0.1µg/ml LPS under basal and angiogenic stimulated conditions (VEGFA and FGF2; both 1ng/ml). (B) Cells treated with 1-10µg/ml LPS under basal and angiogenic stimulated conditions for 9 days.

5.2.1.2 Immunohistochemical analysis for VWF

Fixation and blocking

On day 9 of culture, cells were fixed and permeabillised in acetone: methanol (1:1) at 4°C for 5 minutes, then washed twice with PBS. An endogenous peroxidase block (3% (v/v) hydrogen peroxide in methanol) was applied to the wells for 10 minutes at RT to prevent endogenous peroxidase from being visualised. Then wells were washed with PBS (2 × 5 minutes each). The cells were blocked with 20% (v/v) normal goat serum (NGS) for 30 minutes to prevent any binding to non-target sites.

VWF antibody

Rabbit anti-human VWF IgG (Dako) was diluted in 2% (v/v) NGS (in PBS) to 5µg/ml and 300µl was added to each well and incubated for 2 hours at RT.

Secondary antibody

After two hours incubation with primary antibody, the coverslips were washed with PBS (2 × 5 minutes each). The secondary antibody was used according to the Vector ABC Elite method as follows: biotinylated goat anti-rabbit IgG was diluted 1/250 with 2% (v/v) normal goat serum in PBS and incubated for 30 minutes at room temperature. Then coverslips were washed with PBS (2 × 5 minutes each). This was followed by preparing the avidin-biotin complex as per manufacturer's instruction, which was incubated for 30 minutes at RT. Then coverslips were washed with PBS (2 x 5 minutes each).

Visualisation of the antibody complex, counterstaining and mounting

Visualisation of the bound antibodies was determined by incubating with 3.3'-diaminobenzidine tetrahydrochloride (DAB) for 4 minutes (Section 4.2.4.6). The cells were then counterstained with haematoxylin (Section 4.2.4.7).

5.2.1.3 Image analysis of von Willebrand factor

The method of quantification was developed according to an established protocol, (Robinson et al. 2008). For each treatment in each culture, both wells were selected for analysis. Images acquired from a total of 20 fields of view per coverslip visualised randomly under 5x magnification using Leica DM4000B microscope (Leica Microsystems Ltd., Milton Keynes, UK) and Qlmaging Micropublisher 5.0 RTV colour camera (Qlmaging (UK) Ltd., St Helens, UK). All image analysis was performed using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK). Captured images were tiled to create a composite image for processing. The brown of area (VWF) staining was highlighted. The highlighted brown areas were smoothed and any holes in the staining were removed and any VWF staining area <150mm^2 excluded (to eliminate very small cluster of isolated EC of <3 cells). Only brown areas positively stained with VWF were included in the analysis. Then, the total area of VWF staining, total number of EC islands, the number of EC networks, and the area and length of each individual network was recorded. The number of branch points present in the EC islands undergoing sprouting was determined using an automated measure of branching points (Branch/End feature; Image

Pro-Plus 6.3;(Masri et al. 2007, Woad et al. 2012). Branch points per cluster was calculated by dividing total number of branch points by total number of EC clusters.

5.2.1.4 Progesterone ELISA

Progesterone ELISA (Ridgeway Science; St Briavels, Gloucestershire) was performed according to manufacturer's instructions. Samples were thawed and diluted with PBS. As follows: Day 5 (1:50) and Day 9 (1:150). ELISAs were performed as detailed in Section 3.3.2.4. The lower limit of sensitivity was 0.2ng/ml and the intra- and inter-assay coefficient of variation were 7.2% and 8.8%, respectively.

5.2.1.5 Statistical analysis

Data were checked for normality and homogeneity of variance by using residual plots and Bartlett's test, respectively. Data were log transformed where appropriate. To investigate the effect of CL stages on total EC area and EC branch points, randomised block one-way ANOVA in GenStat 16th Edition was used. In order to minimise the effect of culture, the data were blocked by culture. Additional analysis included investigating the effect of VEGFA/FGF2 on progesterone production and EC network formation. This was compared using randomised block ANOVA. Similarly, the effect of day on progesterone production was analysed by a randomised block ANOVA. For each culture, the EC network variables were converted into % control. The effects of LPS were analysed using randomised block one-way ANOVA in GenStat 16th Edition. Again, in order to minimise the effect of culture, the data were blocked by culture. LPS was added as a factor to the fixed model on the response variable (total area endothelial cell networks, area of individual EC networks, branch points, and progesterone production). Separate analysis was performed to look at the effects of LPS (1) on EC networks from early and mid CLs and (2) under basal and angiogenic stimulated conditions. If there was a significant effect of LPS, then Tukey's multiple comparisons were performed to determine where the difference lay. For all experiments P<0.05 was considered as significant and all data are presented as mean ±SEM

5.2.2 Results of experiment 1

5.2.2.1 Characterisation of the luteal angiogenesis culture system

On day 9 of culture, the cells had formed a confluent monolayer. Numerous endothelial cells EC were stained using VWF as a marker showing that a number of networks had formed in each culture. Each EC network had a central body of endothelial cells, from which a number of branches had developed. These EC networks were different in size and degree of branching depending on the stage of luteal development (Fig.5.3A&B). In addition, other cell types had proliferated with their nuclei abundant across the whole coverslip (Fig. 5.3A&B). The results showed that early CL produced a slightly more extensive EC networks compared to those from mid CL under both basal condition (without VEGFA and FGF-2) (Fig. 5.3A&B) and under angiogenic stimulated conditions (Fig. 5.4A&B). Early CL produced a significantly higher area of branched endothelial cells (Fig. 5.3C and Fig. 5.4C, $P<0.05$), and increased number of branch points (Fig. 5.3D, $P<0.01$) and (Fig.5.4D, $P<0.05$). Progesterone production was significantly increased under angiogenic stimulus (VEGFA and FGF-2) compared to basal conditions (Fig.5.5A, $P<0.001$). Furthermore, progesterone production was higher on day 9 than day 5 of culture by about 2-fold (Fig.5.5B, $P<0.001$).
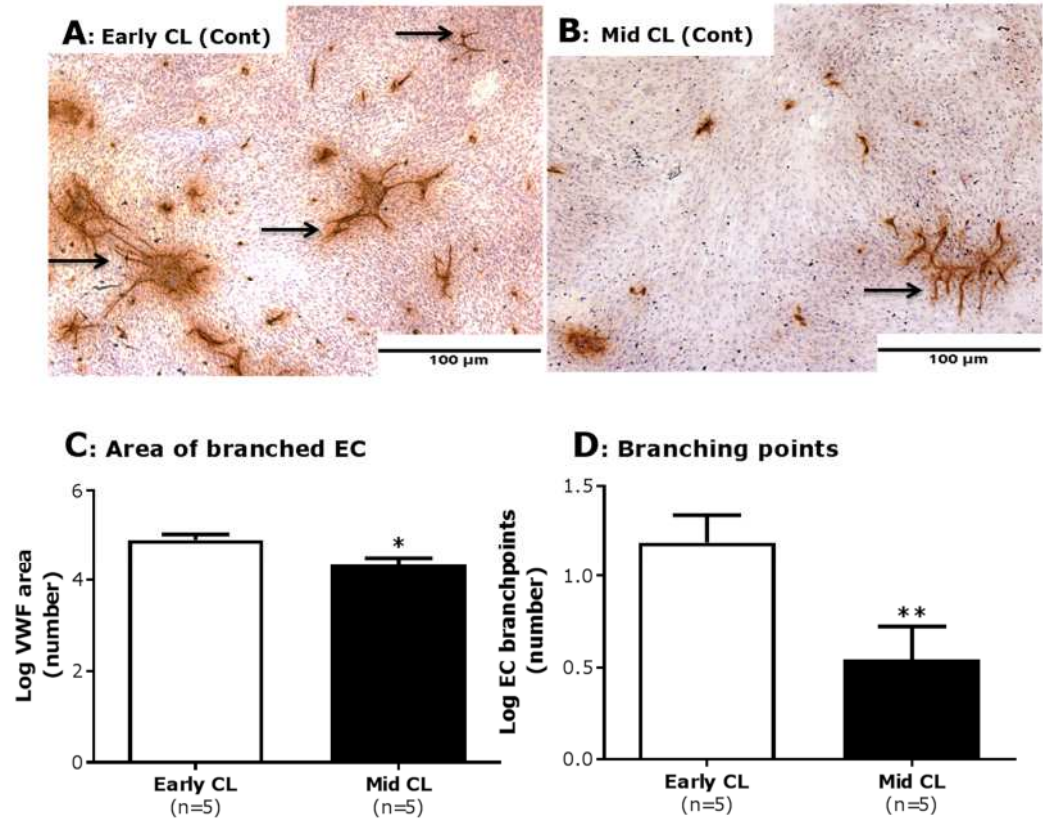
Figure 5.3: Representative images of bovine luteal cells treated with control ECM medium (without FGF2/VEGFA). (A) From early stage CL (days 1-4), (B) from mid stage CL (days 5-7). Endothelial cells were immunostained for von Willebrand factor (brown) after 9 days of culture. Endothelial cells formed organised tubule-like networks when derived from both early stage and mid stage CL. Black arrows indicate EC branch endpoints. (C) There was significant effect of CL stage on area of branched EC. (D) There was significant effect of CL stage on EC branch points (*; $P<0.05$). All treatments within culture were performed in duplicate and the values are mean (n=5 cultures) ± SEM.
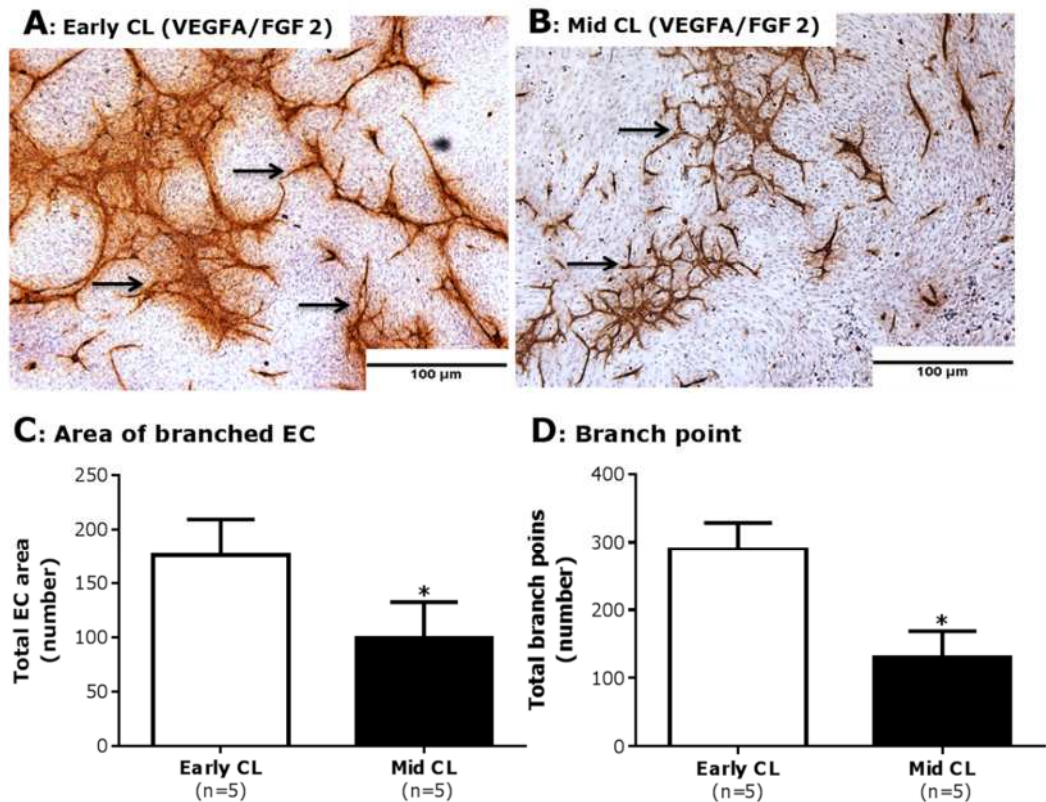
Figure 5.4: Representative images of bovine luteal cells under angiogenic stimulated conditions without LPS treatment. (A) from early stage CL (days 1-4), (B) from mid stage CL (days 5-7). Endothelial cells were immunostained for von Willebrand factor (brown) on 9 days of culture. Endothelial cells formed organised tubule-like networks when derived from early stage CL and mid stage CL. Black arrows indicate EC branch endpoints. (C) There was a significant effect of CL stage on the area of branched EC. (D) There was a significant effect of CL stage on EC branch points (*; *P*<0.05). All treatments within culture were performed in duplicate and the values are mean (n=5 cultures) ± SEM.
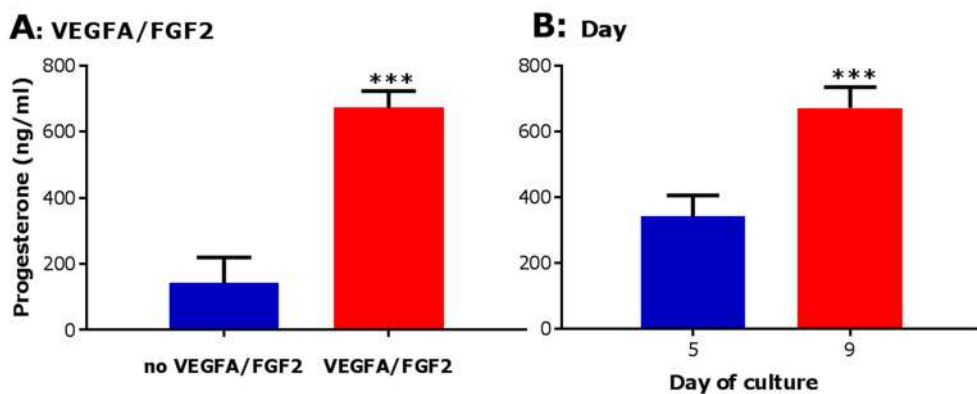
Figure 5.5: Progesterone production by luteal cells in culture. (A) shows that angiogenic stimulation (FGF2 and VEGFA; both 1ng/ml) increased P4 production (***; *P*<0.001). (B) shows that progesterone production across all treatments increased between day 5 and 9 of culture (***; *P*<0.001). The values are mean (n=5 cultures) ± SEM.

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5.2.2.2 The effect of LPS on *in vitro* luteal angiogenesis [early CL]

The patterns of endothelial cells networks that formed under basal angiogenic conditions were greater in control compared to LPS-treated wells (Fig.5.6).
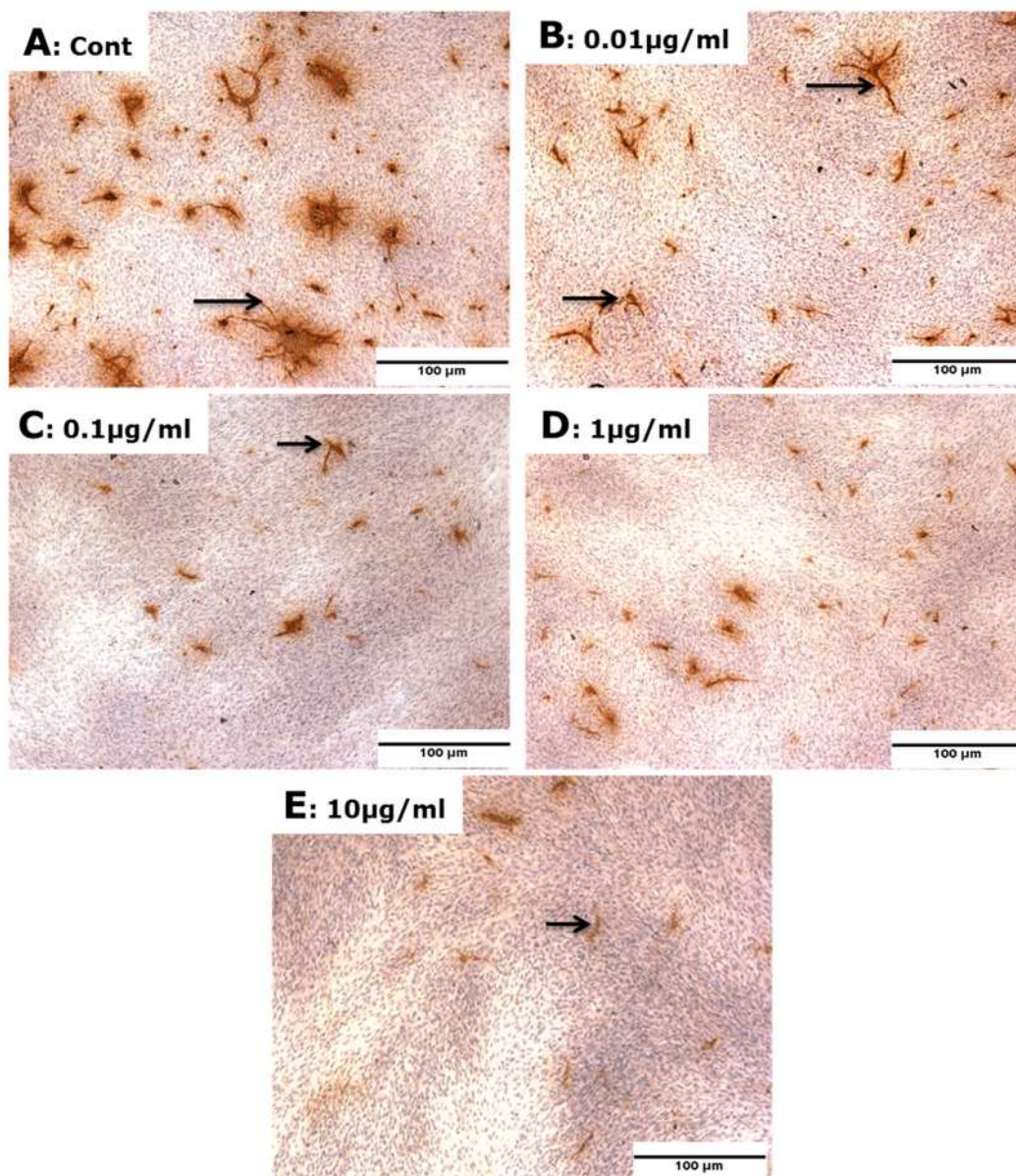
Figure 5.6: Effect of LPS on development of endothelial cell (EC) networks on day 9 of luteal culture from early stage CL under basal angiogenic conditions. (A) In the absence of *E.coli* LPS, endothelial cells formed highly organised tubule-like networks, without angiogenic stimulation. (B) 0.01µg/ml, (C) 0.1µg/ml, (D) 1µg/ml, (E) 10µg/ml LPS. EC were immunolocalised stained with VWF. Black arrows indicate EC branch endpoints.

LPS had adverse effects on EC network formation under both basal and angiogenic-stimulated conditions. In non-LPS treated wells, EC formed highly

organised tubule-like networks, which were dramatically increased under angiogenic-stimulated conditions (Fig.5.7). Reduced numbers of EC networks were observed in LPS-treated wells and at 10µg/ml LPS virtually no EC networks were detected (Fig.5.7).
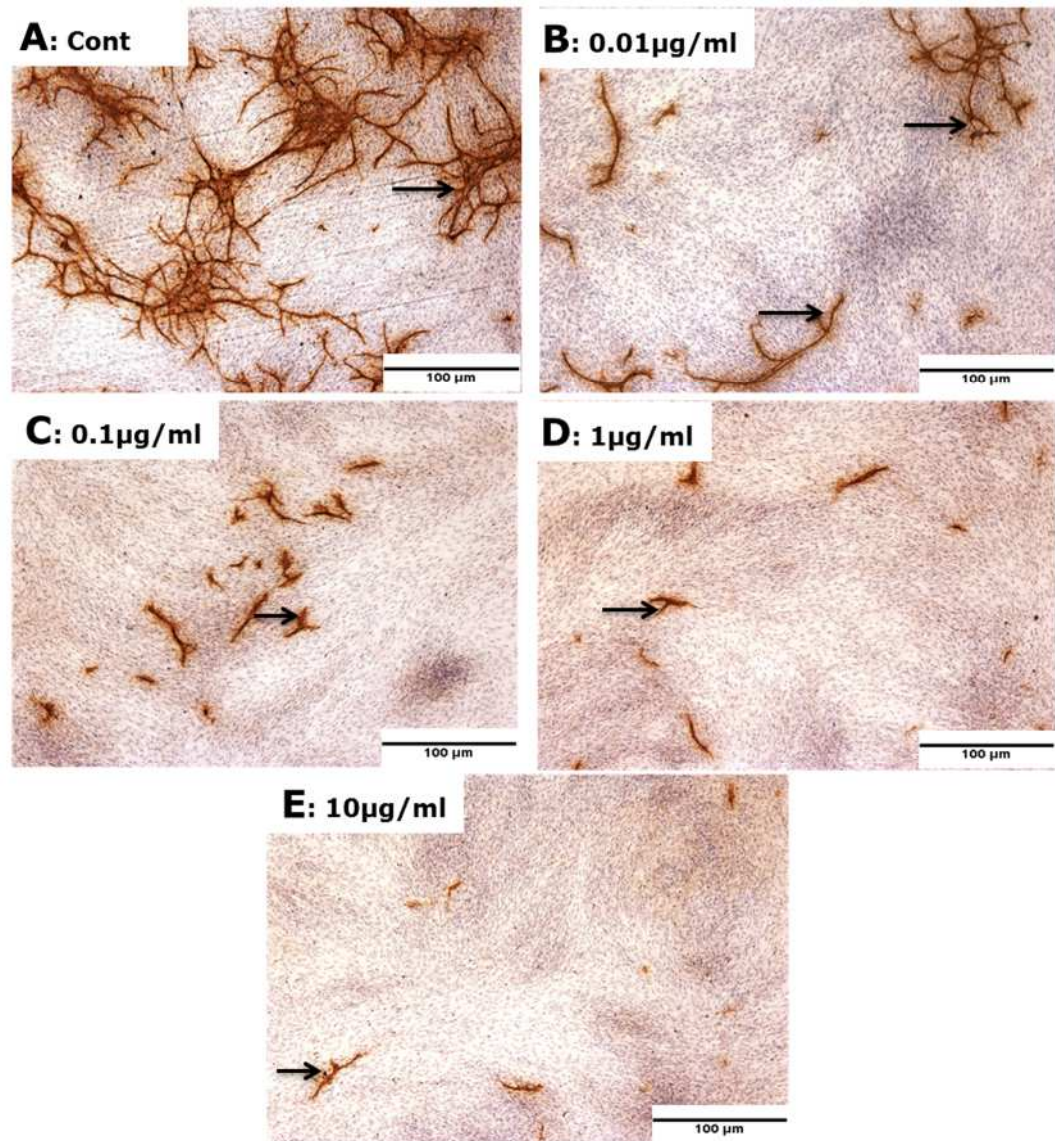
Figure 5.7: Effect of LPS on development of endothelial cells networks on day 9 of luteal culture from an early stage CL under angiogenic stimulated conditions. (A) In the absence of *E.coli* LPS, endothelial cells formed extensive highly organised tubule-like networks, (B) 0.01µg/ml, (C) 0.1µg/ml. (D) 1µg/ml, (E) 10µg/ml LPS. EC networks and cluster were immunolocalised stained with VWF. Black arrows indicate EC branch points.

LPS significantly dose-dependently decreased the area of EC networks, the number of EC clusters and branch points as well as the degree of branching per EC cluster (Fig. 5.8-5.9; $P<0.001$). Significant differences were detected at the different LPS doses ($P<0.001$).
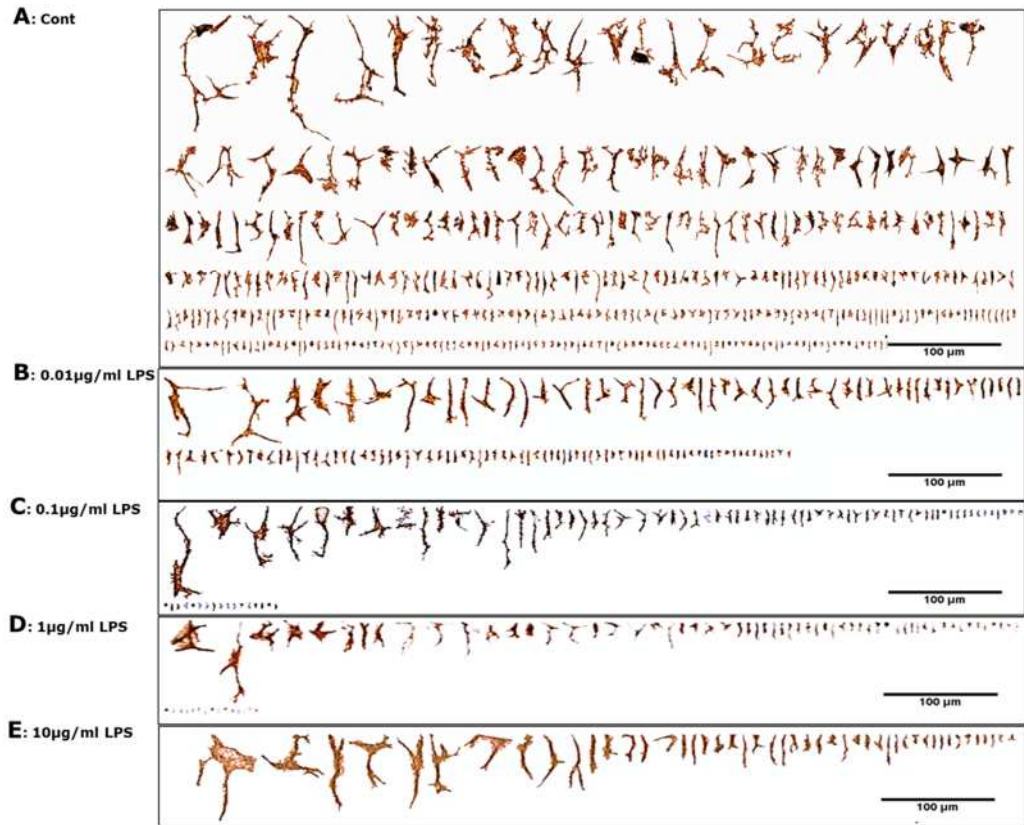
Figure 5.8: Effect of LPS on endothelial cell networks on day 9 of luteal culture under angiogenic-stimulated conditions from an early CL. (A) In the absence of *E.coli* LPS, endothelial cells branching points were highly extensive under angiogenic stimulated conditions (1ng/ml VEGFA & FGF2). (B) 0.01µg/ml LPS. (C) 0.1µg/ml LPS. (D) 1µg/ml LPS. (E) 10µg/ml LPS. EC networks were lower at all the different LPS doses.
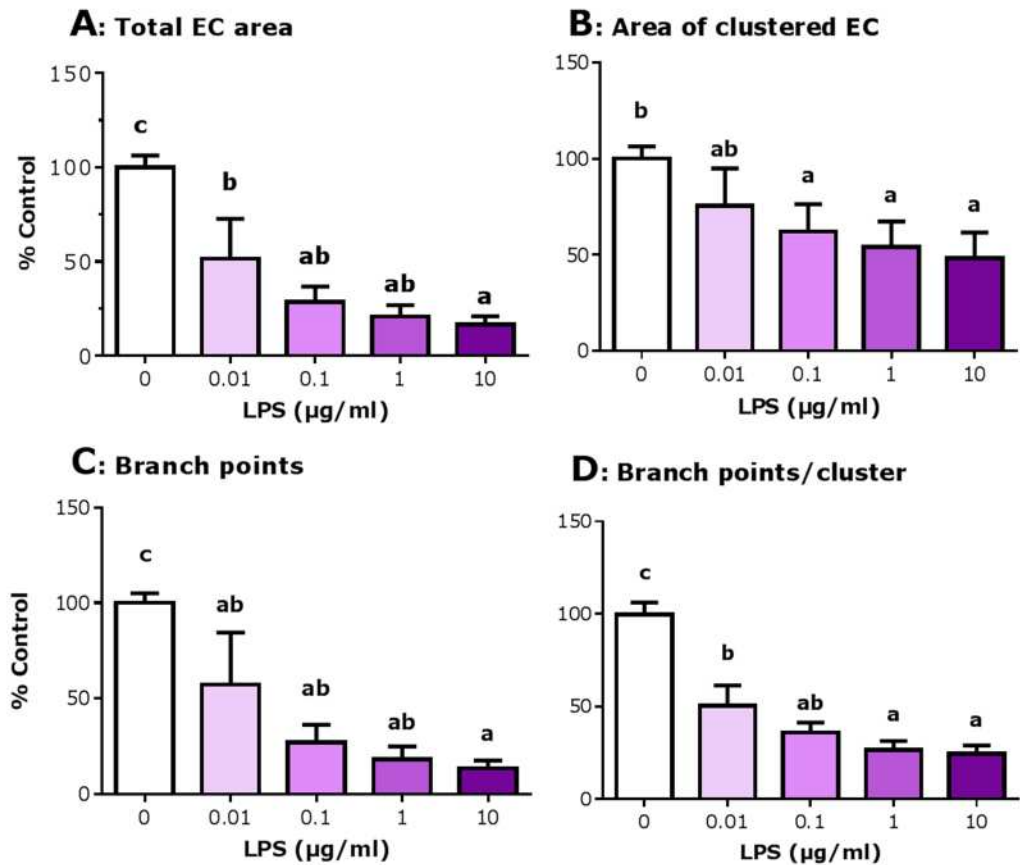
Figure 5.9: Quantification of the effect of LPS on endothelial cell (EC) network formation from early CL after 9 days in culture under angiogenic stimulus (VEGFA and FGF-2). (A) Total EC area. LPS reduced the branched EC area in a dose-dependent manner (P<0.001); (B) area of clustered EC; (C) branch points; and (D) branch point per EC cluster. There was significant effect of LPS on branch points and branch points per cluster in different doses of LPS under angiogenic stimulus (VEGFA and FGF2) (P<0.001). Significances between treatment groups are indicated by different letters a<b<c. Values are mean (n=5 cultures) ± SEM expressed as % control (no LPS) with all treatments within each culture performed in duplicate.

5.2.2.3 The effect of LPS on luteal angiogenesis *in vitro* [mid CL]

There were visibly fewer endothelial cell networks in mid CL developed under basal condition (without VEGFA/FGF2, Fig. 5.10) compared to those under angiogenic stimulus (Fig. 5.11). As for the early stage CL, these EC networks were composed of numerous endothelial cells that formed complex inter-connecting structures.
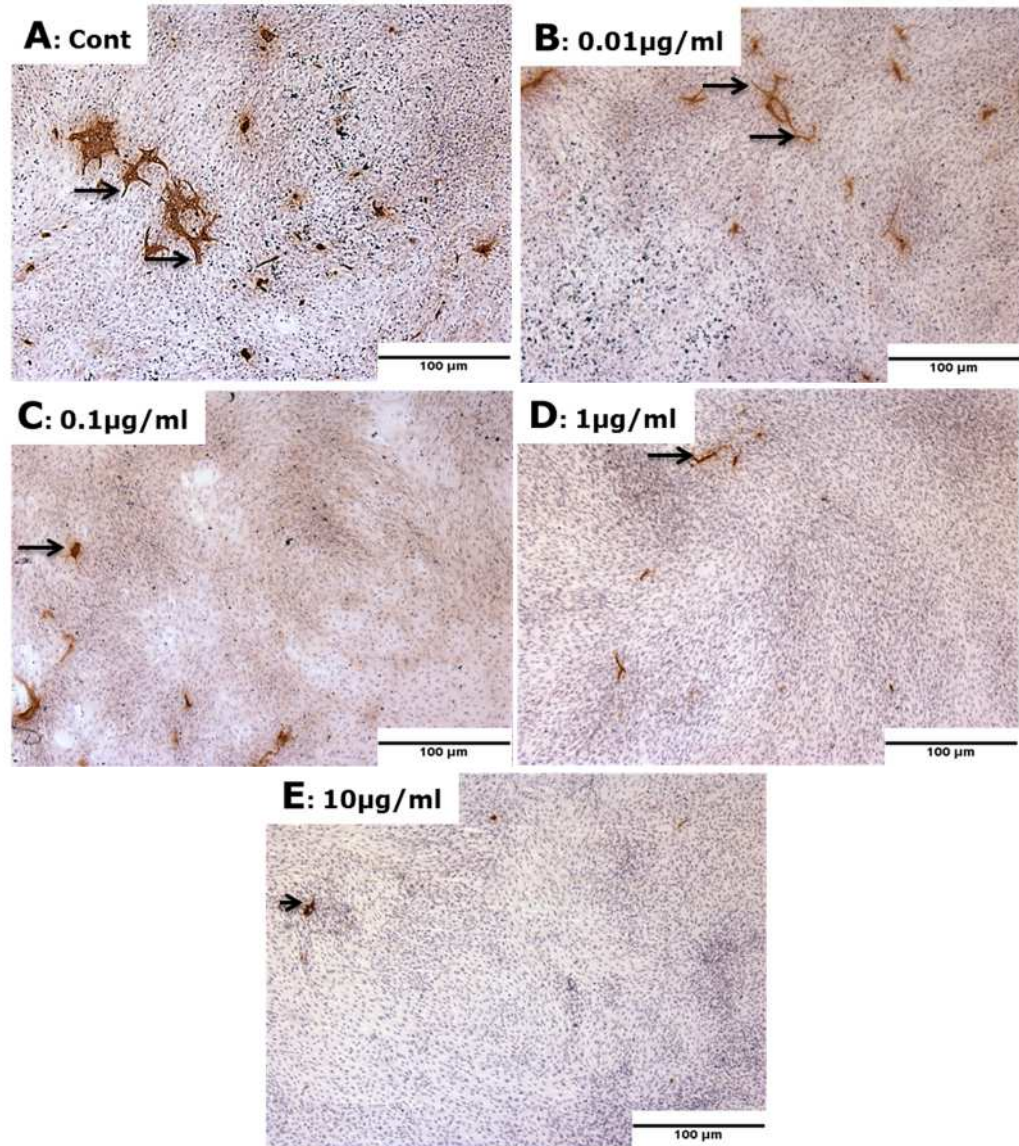
Figure 5.10: Effect of LPS on development of endothelial cell networks *in vitro* on day 9 under basal conditions [mid stage CL]. (A) In non-LPS treated wells, endothelial cells formed interconnecting networks but these were limited. (B) 0.01µg/ml, (C) 0.1µg/ml, (D) 1µg/ml, (E) 10µg/ml LPS-treated wells. EC were stained with VWF, and the EC network formation appeared less with different doses of LPS. Black arrows indicate EC branch points.
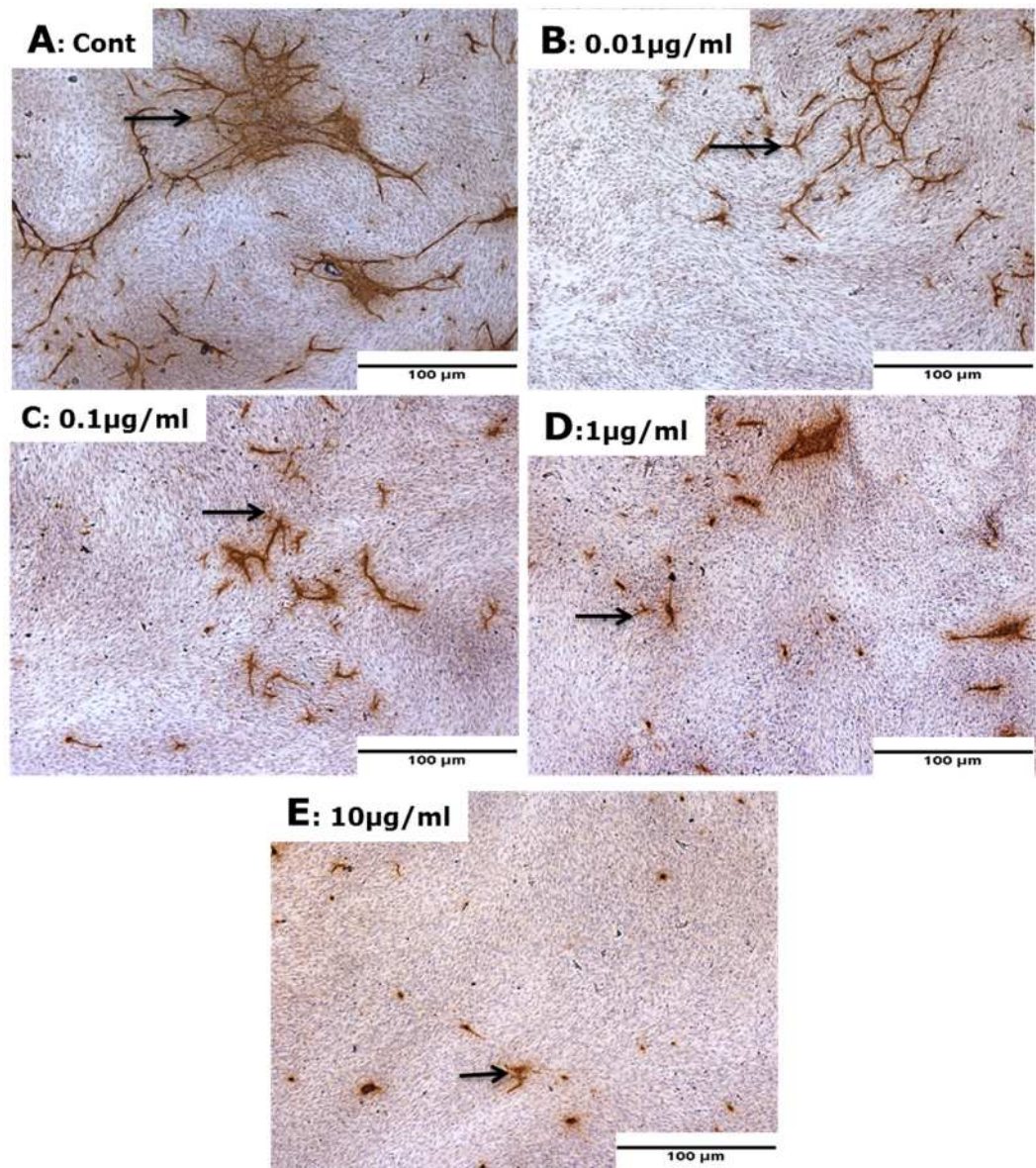
Figure 5.11: Effect of LPS on development of endothelial cell networks on day 9 of luteal culture under angiogenic stimulated conditions [mid-stage CL]. (A) In the absence of *E.coli* LPS, endothelial cells formed highly organised networks. LPS treatments at (B) 0.01µg/ml LPS; (C) 0.1µg/ml LPS; (D) 1µg/ml LPS and (E) 10µg/ml LPS are shown. Area of EC networks were observed in the LPS treated wells but appeared to be lower, particularly at 10µg/ml LPS. Dashed yellow line indicates EC cluster, black arrow indicate EC sprouts and white arrows indicate branch points.

In control wells, EC formed highly organised tubule-like networks, which were dramatically increased under angiogenic-stimulated conditions. In addition, LPS significantly decreased EC network formation, under both basal and angiogenic-stimulated conditions. A reduced number of EC clusters with a smaller number of branches was observed in LPS-treated wells and at the highest dose (10µg/ml LPS) virtually no EC networks (14% of control) were detected (Fig 5.12). LPS significantly decreased the area of EC network, the number of EC clusters and branch points as well as the degree of branch points/cluster (Fig. 5.13A-D, *P*<0.001).
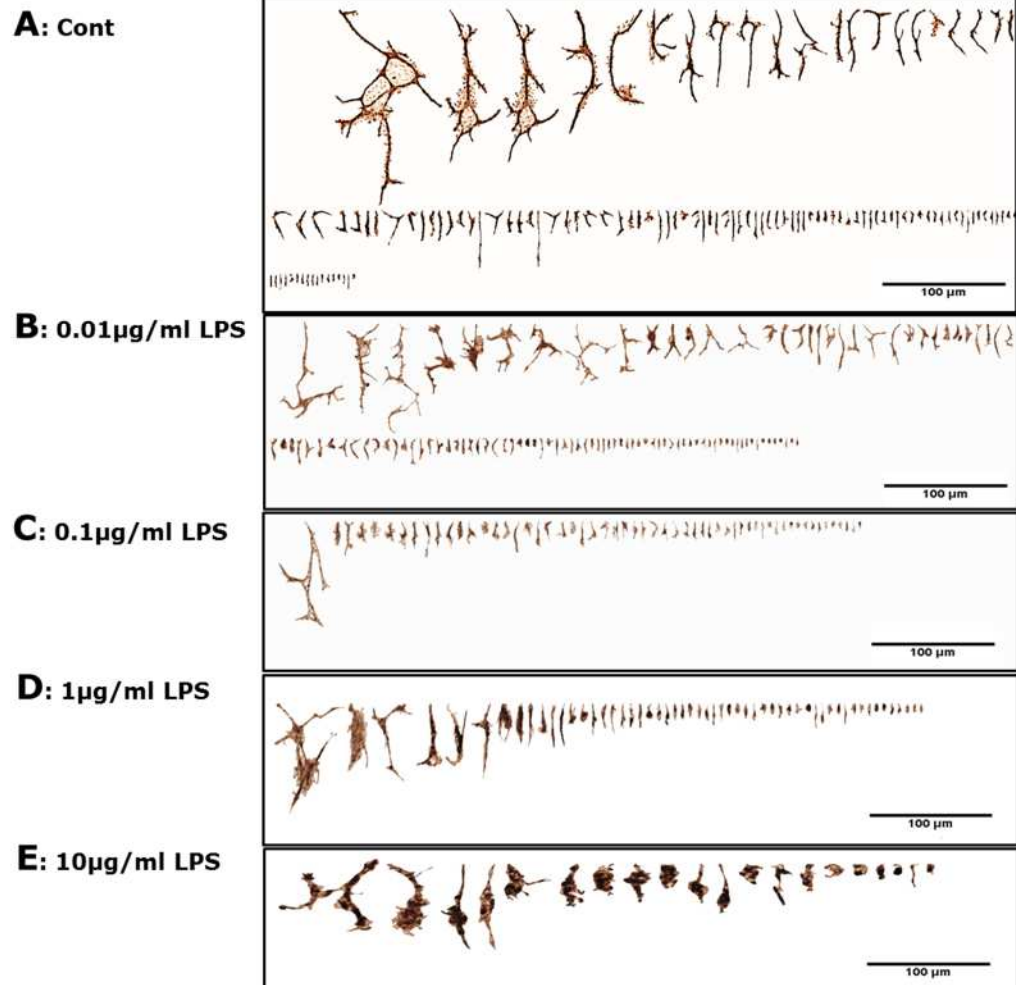
Figure 5.12: Effect of LPS on endothelial cell (EC) network formation from mid stage CL under angiogenic stimulated conditions. (A) in the absence of *E.coli* LPS, EC branching points and branching within each cluster was extensive LPS at all doses appeared to reduce the number of EC clusters and the number of branches per cluster: (B) 0.01µg/ml, (C) 0.1µg/ml, (D) 1µg/ml, (E) 10µg/ml LPS.
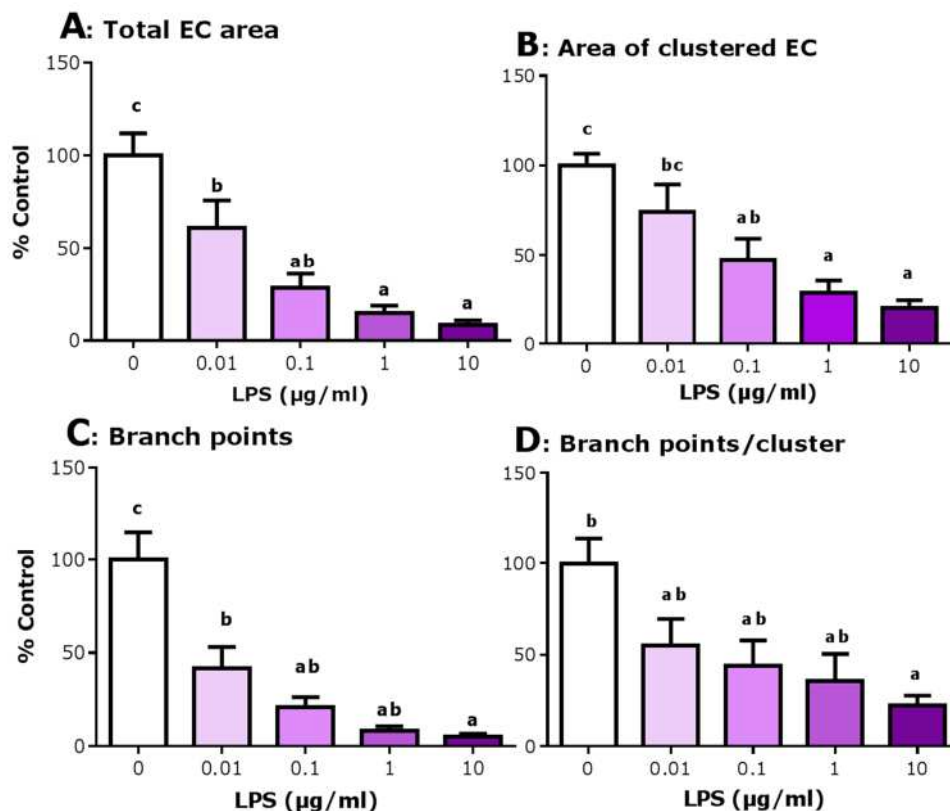
Figure 5.13: Effect of LPS on: (A) total EC area (B) number of EC clusters, (C) total branch points and (D) branch points per culture. Bovine luteal cells (from mid CL) were cultured for 9 days under angiogenic stimulus (mid-stage CL). There was significant dose dependent effect of LPS on all four parameters (P<0.001). Significances between treatment groups are indicated by different letters a<b<c. Values are mean (n=5 cultures) ± SEM expressed as % control (no LPS). All treatments within a culture were performed in duplicate.

5.2.2.4 Effect of LPS on luteal progesterone production in vitro [early CL]

LPS had no effect on progesterone production on day 5 (Fig. 5.14A, *P*>0.05) or day 9 of culture (Fig. 5.14B, *P*>0.05). Furthermore, these results showed that progesterone was not affected by LPS under basal on day 5 of culture (Fig. 5.15A, *P*>0.05) or angiogenic conditions on day 9 of culture (Fig.5.15B, *P*>0.05).
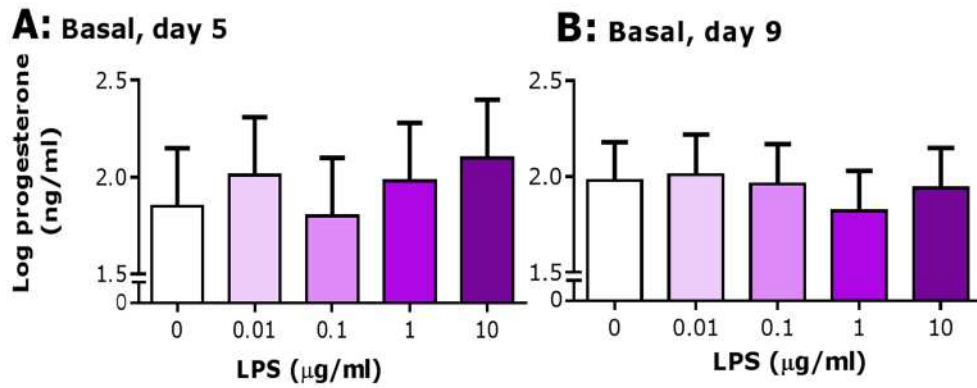
Figure 5.14: Progesterone production by luteal cells cultured from under basal conditions [early CL]. (A) Effect of LPS on P4 production on day 5 of culture. There was no difference in progesterone production between treatments (P>0.05). (B) Effect of LPS on P4 production on day 9 of culture. There was no difference in progesterone production between treatments (P>0.05). The values are mean (n=5 cultures) ± SEM
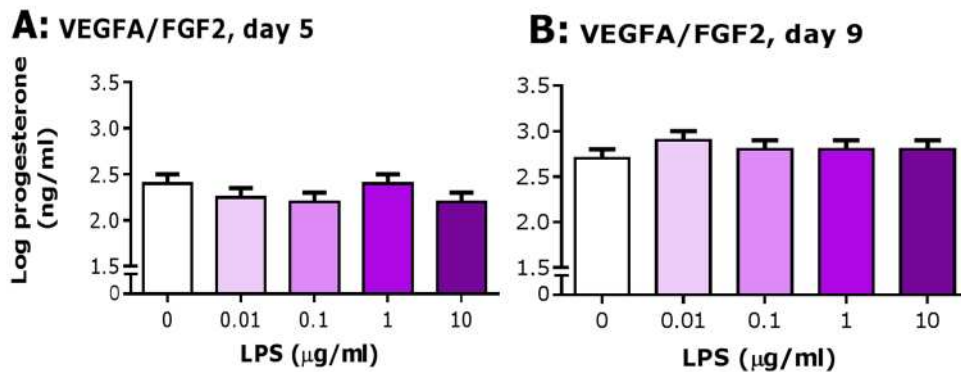
Figure 5.15 Progesterone production by luteal cells cultured from early CL under angiogenic stimulus (VEGFA/FGF-2). (A) Effect of LPS on P4 production in day 5 of culture. There was no difference in progesterone production between treatment (P>0.05). (B) Effect of LPS on P4 production in day 9 of culture. There was no difference in progestogen production between treatment (P>0.05). The values are mean (n=3 cultures) ± SEM.

5.3 Experiment 2: Effect of LPS on luteal EC apoptosis, EC proliferation, progesterone production and expression of steroidogenic enzymes *in vitro*

5.3.1 Materials and methods

The coating of the coverslips, collection of samples, culture media and identification of luteal endothelial cells were described in Section 5.2.1.1. In this experiment, only luteal cells dispersed from early (day 1-4) CL were used.

5.3.1.1. Experimental design

Cells were plated and treated with LPS (0, 0.01 and 1 µg/ml) as described in Fig.5.16. The cells from one plate (A) were subjected to VWF immunohistochemistry; activated caspase-3 (apoptotic marker) immunohistochemistry; Ki67 (proliferation marker) and isolectin (endothelial cell marker) immunofluorescence and dual immunofluorescence for activated caspase-3 with isolectin (Fig. 5.16A). On day 5 of culture, cells were fixed with acetone: methanol for 10 min (Section 5.2.1.1). Then, cells from the other plate (B) collected for protein preparation and Western blot (Fig 5.16B). Additionally, media was collected on days 3 and 5 for progesterone measurement.
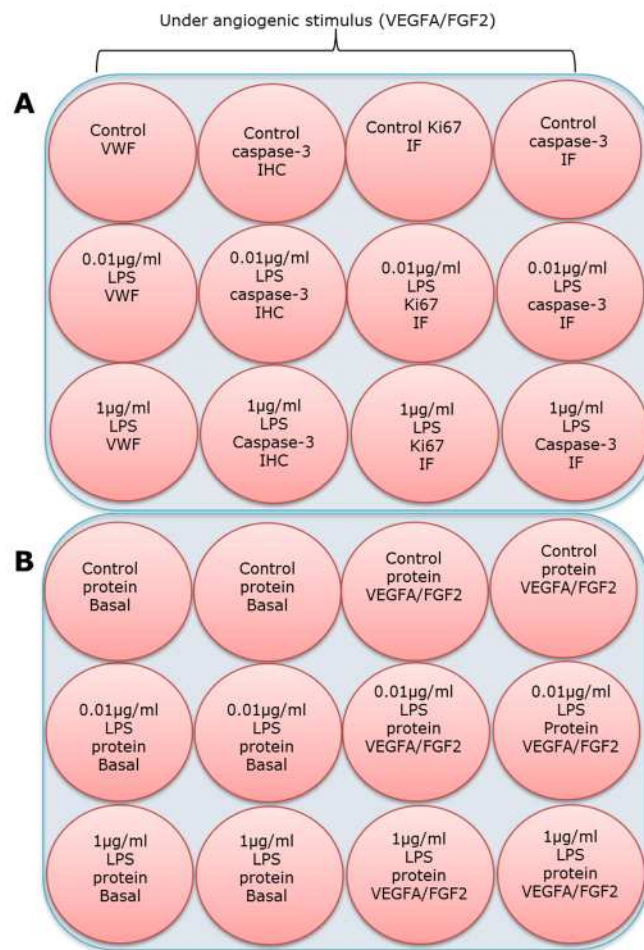
Figure 5.16: The plate layout for Experiment 2. Luteal cell collected from early CL were cultured (A) Cells treated with 0, 0.01 and 1µg/ml LPS under angiogenic stimulation (VEGFA and FGF2) and then fixed on day 5 for immunohistochemistry of VWF [column 1], activated caspase 3 [column 2], dual `immunofluorescence for Ki67 and isolectin [column 3]; dual immunofluorescence of caspase-3 and isolectin [column 4]. (B) Cells treated with 0, 0.01 or 1µg/ml LPS from day 1 to 5 of culture. Samples were used for Western blot.

5.3.1.2 VWF Immunohistochemistry

On day 5 of culture, coverslips were prepared for immunolocalisation for VWF as described in Section 5.2.1.2.

5.3.1.3 Immunohistochemical analysis of caspase-3 in luteal endothelial cells

Caspase-3 is a widely used apoptosis marker (Jeruc et al. 2006, Bressenot et al. 2009). On day 5 of culture, coverslips were fixed with acetone:methanol and blocked with hydrogen peroxide (Section 5.2.1.2). The non-specific binding was blocked using 4% (v/v) NGS diluted with PBS and incubated for 30 minutes at RT. The primary antibody was polyclonal rabbit antihuman cleaved caspase-3 IgG, (diluted 1:300; Cell Signaling, Danvew, USA). An equivalent concentration of rabbit IgG was used for negative control well. The cells were incubated with the primary antibody in a humidifed chamber at 4°C overnight. For the detection of the primary antibody, Vector ABC Complex Elite (Vector Labs, Peterborough, UK) kit was used. This used a biotinylated goat anti-rabbit IgG (diluted 1:250 in 2% (v/v) NGS) and incubated for 30 min at RT. This step was followed by washes in PBS (2 x 5 minutes). The ABC complex was added as per manufacturer's instructions for 30 min at RT followed by PBS washes. Visualisation of this complex was performed using diaminobenzidine (DAB) for 4 min at RT. The coverslips were counterstained with haematoxylin for 20 secs, then dehydrated in ethanol and xylene before being mounted on a microscope slide using DPX mountant. Images of the whole coverslip were taken under brightfield with a Leica DM 4000B under 40x objective lens.

5.3.1.4 Dual immunofluorescence staining of Ki67 with isolectin

To determine the proliferation index of endothelial cells, a dual immunostaining was performed with Ki67 (proliferation maker) and isolectin-B4 (marker for endothelial cells). The coverslips were fixed and blocked (Section 5.2.1.2). The primary antibody was mouse anti-human Ki67 (Clone MM1, Vector KP452, diluted 1:80 in 1xPBS) and biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4 (20µg/ml, diluted 1:25 in PBS, Vector, cat no B1205). The primary antibody and isolectin were incubated in a humidifed chamber at 4°C overnight. On the next day, the cells were washed with PBS (2 x 5 minutes each). Next, Texas-Red labelled anti-mouse IgG

(20µg/ml) and Avidin-labelled with Flourescence DCS (20 µg/ml) were applied to the wells and incubated for 1h at RT. The coverslips were washed with PBS (2 x 5 minutes each). The coverslips were mounted by adding 3 drops of Vectashield with DAPI, then sealed with nail varnish and stored 2-3h at -20°C. Five pictures under the 40X objective lens were taken using a Leica DM 5000 B florescence microscope, equiped with a Leica DFC 350fx camera (Leica Microsystem (UK), Ltd, Milton Keynes, UK) for each coverslip.

5.3.1.5 Dual immunoflurescence staining of caspase-3 with isolectin

To determine the number of endothelial cells that were apoptotic, caspase-3 was co-localised with isolectin B4. The coverslips were fixed with acetone: methanol for 5 min at 4°C, then followed by two washes with PBS for 5 minutes each. The non-specific binding was blocked using 4% (v/v) normal goat serum (NGS) and incubated for 30 min at RT. The primary antibody was rabbit anti-human caspase-3 (diluted 1:300) and biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4 (20µg/ml, Vector, diluted 1:25 in 1xPBS). These were incubated in a humidifed chamber at 4°C overnight. On the next day, cells were washed with PBS (2 x 5 minutes each). Then, Texas-Red avidin IgG (25µg/ml; Vector) and FITC-labelled anti-rabbit IgG conjugate (20µg/ml; Vector) mixed together were applied to the cells and incubated for 1 hour at RT. The coverslips were mounted by adding 3 drops of Vectashield with DAPI, then sealed with nail varnish and stored 2-3h at -20°C. Five pictures under the 40X objective lens were taken using a Leica DM 5000 B florescence microscope, equiped with a Leica DFC 350fx camera (Leica Microsystem (UK), Ltd, Milton Keynes, UK) for each coverslip.

5.3.1.6. Protein extraction from cell culture

On day 5, culture media was collected and 500µl of 1xPBS was added to each well. Cells were scraped off using the end of the pipette, and then pipetted up and down to help dispersion. Samples were centrifuged at 13000**g** for 5 minutes, and supernatant was removed. Then 50 µl of lysis buffer was added and samples stored at -70°C until analysis. Lysis buffer composed of [0.1 M Tris pH7.4, 0.1% (w/v) dithiothreitol (DTT), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.18 mg/ml sodium orthovanadate plus protease inhibitors (25 x (v/v), Complex protease inhibitor cocktail; Roche Diagnostic Ltd, East Sussex, UK). Samples were taken out and thawed on ice, then centrifuged at 13000**g** for 2 minutes and supernatant collected and added a further 100µl of

cell lysis buffer (0.18mg/ml sodium orthrovandate, 0.01% SDS) and stored on ice. Samples were rapidly frozen in methanol in dry ice for 2 minutes and then rapidly thawed for 1 minute using a hot block at 50°C. This step was repeated 5 times. Samples were sonicated in water bath for (4× 20 seconds in ice) then with 1 minute on ice in between sonications. This was followed by centrifugation at 13000**g** for 5 min. The supernatants were collected and 30μl from each sample was used for protein measurement, and the remaining aliquots 70μl were stored at -70°C until Western blot was performed.

5.3.1.7 Protein determination and Western blotting

Protein concentrations were determined using a Bradford assay (Section 4.2.3.3) and then Western blot was performed using 10μg protein for HSD3B, STAR, ACTA2 and 20μg protein CYP11A (Sections 4.2.3.3-8). Protein was only used from cells under angiogenic stimulation (VEGFA/FGF2) because insufficient protein was collected from cells cultured under basal conditions.

5.3.1.8 Progesterone analysis

Progesterone ELISA (Ridgeway Science) was performed according to manufacturer's instructions (Section 5.2.1.4). Samples collected on day 3 were diluted 250 fold; whereas, samples collected on day 5 of culture were diluted 500 fold. The lower limit of sensitivity was 0.2ng/ml and the intra and inter assay coefficient of variation were 6.3% and 12.1%, respectively.

5.3.1.9 Image analysis for caspase-3 and Ki67

All image analysis was performed using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK). A total of 5 images were captured for each coverslip under 20x objective lens and the EC area highlighted. Cells with nucleus immunostaining were visually counted using Image Pro-Plus 6.0 software. Brown stained nucleus with caspase-3 IHC were visually assessed; green stained with capase-3 and red stained nucleus with Ki67 with immunofluorescence were visually assessed. The total number of counted cells was recorded automatically. The proliferation and apoptotic index were calculated as follows:

- Proliferative rate =100 x (Number of nuclei stained positive with Ki67/Total number of nuclei present).

- Apoptotic rate= 100 x (Number of nuclei stained positive with caspase-3 /Total number of nuclei present).

5.3.1.10 Statistical analysis

Data were checked for normality and homogeneity of variance by using residual plots and Bartlett's test, respectively. Data were log transformed where appropriate. The association between LPS treatment and percentage of area of von Willebrand factor, area of clustered EC, branch points and branch points per cluster were analysed by randomised block one-way ANOVA. If there was a significant effect of LPS, the multiple comparisons (Tukey) were used to determine where the differences lay. Furthermore, the effect of day on progesterone production was analysed by randomised block ANOVA. For all experiments $P<0.05$ was considered as significant and all data are presented as mean±SEM.

5.3.2 Experiment 2, Results

5.3.2.1 Immunolocalisation of endothelial cells

In this experiment, luteal cells were cultured under angiogenic stimulation for 5 days. The patterns of endothelial cells networks was different on day 5 of culture to that observed for day 9 in the Experiment 1. In the control wells, some of EC islands had begun to branch away from the island (Fig 5.17A, 5.18A). However, the majority of the endothelial cells remained in large clusters. Additionally, the number and size of these EC islands were visibly reduced in those wells treated with LPS at both doses (Fig. 5.17, 5.18).
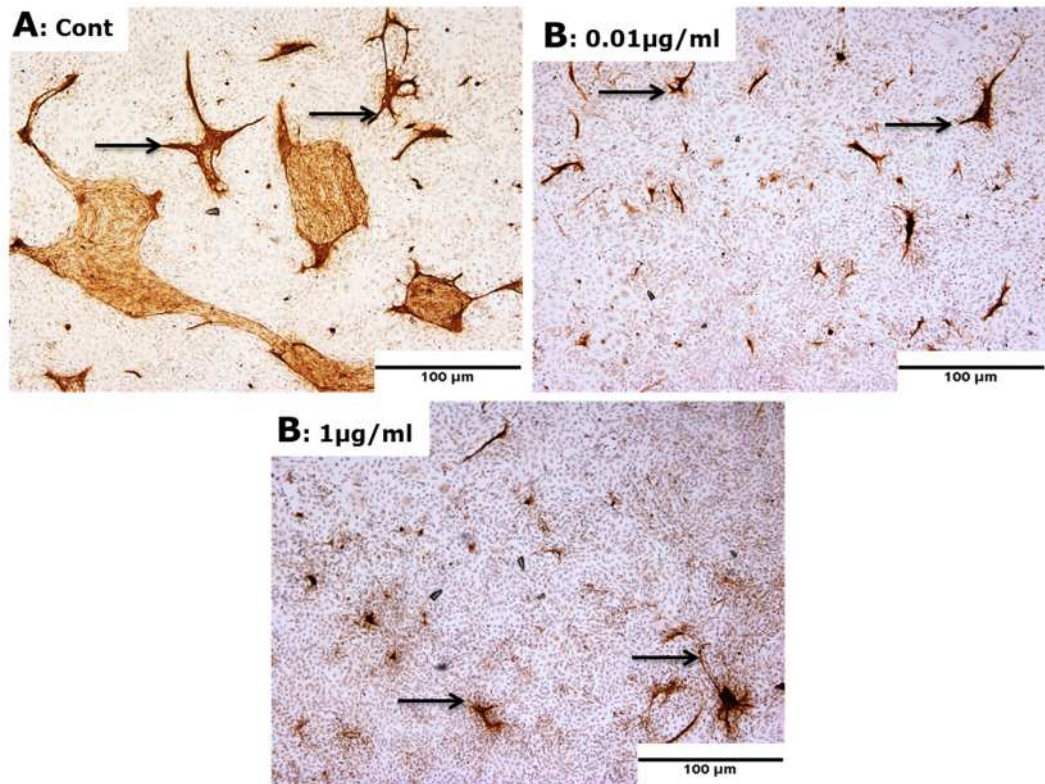
Figure 5.17: Effect of LPS on development of luteal endothelial cell networks on culture day 5 under angiogenic stimulation. (A) In the absence of *E.coli* LPS, endothelial cells formed large islands of 'poorly developed' endothelial cells. These had partly begun to sprout from the EC island and form network-like structures. Cells treated with (B) 0.01µg/ml LPS or (C) 1µg/ml LPS had reduced number and size of EC islands and networks were noted. Black arrow indicates branching in the EC islands.
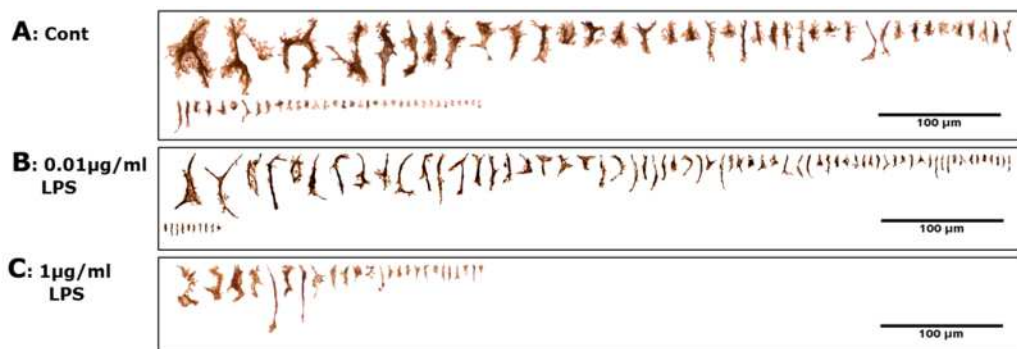
Figure 5.18: Effect of LPS on number of endothelial cell networks and their degree of branching on day 5. (A) In the control wells, moderate level of EC networks with branching was observed. This appeared to be reduced when cells were treated with (B) 0.01µg/ml LPS and particularly (C) 1µg/ml LPS.

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Furthermore, quantification showed that even in the presence of FGF2/VEGFA, the total area of these EC islands was significantly reduced by LPS treatment at both doses (Fig.5.19A, *P*<0.001). The total area of clustered EC (Fig. 5.19B; P<0.01) and the total branched points/cluster were significantly reduced by LPS treatment at both doses. However, total number of branch points was only significant different at 1µg/ml (Fig.5.19 C; *P*<0.05).
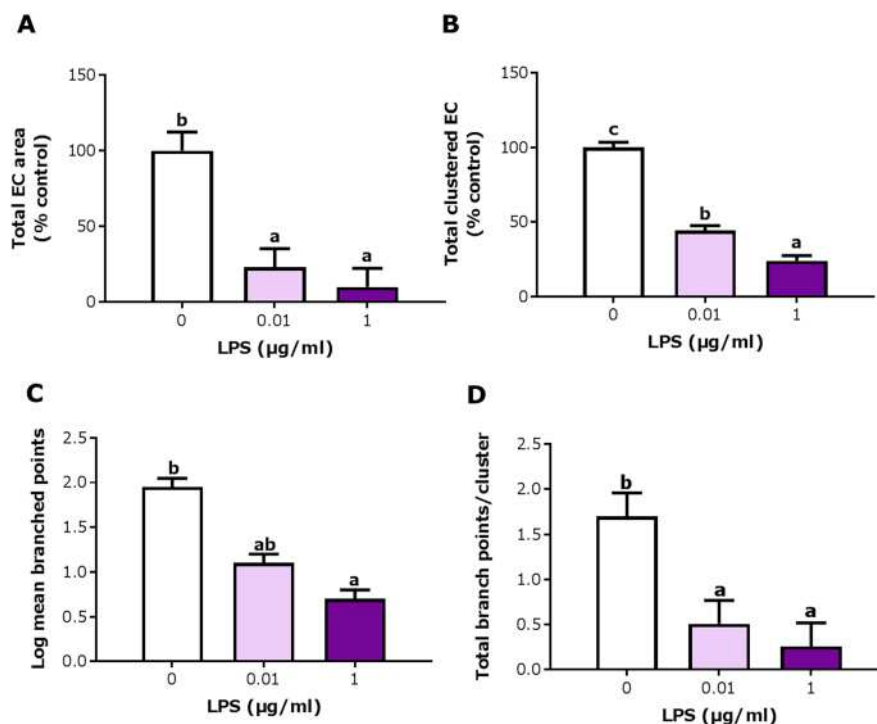
Figure 5.19: Effect of LPS on: (A) total area of VWF staining; (B) total area of clustered EC networks; (C) total number of branch points and (D) total number of branch points per cluster in luteal EC after 5 days in culture. There was a significant effect of LPS on each parameter (P<0.01). Significances between treatment groups are indicated by different letters a<b<c Values are mean (n=3 cultures) ± SEM.

5.3.2.2 Effect of LPS on endothelial cell proliferation

Ki67 was present in the cell nucleus across the well of all treatments (Fig 5.20). Islands of endothelial cells were also identified with isolectin staining and had a similar appearance to that observed with VWF. These were particularly evident in the control wells. In the control well, endothelial cells were positive for Ki67 (proliferation index = 30%) throughout the islands (Fig.5.20). Non-endothelial cells were also positive for Ki67. In the LPS-treated cells, the EC islands were smaller but a few endothelial cells were Ki67-positive (Fig.5.20B&C). The quantification of EC Ki67-positive cells showed that LPS had significantly decreased proliferation index of endothelial cells by 2-fold (Fig. 5.20D, *P*<0.01).
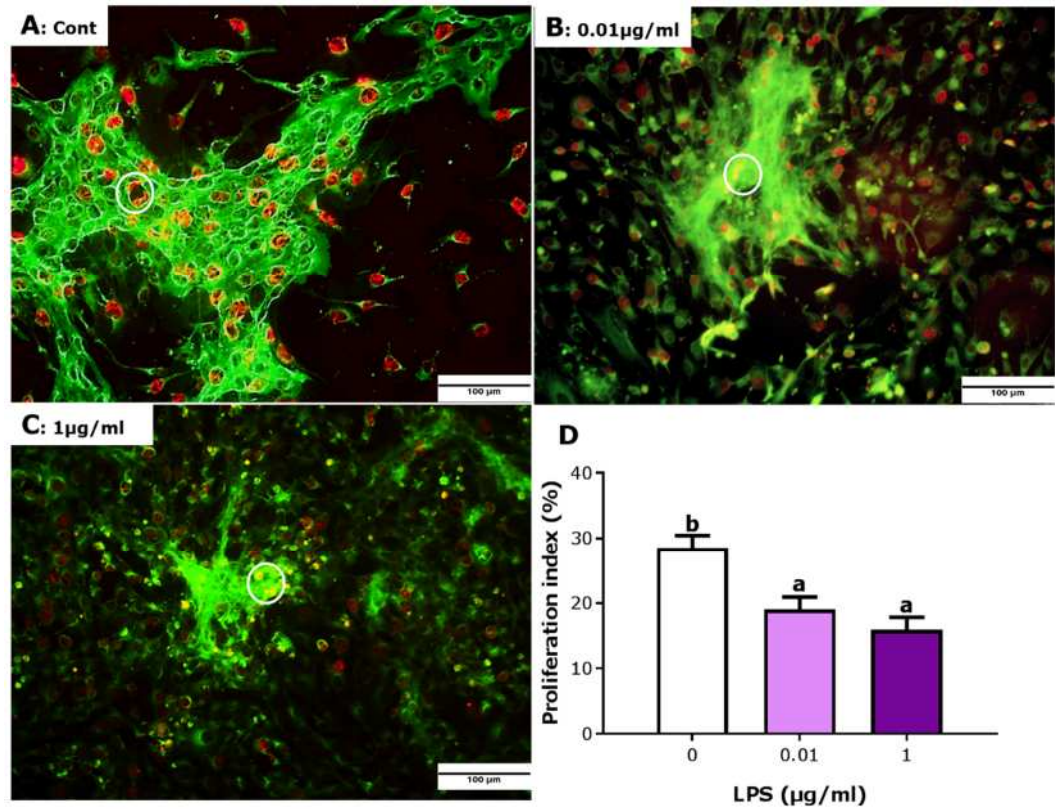
Figure 5.20: Effect of LPS on luteal endothelial cell proliferation on culture day 5; (A-C) Immunofluorescence showed that expression of Ki67 (red nucleus) in endothelial cells (isolectin staining; green) was higher in control well compared to wells treated with LPS. The white circles indicate Ki67 positive cells within the EC islands; the cells were counterstained with DAPI (blue); (D) The quantification of proliferation index within EC island showed that it was significantly inhibited (a<b; $P<0.05$) by treatment with LPS. Values are mean (n=3 cultures) ± SEM.

5.3.2.3 Effect of LPS on apoptosis of endothelial cells

Immunostaining for activated caspase-3 was observed in the cell nucleus. In the control wells, few endothelial cells and non-endothelial cells were positive for caspase-3 (Fig 5.21A). The caspase 3 staining was more evident in the cells treated with 0.01µg/ml or 1µg/ml LPS (Fig.5.21B&C). The quantification showed that LPS significantly increased the apoptotic index in luteal endothelial cells in a dose depended manner (Fig. 5.21D; $P<0.001$).
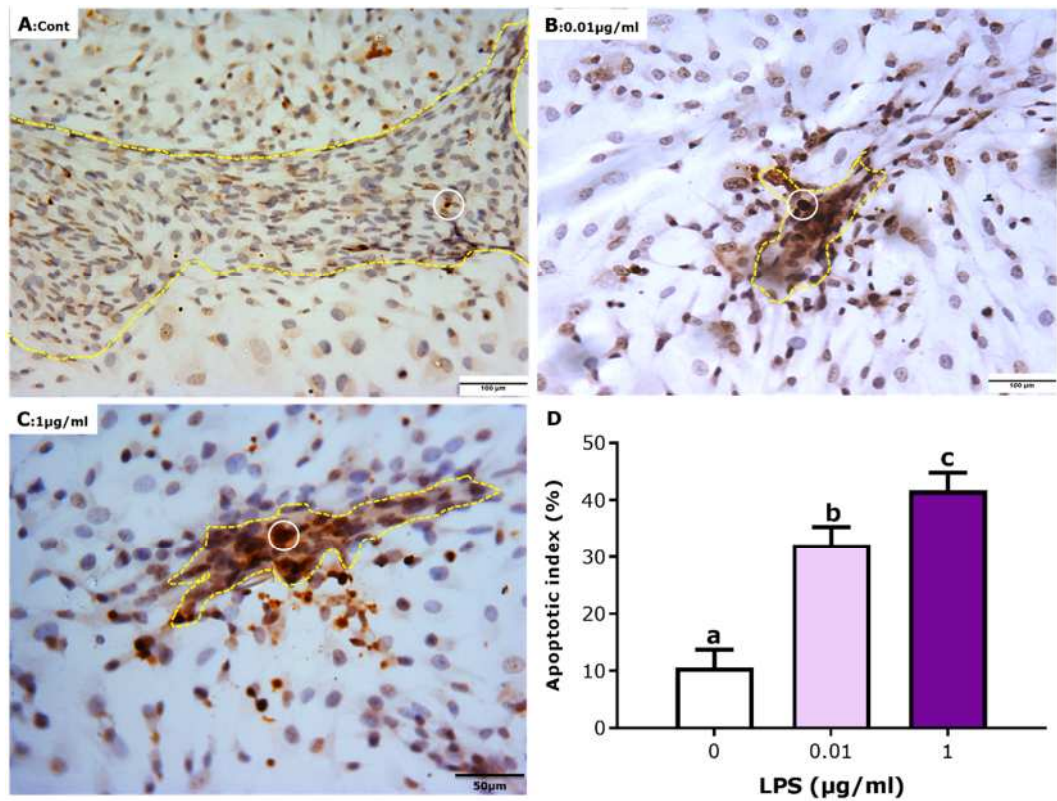
Figure 5.21: Effect of LPS on apoptosis in luteal endothelial cells on culture day 5. (A-C) Immunohistochemistry showed expression of caspase-3 (brown staining) in presumptive endothelial cell islands (indicated by dashed yellow line). The white circles highlight caspase 3 positive cells in these EC islands. The smaller EC islands in the LPS-treated wells had visibly higher expression of caspase 3 staining. (D) The quantification of caspase 3 staining showed that it was significantly inhibited (a<b<c; $P<0.05$) in cells treated with LPS. Values are mean (n=3 cultures) ± SEM.

These observations were confirmed with immunofluorescent dual staining of endothelial cells (with isolectin) and apoptosis (activated caspase-3). Again the number of endothelial cells that were caspase-3 positive was lower in control cells compared to those exposed to LPS (Fig. 5.22A-C). Quantification revealed that LPS had increased luteal endothelial cell apoptosis by 2-fold, (Fig. 5.22D, $P <0.01$).
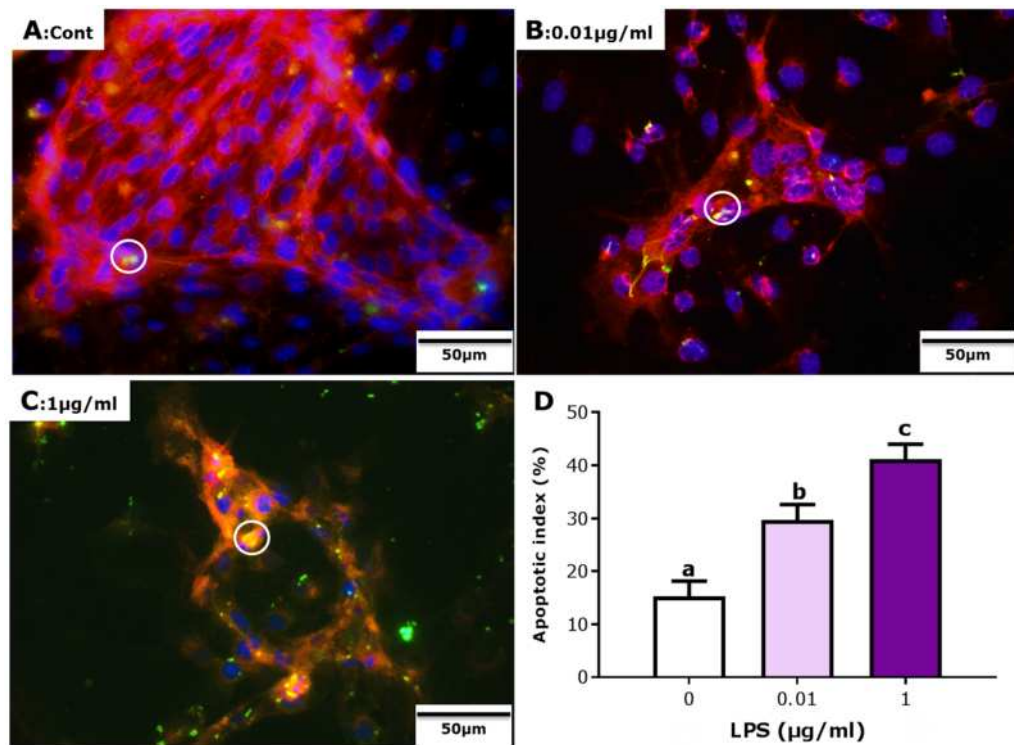
Figure 5.22: Effect of LPS on luteal endothelial cell apoptosis on culture day 5; (A-C) Immunofluorescence showed expression of caspase-3 (green) in endothelial cells (isolectin; red). These cells are highlighted with white circles. This was more evident in cells treated with LPS. The cells were counterstained with DAPI (blue); (D) The expression of caspase-3 was significantly increased (a<b<c; $P<0.05$) in cells treated with LPS. Values are mean (n=3 cultures) ± SEM.

5.3.2.4 Effect of LPS on progesterone production

Progesterone production was affected by culture day with P4 concentration being higher on day 5 versus day 3 of culture (Fig. 5.23, $P<0.05$). LPS had no effect on P4 production on days 3 and 5 of culture (Fig. 5.24, $P>0.05$).
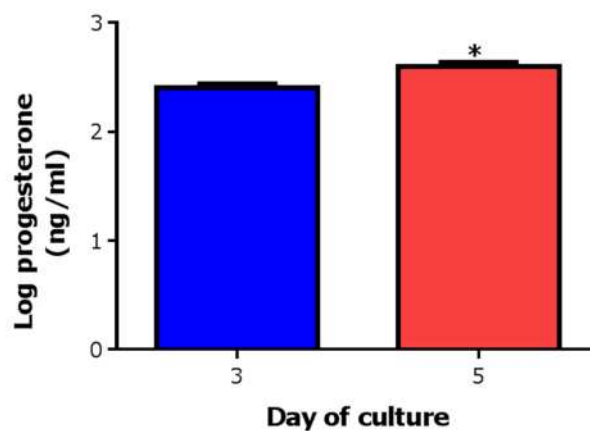
Figure 5.23: The effect of culture day on P4 production. There was a significant difference in progesterone production between days of culture (*; $P<0.05$). The values are mean (n=3 cultures) ± SEM.
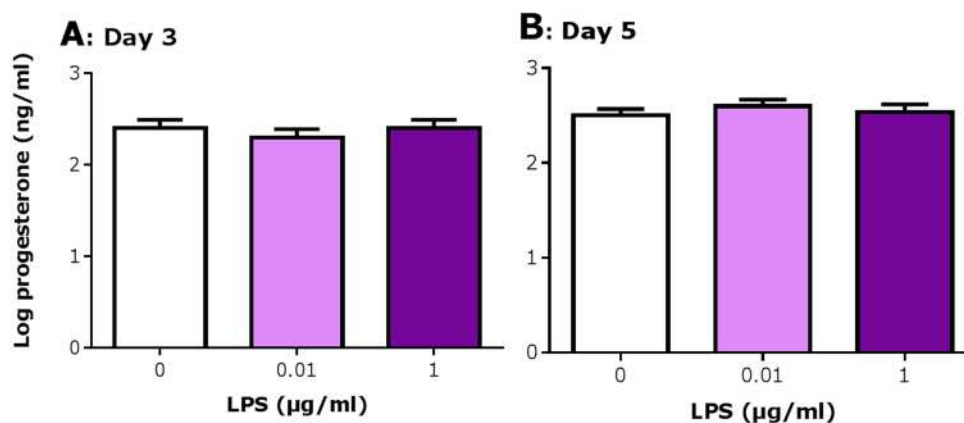
Figure 5.24: Effect of LPS on P4 production by bovine luteal cells on day (A) 3 and (B) 5 of culture. There was no treatment effect of LPS on progesterone production on either day 3 ($P > 0.05$) or day 5 ($P > 0.05$) of culture. The values are mean (n=3 cultures) ± SEM.

5.3.2.5 Western blot quantification of progesterone production proteins

Western blot showed that there were two bands present for STAR (Fig 5.25). A dominant band was detected at 25kD and lower abundance band at 32 kD. Single bands at the expected molecular weight were detected for HSD3B (42kD; Fig 5.26) and CYP11A (61KD; Fig 5.27). Quantification demonstrated the expression of all three proteins was unaffected by LPS treatment ($P > 0.05$; Fig.5.25-5.28).

Western blot for ACTA2 showed a single band at the expected molecular weight of 42kD. Quantification showed that expression of ACTA2 protein was significantly reduced in cells treated with 0.01µg/ml LPS by about 2-fold and 4-fold for those treated with 1µg/ml LPS compared to control cells ($P < 0.01$; Fig.5.28).
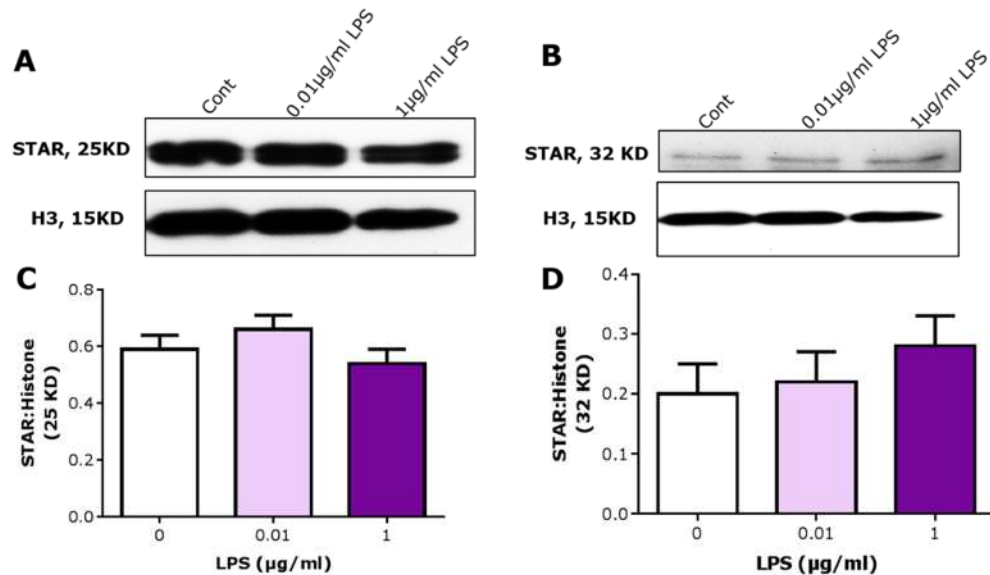
Figure 5.25: The expression of steroidogenic acute regulatory (STAR) protein in the bovine luteal cells *in vitro*. (A) Western blot of STAR revealed two bands at (A) 25kD and (B) 32kD. There was a single band for histone H3 at 15 KD. The housekeeping protein, histone 3 (H3) was stable across groups. The expression of STAR (normalised to histone H3) band at (C) 25kD and (D) 32 kD was not different between control and LPS treated well (P>0.05). The values are mean (n=5 cultures) ± SEM.
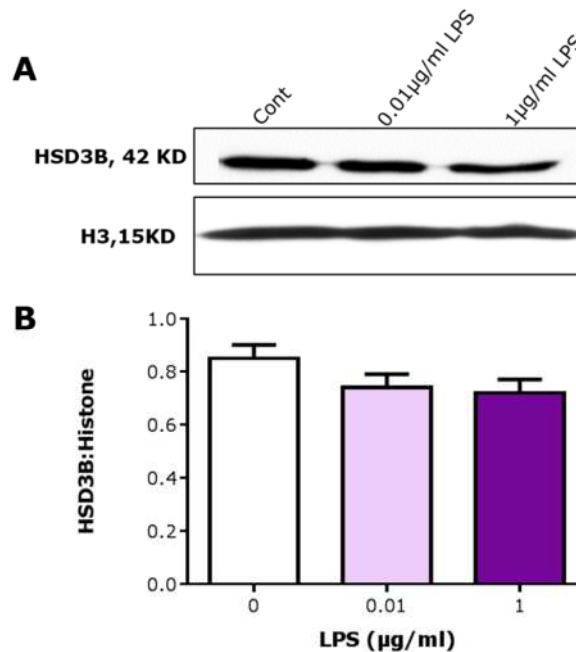
Figure 5.26: The expression of 3β-hydroxysteroid dehydrogenase (HSD3B) protein in the bovine luteal cells *in vitro*. (A) Western blot analysis of HSD3B protein showed a single band for HSD3B at 42kD (B) The expression of HSD3B (normalised to histone H3) was not different between control and LPS treated wells, (P>0.05). The values are mean (n=4 cultures) ± SEM.
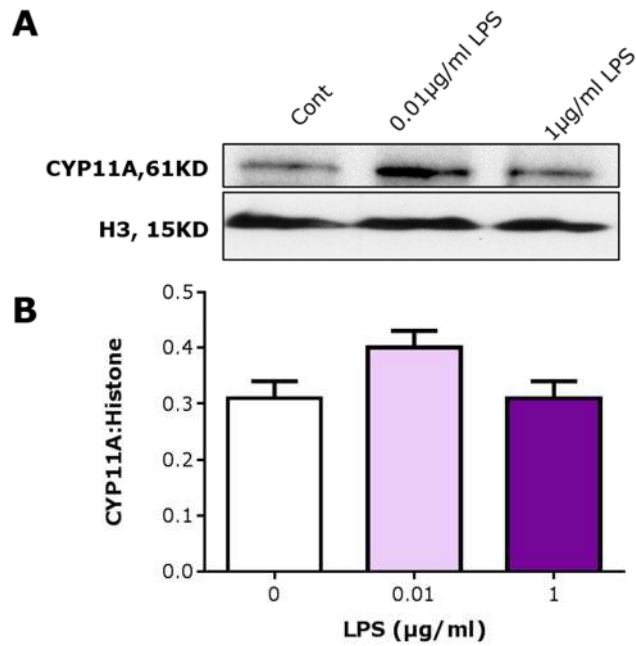
Figure 5.27: The protein expression of cholesterol side-chain cleavage enzyme (CYP11A) in the bovine luteal cells *in vitro*. (A) Western blot analysis of CYP11A protein expression showed a single band for CYP11A at 61kD (B) The expression of CYP11A (normalised to histone H3) was not different between control and treatments wells (P=0.12). The values are mean (n=3 cultures) ± SEM.
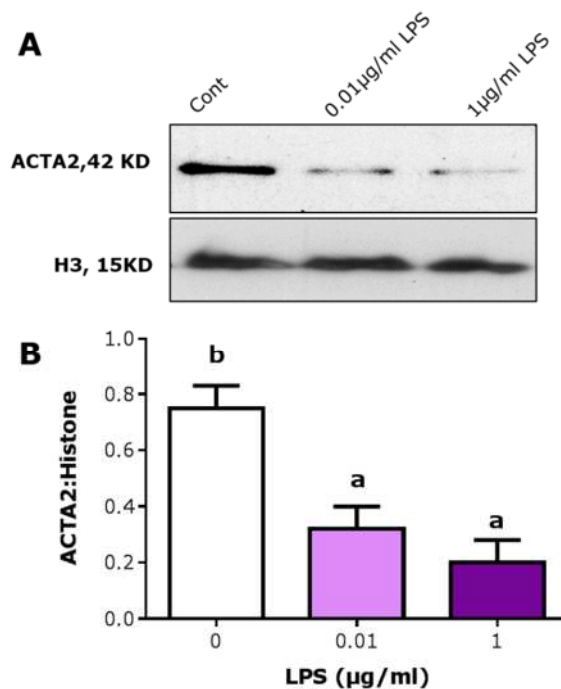
Figure 5.28: The effect of LPS on expression of α-smooth muscle actin protein (ACTA2) in the bovine luteal cell culture after 5 days *in vitro* under angiogenic-stimulated conditions. (A) Western blot analysis showed a single band for ACTA2 at 42kD. (B) The expression of ACTA2 (normalised to histone H3) was reduced by LPS at both doses (P<0.01).   Significances between treatment groups are indicated by different letters a<b. Values are mean (n=5 cultures) ± SEM.

5.4 Discussion

This chapter aimed to determine the effect of *E.coli* lipopolysaccharide (LPS): a) on luteal endothelial cell (EC) network formation, progesterone production and expression of steroidogenic proteins (STAR, 3BHSD, CYP11A); b) on luteal endothelial cells proliferation and apoptosis and on expression of ACTA2 (smooth muscle actin). To the best of our knowledge, this is the first study on the direct effect of LPS on the ability of luteal endothelial cells to form networks, proliferate and undergo apoptosis *in vitro*. LPS dose-dependently suppressed luteal endothelial network formation, by suppressing proliferation and increasing apoptosis.

The present study reported that LPS adversely effected luteal EC network formation *in vitro* even at low dosage of LPS (0.01µg/ml). Conversely, LPS has been shown to stimulate endothelial cells to promote angiogenesis in other systems. It has been demonstrated that LPS increased angiogenesis in different tissues (e.g. human pulmonary microvascular endothelial cells (Menden et al. 2015); human dermal microvascular endothelial cells *in vitro* (Pollet et al. 2003) and outgrowth endothelial cells (Ma et al. 2015). The mechanism by the LPS increased angiogenesis in these studies might be associated with the use of different endothelial cell phenotypes compared to the present study.

The present study demonstrated that LPS exerted an effect on luteal endothelial cells in the presence of pro-angiogenic factors. This inhibition could be due to LPS adversely affecting (1) endothelial cell survival (2) interfering with the stimulatory effects of VEGFA or FGF2. These factors are potent stimulators of both endothelial cell proliferation and migration (Ferrara et al. 2003, Carmeliet 2003). Indeed, in this exact model system, the development of endothelial cell networks was dependent on FGF2 and VEGFA (Robinson et al. 2008, Woad et al. 2009). 3) LPS could induce the production of factors (e.g. member of the TGFB superfamily) that could inhibit EC function and proliferation. TGFB plays a key role in the process of the angiogenesis (Lebrin et al. 2005, ten Dijke and Arthur 2007, Goumans et al. 2009). TGFB1 promotes endothelial cell apoptosis in bovine aortic endothelial cells (Pollman et al. 1999) and human umbilical vein endothelial cells (Ferrari et al. 2009). Furthermore, TGFB1 reduced luteal endothelial cell growth and network formation (Maroni and Davis 2011). There is very limited evidence to support LPS increasing expression of TGFB in luteal tissue. Previous studies reported that LPS stimulated the expression of TGFB1 by human and rat bile

duct epithelial cells *in vitro* (Kassel et al. 2012), they reported a potential mechanism through which LPS could enhance TGFB1-promoted PDGFB expression by increasing activation of SMAD transcription factors. Furthermore, Seki et al. (2007) demonstrated that TLR4 stimulated by LPS induced expression of TGFB1 *in vitro* culture of mice hepatic cells.

LPS reduced luteal EC proliferation during the earlier stages of EC network formation (decreased number of Ki67-positive cells) as well as increasing EC apoptosis (caspase3 positive cells). This occurred across the range of concentrations even at the lower concentration of 0.01µg/ml. This is not dissimilar to the concentration (0.04-0.8 µg/ml) of LPS measured in follicular fluid (Herath et al. 2007) but was greater than LPS concentrations of <10 ng/ml measured in plasma of cows with uterine infection postpartum (Mateus et al. 2003). It has been shown that LPS contributed to vascular damage in various types of endothelium (Frey and Finlay 1998, Fujita et al. 1998, Choi et al. 1998). However, other types of cells (e.g. steroidogenic (large and small luteal cells), LPS did not have a toxic effect.

It has been shown that LPS contributes to the damage observed in various types of endothelium in mice (Fujita et al. 1998), humans (Hotchkiss et al. 1999, Wort and Evans 1999, Koide et al. 1996, Frey and Finlay 1998), and in the bovine (Frey and Finlay 1998). LPS induced EC apoptosis *in vitro* and *in vivo*, in the development of diseases including septic shock and its syndromes (Hotchkiss et al. 1999, Wort and Evans 1999, Koide et al. 1996). Frey and Finlay (1998) stated that LPS at 10µg/ml induced apoptosis in bovine pulmonary artery the endothelial cells through fragmentation of DNA. The same study has shown that VEGFA protects the endothelium against LPS-induced apoptosis. However, the present study demonstrated that LPS induces luteal endothelial cells apoptosis through increased expression of caspase-3 in a dose dependent manner, even in presence of VEGFA.

It is feasible that LPS stimulated apoptosis by inducing oxidative stress and, inhibiting cellular antioxidant systems. Indeed LPS caused apoptosis through the mitochondrial apoptotic pathway in buffalo luteal cells (Mishra and Dhali 2007). Similarly, *in vivo* infusion of LPS increased caspase-3 mRNA and protein expression in the CL within 12 hours of administration (Herzog et al. 2013). However, they also found that LPS only caused a temporary suppression of CL function and morphology, without complete luteolysis.

It has been demonstrated that VEGFA can inhibit LPS-induced endothelial cell apoptosis in human umbilical vein endothelial cells (HUVEC) (Munshi et al.

2002). It is not surprising since VEGFA acts as a survival factor for endothelium. This is in contrast to the present study, where LPS increased apoptosis when cells were treated with exogenous VEGFA and FGF2. This study demonstrated that in bovine luteal endothelial cells that VEGFA/FGF2 co-treatment did not protect endothelium against LPS-induced apoptosis.

LPS had a large effect on the expression of α-smooth muscle actin (ACTA2), which is present in vascular mural cells including pericytes. This is in agreement with Sandbo et al. (2007) who observed that expression of ACTA2 in aortic smooth muscle cells was inhibited after 24 hours treatment with higher doses of LPS (10μg/ml) *in vitro*. Furthermore, LPS (10μg/ml) caused pericyte loss and microvascular dysfunction by disrupting angiopoietin expression in lung microvascular endothelial cells co-cultured with human coronary artery smooth muscle cells (Zeng et al. 2016). The primary role of perivascular cells is to regulate endothelial cell function and angiogenesis during tissue growth and development (Folkman and D'Amore 1996, Hirschi and D'Amore 1996). Furthermore, vascular smooth muscle cells and pericytes produce VEGFA which is increased under hypoxic conditions (Shifren et al. 1994, Nomura et al. 1995, Stavri et al. 1995). Thus pericytes, like endothelial cells, could sense and react to low tissue oxygen and initiate angiogenesis.

Reproductive tract infections in dairy cows lead to endocrine dysfunction and ultimately infertility (Herath et al. 2007, Williams et al. 2008a, Dohmen et al. 2000). Gram negative *E.coli* is regarded as the most prevalent pathogenic bacteria isolated from the uterus during the post-partum period (Herath et al. 2007, Sheldon et al. 2010). Furthermore, uterine diseases negatively affect the production of androstenedione by bovine theca cells (Herath et al. 2007, Shimizu et al. 2012, Magata et al. 2014). *In vivo*, LPS inhibited progesterone and androstenedione production by suppressing mRNA expression of *StAR* and CYP17 from bovine theca cell (Magata et al. 2014). However, in this study in two separate experiments *E.coli* LPS had no effect on progesterone production by bovine luteal cells *in vitro* even at high doses (10μg/ml). This was supported by the Western blot, which showed that LPS did not affect the expression of STAR, HSD3B or CYP11A. Similarly, it was reported that LPS had no effect on P4 production by bovine granulosa cells, with STAR and HSD3B expression not being affected by LPS, even at high concentration of LPS (10μg/ml) (Shimizu et al. 2015). Similar observations were made in rat granulosa cells (Taylor and Terranova 1996). In contrast, Grant et al. (2007) reported that LPS increased progesterone production from bovine luteal cells

in culture in response to LPS during the first 48h, however, progesterone production was not increased after 48h, even at 3μg/ml.

It is interesting to note that, this study demonstrated that luteal cells were not affected by LPS across the range of concentrations tested, even though the higher concentrations (10μg/ml), were much greater than the LPS concentrations <10 ng/ml (Mateus et al. 2003) and <1μg/ml in follicular fluid (Herath et al. 2007) measured in postpartum cows with uterine disease. Unlike endothelial cells, steroidogenic cells still proliferated, even at the high concentrations of LPS (10μg/ml). This is in contrast to, Grant et al. (2007) who demonstrated that 1 μg/ml of LPS induced cell death after 48h, which is lower than present study in which 10 μg/ml used at high concentration but did not affect luteal cells.

The present study found that progesterone was increased by addition of VEGFA/FGF-2. This is in agreement with previous reports (Kobayashi et al. 2001, Miyamoto et al. 2009, Robinson et al. 2008) which reported that VEGFA promotes progesterone production *in vitro*. Similar results have found by Woad et al. (2009) using the same bovine luteal angiogenesis system, where progesterone production was decreased when VEGFA signaling was inhibited. Moreover,Yamashita et al. (2008) have shown that local immunoneutralisation of VEGFA reduced luteal development and progesterone production. In contrast, Laird (2010) reported that VEGFA had no additive effect on progesterone production in a bovine follicular angiogenesis culture system. The present study found that progesterone production was increased throughout the culture period even in the presence of the high LPS dose. This suggests that the steroidogenic cells underwent further luteinisation and/or proliferation in the presence of LPS. Thus, the doses of LPS were not generally cytotoxic and that the EC appear to be particularly sensitive to LPS challenge.

5.5 Conclusion

We have shown repeatedly that LPS inhibited *in vitro* luteal angiogenesis from CL from both the early and mid-luteal phase. LPS exerted its action by significantly increasing apoptosis in luteal endothelial cells and suppressing their proliferation. In addition, LPS significantly decreased ACTA2 expression, which is likely to reflect decreased number of vascular mural cells or altered phenotype. This might be a mechanism by which LPS elicited its inhibitory effect on luteal endothelial cells. Surprisingly, LPS had no effect on progesterone production and expression of progesterone production proteins. Thus, it can be speculated that *in vivo* negative effects of LPS on progesterone level is as a consequence of attenuated vasculature rather than direct effects on luteal steroidogenic cells.

6 General discussion

Declining fertility in dairy cows is a global problem that is related to the genetic selection for high milk production recovery little selection for other traits (Royal et al. 2000, Butler 2003, Pryce et al. 2004). The central theme of this thesis to improve our understanding of the mechanisms behind subfertility and in particular the way in which uterine infections affect fertility in high yielding dairy herds. This was achieved by; firstly comparing reproductive performance in cows that experienced uterine disease with others that did not in a large scale multi-herd study. Secondly, determining the association between uterine infection and post-partum ovarian cyclicity in high yielding dairy cows at the Nottingham dairy centre. Thirdly, assessing the impact of uterine infection on bovine CL vascularisation and steroidogenesis *ex vivo*. Finally, determining the effect of *E.coli* lipopolysaccharide (LPS) on bovine luteal endothelial cell network formation and steroidogenesis *in vitro,* and the underlying mechanism by which LPS exerts its action.

The primary production goal in postpartum dairy cow management is to establish a pregnancy in an efficient manner and at a profitable interval after calving. However, while fertilization rates in cows may be as high as 90%, conception rates much are lower (Pomar et al. 2005). The common reasons for reproductive failure in high yielding dairy cows are poor detection of oestrus (Van Eerdenburg et al. 2002) and early embryonic losses (Humblot 2001, Wathes et al. 1998, Mann and Lamming 1999). Opsomer et al. (2006) stated that good oestrus detection, high semen quality, good insemination technique and a healthy uterine environment are essential for high reproductive performance in dairy cows. Uterine disease such as endometritis can have dramatic effects on reproductive performance of high milk-yielding cows due to persistent bacterial infection causing uterine inflammation and damage to the endometrium thereby, prolonging uterine involution and impairing fertility (Fourichon et al. 2000, Gilbert et al. 2005, Kasimanickam et al. 2004).

Sizeable and significant associations between the occurrence of reproductive tract inflammatory disease (RTID) and reproductive outcomes were established at the level of a unit of time within lactation (Chapter 2 and 3). Chapter 3 further demonstrated that RTID had negative effect on post-partum reproductive performance in dairy cows particularly day to first service and increased incidence of prolonged luteal phase.

The present study showed that reproductive tract inflammatory disease (RTID) is present in 12% of dairy cows. This was extracted from a large-scale multi-commercial dairy herds across England and Wales. Similarly, in dairy herds in the UK, 15% annual incidences of vulval discharge/endometritis have been reported (Esslemont and Peeler 1993, Esslemont and Kossaibati 1996). It is worthy while noting that these studies were carried out in 90 herds and involved >14,000 cows, (average 150 cows per herd), which is fewer than in the present study. Various factors, such as efficiency and method of examination of disease, size of herd, level of production, incidence of other diseases, genetics, housing, and management could increase the prevalence of reproductive disorders, which might have contributed to the observed variations among the different studies.

Although many diagnostic methods are applied in the diagnosis of RTID within 3 weeks and onwards, all of them vary and are applicable for RTID in practice. These depends on the sensitivity and specificity on the methods. For example, trans-rectal palpation has been used extensively but is not considered an accurate method because the assessment of uterine size and palpable content might be varied between people. Vaginoscopy is a routine diagnostic method used by a veterinarian, particularly when rectal palpation is the only other diagnostic modality being used (LeBlanc et al. 2002). However, vaginoscopy is not as effective as endometrial cytobrush cytology for both clinical and subclinical endometritis (Barlund et al. 2008, Kasimanickam et al. 2004). However, vaginoscopy did support for diagnosis >4 week post-partum period (Barlund et al. 2008). It would be beneficial to use all methods (ultrasound, endometrial cytological and uterine biopsy), but this is time consuming and uterine biopsy is invasive and expensive (Sheldon 2004, Bonnett et al. 1993).

Current state of dairy cow fertility across England and Wales

The current state of dairy cow fertility in England and Wales was assessed. The present study found that the observed overall mean estimates of three intervals namely days to first service, calving to conception interval and calving interval fall within the ranges reported previously (Mayne et al. 2003). Although the present mean intervals were within the range of previous studies, they are considered unfavourable and extended compared with the optimum CCI (80–85 days) recommended achieving the commonly accepted 360-365 days calving interval (Ball and Peters 2004, Arbel et al. 2001). The results of this thesis (Chapter 2) with previous similar reports from UK

suggest that the efficiency of reproduction in dairy herds is still unsatisfactory and hence deserves attention.

Effect of RTID on reproductive performance

The results of Chapter 2 and 3 have shown that the three intervals (DFS, CCI and CI) were significantly longer in those cows that experienced RTID than in those that did not. Cows with RTID had 5 days longer DFS. Causes of extended DFS in cows with RTID might be due to: 1) ovarian pathologies such as prolonged anovulatory anoestrus (Sheldon et al. 2009b), ovarian cysts (Tsousis et al. 2009, Vacek et al. 2007) or persistent CL (Sheldon et al. 2009b). 2) cows failed to respond to the fertility treatments; 3) cows have lower peripheral plasma oestradiol concentration due to slower growth of the first postpartum dominant follicles which has been associated with lower peripheral plasma progesterone concentration after ovulation (Sheldon et al. 2002b, Williams et al. 2007); 4) bacterial LPS and/or various mediator cytokines (e.g. TNFA and IL-1) entering the circulation and blocking the secretion of GnRH from the hypothalamus (Rivest et al. 1995, Battaglia et al. 2000, Williams et al. 2007). Importantly management factors such voluntary waiting period, silent heat, and poor oestrus detection, herd size, and farmer's decision, could further contribute to such extended intervals.

Similarly, the results of survival analysis (Chapter 2) indicated that cows with RTID had a reduced chance of becoming pregnant in the 85-day period after calving compared to their respective control counterparts. The presence of RTID can not only influence the resumption of normal ovarian cyclicity, but also could have influence on sperm transport, sperm death and finally reduce conception rate (Rahman et al. 1996). In another way, the presence of pus in the uterus alters immune responses which are crucial at the interface between the endometrium and the embryo and finally leads to repeat breeding. Another reason for extend CCI in cows with RTID might be due to poor CL formation after the first ovulation during the post-partum period resulting in inadequate P4 production to establish pregnancy.

Cows with RTID had an increased number of services per conception and had lower conception rate at first service compared to cows without RTID. This reduction in the reproductive efficiency of cows could be associated with uterine tissue damage, delayed uterine involution (Shrestha et al. 2004b, Sheldon et al. 2003), disruption of endometrial function, reduction of steroid concentrations of ovarian follicles (Green et al. 2011), and disruption of ovarian cycles (Opsomer et al. 2000, Herath et al. 2009). Furthermore, this

study has shown that cows with RTID (Chapter 2) had a 22 day longer CI compared to cows without RTID. This might be because that cows with RTID had a longer CCI and took longer to get pregnant since CCI is regarded as a major component of CI. It should not be neglected that others factors could contribute to extended CI include management expertise, environmental constraints, herd size, and labour availability.

At the herd level, RTID was associated with subfertility in dairy cows, and this was characterised by longer DFS, CCI or conception for cows experienced with RTID, and more cows exited for failure to conceive in a timely manner. These effects on fertility and the costs of treatment mean that RTID is one of the most expensive conditions challenging the dairy industry (Sheldon et al. 2008). Thus, substantial effort is required to further understand the risk factors for RTID. Fertility can account for one of the major costs of production. Inadequate dairy herd fertility is known to have many consequences, both direct and indirect (DairyCo 2014b). The loss of mature dairy cows due to early culling has a significant adverse financial impact (DairyCo 2014b). Currently, the costs of replacement is around £14,400 per annum per 160 cows herd, this equates to £90 per cow (DairyCo 2014a). It has been reported that the direct cost associated with treatment for RTID was approximately £72 per cow (Arnott et al. 2015). This, however, does not include the indirect costs associated with poor subsequent reproductive performance.

Diagnosis and treatment of RTID are crucial components of fertility programs. It is assumed that cows with reported RTID in this study were treated, however, the type of treatments and doses were unknown. In addition, the efficiency of any treatment used was unknown, but still adverse effects of RTID were observed. Indeed, type of treatments, dose of treatment and time of administration, can influence the efficiency of control systems (Dolezel et al. 2008).

In the 170 cows, whose milk was analysed for progesterone (Chapter 3), the mean onset of luteal activity (OLA) after calving was 28 days. Moreover, 16% exhibited a delay to 1st ovulation (>45 days); 19% had short ovarian cycles; 18% cessation of cyclicity and 47% had prolonged luteal phase. The presence of endometritis significantly impaired post-partum ovarian cyclicity in Holstein dairy cows. Cows exhibiting endometritis had a higher incidence of atypical ovarian cycles, particularly prolonged luteal phase (65.8%). The most prevalent pathology was a persistent CL and is likely to indicate altered

endometrial prostaglandin production. The endometrium expresses specific receptor complexes for the detection of LPS, and LPS switches prostaglandin secretion from PGF2α to PGE2 (Herath et al. 2009). This would lead to disrupted luteolysis (Herath et al. 2009, Sheldon and Dobson 2004). Cows with normal ovarian cycle had shorter DFS and were 1.8 times more likely to become pregnant at first service.

Reproductive tract inflammatory diseases (RTID) might have had an affect either locally or systemically. Bacterial endotoxin such as LPS infused into the uterus or peripheral circulation disrupted LH secretion from the pituitary (Battaglia et al. 1999, Peters et al. 1989). The mechanism by which LPS enters into the peripheral circulation from the uterus is poorly understood; however, it has been suggested that following escape through the oviduct into the peritoneal cavity, LPS might then enter the bloodstream by transmural movement in the peritoneal cavity (Peters et al. 1989). On the other hand, LPS can be absorbed from the uterine lumen into the peripheral circulation (Peters et al. 1990, Mateus et al. 2003). Moreover, LPS may signal indirectly from the uterus to the ovary via altered prostaglandin production (Herath et al. 2006a).

Lipopolysaccharide accumulation in the follicular fluid has been demonstrated and one candidate molecule that could contribute to this accumulation is LPS binding protein (LBP). LBP is an acute protein produced by liver, which enters into circulation where it detects and forms a high affinity complex with LPS (Schumann et al. 1990). During the acute phase of inflammation, LBP has been detected in serum and in such situation is increased by about 50 fold in human (Zweigner et al. 2001). Therefore, it is probable that LBP is produced in response to acute uterine inflammation and binds with LPS, then would accumulate in the follicular fluid.

In Chapter 4, it was demonstrated that endometritis/metritis significantly reduced CL size. This reduction might be due to a reduction in the degree of vascularisation and estimated total luteal vasculature. Furthermore, there was reduced expression of α-smooth muscle-actin (ACTA2) reflecting pericyte/smooth muscle cell coverage in the CL, which plays an important role in the function of CL vasculature. The logical extension of this observation would be markedly reduced luteal blood flow, and the ability to exchange nutrients and supply steroidogenic cells with cholesterol/LDL for progesterone production. Indeed, decreased blood flow after LPS treatment has been observed in cows (Herzog et al. 2012).

It is important to know the mechanism by which endometritis and/or LPS affect on the uterine angiogenesis, however, according to our best knowledge there are not studies have been reported in this situation. Uterine blood flow was affected by uterine disease, this was demonstrated by an increased blood flow volume and a decreased pulsatile index in the uterine arteries of cows with uterine disease, 8 days post-partum, as assessed by Doppler sonography (Heppelmann et al. 2013). The same study established that blood flow volume in the contralateral artery of affected cows was greater than in healthy cows during the entire examination period. This might also be a key to decreasing blood flow to the follicle and CL vasculature in cows with RTID. LPS increased mRNA expression encoding Caspase-3 in CL, which cause suppression of luteal function and morphology (Herzog et al. 2012, Debertolis 2015). This could contribute to reduction of P4 production in cows with endometritis.

Cows with endometritis/metritis had reduced luteal P4 content, as measured from homogenised CL. This was confirmed by markedly reduced protein expression of key proteins involved in progesterone synthesis (STAR, CYP11A and HSD3B) *in vivo*. However, surprisingly neither progesterone production nor expression of progesterone synthesis enzymes were affected by LPS treatment of luteal cells even at high doses of LPS (10µg/ml) *in vitro.* Surprisingly LPS had minimal effect on P4 production even at 10 µg/ml of LPS on day 3, 5 and 9 of culture and protein expression of steroidogenic enzymes on day 5. The explanation for this could be due to that 1) all cells were supplemented with 5ng/ml LH, and fetal bovine serum; 2) P4 concentrations increased in control wells and LPS treated wells, due to increased luteinisation of steroidogenic cells proliferation and; 3) cells being provided with all the required nutrients for progesterone production.

The mechanism through which LPS inhibits steroidogenic enzyme activity in bovine luteal cells *in vivo* might be due to: 1) endometritis suppressing hypothalamic release of GnRH, and hence pituitary secretion of luteinising hormone (LH) (Peters et al. 1989, Battaglia et al. 2000). Any suppression of GnRH-induced LH surge by LPS could play an important role in diminished stimulation of angiogenesis during the follicular luteal transition (Robinson et al. 2009). LH has been shown to stimulate luteal hyperaemia and increases blood flow to the CL (Niswender et al. 1976), 2) suppression of CL vasculature through which the CL is supplied with oxygen and nutrients as discussed above; 3) increased endothelial cell apoptosis and decreased proliferation, as confirmed in the *in vitro* study.

A focus of this thesis was to elucidate the mechanism by which endometritis could affect the function of the CL. In this regard, the effect of the bacterial endotoxin, LPS, on luteal angiogenesis and steroidogenesis *in vitro* was investigated. Firstly, it was important to verify that the LPS receptors TLR4, CD14 and MD-2 were present in the bovine CL. Indeed, all three mRNAs were identified in the CL at all stages of oestrus cycle using multiplex PCR. Additionally, the mRNA encoding the LPS receptors (*TLR4*, *CD14* and *MD2*) was present in the CL from cows with endometritis. Further investigation using qPCR is required in order to precisely quantify their expression. Additionally, the cellular localisation of these receptors would confirm whether LPS could have a direct effect on luteal development via endothelial and/or steroidogenic ells).

On both day 5 and 9 of culture, LPS significantly inhibited luteal endothelial cell network formation, even at the lowest dosage of LPS (0.01µg/ml). To our knowledge, this is the first study investigating the effect of LPS inhibits on luteal endothelial cells network formation *in vitro.* Even at low dose of LPS 0.01 µg/ml luteal endothelial cell network formation was decreased by about 50%. This study has shown that LPS decreased luteal vasculature thorough increased luteal endothelial cell apoptosis and decreased proliferation in a dose dependant manner. The pattern of caspase3 and Ki67 staining was more abundant in the EC islands compared with the other regions on the coverslip. The inhibition of luteal endothelial cells angiogenesis is confirmed by immunolocalisation of caspase-3 to presumptive EC islands and dual immunofluorescence of Caspase-3 and Ki67 to luteal endothelial cells on day 5 of culture. This study has shown that LPS *in vitro* reduced expression of ACTA2, and this could be another important factor by which LPS inhibits luteal endothelial cells angiogenesis. Reduced levels of ACTA2 expression and associated with diminish cellular contraction (Wylam et al. 2001).

6.1 Conclusion

This thesis has improved the understanding of how uterine infection can affect high yielding dairy cow fertility by: 1) RTID had negative effects on reproductive performance in high yielding dairy herds, through extension of DFS, CCI, CI, higher SPC, lower conception rate at 1st AI and increased exit rate; 2) Endometritis has potential effects on post-partum ovarian activity, and is associated with an increased rate of abnormal progesterone profiles; particularly prolonged luteal phase; 3) Endometritis decreased luteal P4 content and protein expression of steroidogenic enzymes including STAR,

HSD3B and CYP11A *in vivo*. However, neither P4 concentration nor protein expression of steroidogenic was affected by LPS treatment of luteal cells *in vitro*; 4) LPS significantly inhibited luteal endothelial cells network formation, and decreased protein expression of α-smooth muscle-actin (ACTA2); 5) LPS increased endothelial cell apoptosis and reduced EC proliferation.

Future work

There is no doubt that it is the interaction of several systems which ultimately determines luteal function and subfertility in dairy cows. Uterine infection is regarded as the primary factor affecting reproduction in dairy cows and could exert effects on ovarian function. The studies in this thesis have established that endometritis decreased CL angiogenesis and luteal function *in vivo*. Further work is required to measure the effect of endometritis on both uterine and CL blood flow using colour Doppler ultrasound. In order to determine whether LPS directly affects luteal endothelial cells *in vitro*. Future work requires isolation of endothelial cells (devoid from contamination with other cells) from an early stage CL and treatment with LPS. The following procedures required to perform such as:

1) Quantification of LPS complex receptors (TLR4, CD14 and MD-2) from isolated endothelial cells using Multiplex qPCR.
2) Immunohistochemistry for caspase-3 and Ki67 from isolated endothelial cells and treatment with LPS at 0-10μg/ml on day 3, 5 and 9 of culture, to identify the pathway by which LPS causes EC apoptosis.
3) Expression of mRNA encoding TGFB from CL of cows positive with endometritis *in vivo* and *in vitro* by using co-culture of luteal endothelial cells.

TGFB has also been related to the interaction between pericytes and endothelial cells. TGFB localise in CL and ovarian vasculature of most species (Gangrade et al. 1993, Zheng et al. 2008, Maroni and Davis 2011). TGFB reduce bovine luteal endothelial cells migration and supress endothelial network formation (Maroni and Davis 2011). Furthermore, TGFB increase apoptosis in bovine aortic endothelial cells (Pollman et al. 1999) and in human umbilical vein endothelial cells (Ferrari et al. 2009). It has been reported that endotoxins are capable of inducing the expression and secretion of TGF-B1 and TGFB as amechanisms to stimulate endotoxin-induced endothelial fibrosis (Echeverría et al. 2014). Therefore, TGFB will be a prime target for future work into effect of role of TGFB on luteal endothelial cells angiogenesis after treated with LPS.

4) The measurement of TNFA and IL-1 from culture media of isolated EC from day 1-9 using ELISA. The aim of this step to determine whether LPS increase expression of TNFA from isolated EC. In addition, qPCR and Western blot to determine the expression of of TNFA receptors *in vitro.*

5) Identify the relationship between bovine single nucleotide polymorphisms (SNPs) in candidate immune and angiogenesis-related genes and susceptibility to uterine inflammatory disease.

At the level of the investigations conducted and results in this thesis established that endometritis during postpartum adversely affected fertility in dairy cows and luteal angiogenesis. Angiogenesis plays a key role in the growth of tumour and its induced by the same cells and factors involved in the physiological ovarian angiogenesis (Tamanini and De Ambrogi 2004). Therefore, the roles of LPS and the association between LPS and luteal endothelial and vascular smooth cells may provide useful treatment for further research into ovarian cancer. The types of approaches outlined above might lead to the possibly new findings which will help to understand the impact of LPS on luteal cells

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Appendices

Appendix I: Preparation of solutions

Immunohistochemistry

Phosphate buffer saline (PBS)

1x PBS solution was prepared by adding 10 L of distilled water to the following:

Amounts (g)	Name of chemical product
85	Sodium chloride (NaCl)
2.5	Potassium dihydrogen phosphate (KH2PO4)
17.8	Sodium phosphate dibasic dihydrate (Na2HPO4.2H2O)
The pH was then adjusted to 7.4	

10mM Citrate buffer (Antigen retrieval)

Two solutions were prepared as follows:

Solution	Chemical	Amount (g)	Volume of distilled water
A	Citric acid	4.2	200 ml
B	Sodium citrate	14.7	500ml
16ml of solution A was mixed with 84ml of solution B and the volume was then made up to 1 L.			
The pH was then checked to be 6 and adjusted as appropriate			

0.4mg/ml trypsin and 0.25mg/ml CaCl2 (antigen retrieval)

This was prepared by adding 120mg of trypsin and 75mg of anhydrous CaCl2 to 300ml of 1XPBS.

Acetone/Methanol Fixative

This was prepared by adding 5ml of acetone to 5ml of methanol

<u>*Peroxidase Block*</u>

This was prepared by adding 2ml of Hydrogen peroxide (H_2O_2) to 18ml of methanol

<u>20% (V/V) normal goat serum</u>

1ml of normal goat serum (Sigma) was diluted in 4 ml of 1XPBS solution. The normal goat serum was then dispensed into 1ml aliquots and stored at -20˚C.

Primary Antibodies

<u>*VWF antibody*</u>

Polyclonal Rabbit anti-human VWF (5 μg/ml, A0082, Dako). Prior to use the antibody was diluted 1:1000 in 2% NGS (f.c. 5 µg/ml) as follow:

5µl of VWF into 100µl of normal goat serum, and volume made up to 5ml with PBS

<u>*Rabbit anti-human cleaved caspase3 (Cell Signalling, cat no 9661)*</u>

Dilute caspase3 antibody 1 in 300 (diluted 3.3µl into 1ml 1x PBS)

Secondary Antibodies

<u>*Biotinylated Goat anti-rabbit (Vectastain kit)*</u>

Biotinylated goat anti-rabbit was diluted 33µl in 100µl of normal goat serum+ PBS

Immunofluorescence

Primary antibodies

<u>*Mouse anti-human Ki67 (Clone MM1, Vector KP452)*</u>

This antibody was diluted 1:80 (12.5µl of Ki67 into 1ml of 1XPBS).

<u>*Biotinylated Griffonia (Bandeiraea) simplicifolia lectin I isolectin B4 (Vector, cat no B1205)*</u>

Isolectin (500μg/ml) was diluted by 1/25 to concentration of 20μg/ml in 1× PBS buffer and to this other primary antibodies were added.

Rabbit anti-human caspase 3

Caspase 3 was diluted 1/300 (3.3µl in 1ml of 1XPBS).

Secondary antibodies

Avidin labelled with fluorescein DCS and Texas Red anti-mouse IgG

Avidin-FITC was diluted 1/100 to a working concentration of 20µg/ (Diluted by adding 10µl into 990µl of 1XPBS to give final volume 1ml of 1XPBS).

Texas Red anti-mouse IgG

Texas red anti-mouse IgG was diluted 1/100 (f.c. 10µg/ml) in 1× PBS Buffer (It was diluted as follows 10µl of Texas red anti-mouse IgG into 990µl of 1XPBS to give final volume 1ml).

Texas Red avidin and FITC anti-rabbit IgG 1/75

Texas-Red avidin was dilute 1/100 (added 10µl into 990µl of 1XPBS)

FITC anti-rabbit IgG

FITC anti-rabbit IgG was diluted 1/75 (13µl into 987µl of 1XPBS)

Haematoxylin & Eosin staining of paraffin embedded sections

1%Acidified IMS (fume hood)

It was prepared as follows:

280ml Industrial Methylated Spirit (IMS)

4ml HCL

116ml dH$_2$0

Ammoniated Water

It was prepared as follows:

300ml dH$_2$0

3ml of Ammonia Solution

1.5% Eosin Stain

It was prepared as follows:

3g Eosin Powder

60μl Acetic acid

300ml dH$_2$0

H&E Procedure

1- Xylene (2 x 5 mins)
2- 100% IMS (5 mins)
3- 95% IMS (5 mins)
4- 70% IMS (5 mins)
5- Harris's haematoxylin (Filtered) (2 min)
6- Tap water until runs clear
7- 1% acid IMS (2 dips)
8- Rinse in tap water
9- Ammoniated water (2 Dips)
10- Rinse in tap water
11- Eosin (1.5% (w/v; filtered) (5 min)
12- Tap water until runs clear
13- 70% IMS (2 min)
14- 90 % IMS (2 min)
15- 100% IMS (2 min)
16- Xylene (2 × 5 mins)
17- Mount in DPX mountant, add coverslip and allow to air dry

Tissue culture

Fibronectin (1mg/ml)

For 12 wells was prepared as follows:
Added 200μl of 1mg/ml Fibronectin into 20ml of sterile distilled water.

Dissociation solution (make up just before culturing)
40 ml DMEM/Ham's media (with no FCS/BSA)
0.8 ml of 100 mg/ml Collagenase I (Dissolved 1g pot in 10ml DMEM/Ham's media stored at -20°C)
250 μl DNase I (dissolve one vial (5mg) in 1 ml)
_500 ng/ml LH_
From 250 μl aliquot of 1000 μg/ml LH

Dilute 1:2000 i.e. 20 μl in 40 ml media $\rightarrow$ 500 ng/ml LH solution (100x) Sterile filter

Cell culture medium

500 ml of Endothelial cell media (ECM-complete, Lonza)

The following products were added:

5 ml of 500 ng/ml LH
5 ml of 100$\times$ ITS
5 ml of 100$\times$ pen/strep
500 μl hEGF
500 μl GA-1000
500 μl heparin
500 μl R3-IGF-1
500 μl Absorbic acid
200 μl Hydrocortisone
10 ml FBS (Fetal bovine serum)

Western blot

Tissue lysis buffer

0.5g of Dithiothreitol (DTT, Sigma)

0.5ml of 10% Sodium dodecyl sulphate (SDS)

1 tablet of protease inhibitor cocktail (Roche)

50 ml PBS

25x tissue lysis buffer

0.5g DTT

0.5ml of 10% SDS

1 tablet of protease inhibitor cocktail (Roche)

1.5ml of 1xPBS

Store in 400µl aliquot and dilute with 960µl of 1xPBS

10% Ammonium per sulphate (APS)

This was prepared as follow: 100µg added into 1 ml of dH2O

10% (w/v) Sodium dodecyl sulphate (SDS) solution

 10g into 100ml dH$_2$O

Blotting buffer

50ml of 1XTGS (Tris Glycine SDS, Biorad, cat no 191-0772)

450ml dH$_2$O

Transfer buffer

Transfer buffer was prepared as follow:

100ml of 10x Tris Glycine (TG)

200ml methanol

700ml dH$_2$O

Ponceau S stain

0.1g Ponceau s

5ml   acetic acid

95ml dH$_2$O

Blocking solution (Phosphate Suffered Saline with Tween® 20)

1ml of Tween20

1000ml 1xPBS

Primary Antibodies

Steroidogenic enzymes protein (STAR, Abcam, ab96637, Rabbit)

It was diluted 1:3000 (added 1.33 µl of STAR into 4ml blocking solution)

3beta-Hydroxysteroid dehydrogenase (HSD3B, Richard Parker, Rabbit)

It was diluted 1:200 (added 10µl of HSD3B (diluted already 1:20) into 4ml blocking solution)

Cholesterol side-chain cleavage enzyme (CYP11A, Abcam, ab75497)

It was diluted 1:1000 (added 4µl of CYP11A into 4ml blocking solution)

Histone 3 (H3, Abcam, ab1791, Rabbit)

It was diluted 1:1000 (added 4µl of H3 into 4ml blocking solution)

α-smooth muscle actin (α-SMA, mouse, Sigma, A2547)

It was diluted 1:1000 (added 4µl of α-SMA into 4ml blocking solution)

Secondary Antibodies

Goat anti-rabbit HRP (Sigma Cat NO. A9169)

It was diluted 1/8000 in blocking solution (5 µl in 40ml blocking solution)

Sheep anti-mouse HRP (Amersham, Cat NO. NXA931)

It was diluted 1/50000 in blocking solution (1µl in 50ml blocking solution)

Appendix II

Lipopolysaccharide receptors sequences and primer sites

There are 3 LPS receptors genome sequences from bovine (*Bos-Taurus*) with their accession numbers obtained from National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The top red and bottom green highlighted fragments were used for designing forward and reverse primers, respectively, to amplify the large amplicon of each gene. The forward primer site used only to sequence the middle part of TLR4, CD14, MD-2 and Suz14 (House keeping gene) are highlighted with yellow.

Toll like receptor4 (TLR4), accession number, NM_174198.6, size 224 bp

AGATAAATATCAGAGAAACGAAAGTCCAGAAAGCTAAGGCTGCCCCTCTCACTTCCTCTCCTCCCGTAACCCAG
CACTGCTTTGAATAGGGGCAGTGCCCTGGGGCACCAAGCCGGGGAGAGACGACACTACAGTGCCTCGGAGGC
TGCCCTGGCCGGCACAGACAGAGGGTTATGCTTTCACAGAGCCACTTCTGGTCACAGAAAATGCCAGGATGAT
GGCGCGTGCCCGCCTGGCTGCGGCTCTGATCCCAGCCACGGCCATCCTCTCCTGCCTGAGAACCGAGAGTTGG
GACCCTTGCGTACAGGTTGTTCCTAACATTAGTTACCAGTGCATGGAGCTGAATCTCTACAAAA<mark>TCCCCGACAAC</mark>
<mark>ATCCCCATA</mark>TCAACCAAGATGCTGGACCTGAGCTTTAA<mark>CTACCTGAGACATTTAGGCAGCCATAACTTCTCCAG</mark>C
TTCCCAGAACTGCAAGTGCTGGATTTATCCAGATGTGAAATTAAGATTATTGAAGACGACACATTTCAGGGCCTA
AACCACCTCTCCACCTTGATACTGACGGGAAACCCTATCCAGA<mark>GTTTAGCCTGGGGAGCCTTT</mark>TCTGGGCTATC
AAGTTTACAGAAGCTGGTGGCCGTGGAGACAAACCTAGTCTCTCTAAATGACTTCCCCATTGGACATCTCAAAA
ACTTGAAAGAGCTTAATGTGGCTCACAATTTTATCCATTCCTTCAAGTTACCTGAATATTTTTCTAACCTGCCCAA
CCTGGAGCACTTGGATCTTTCTAACAACAAAATCCAAAATATTTATTATGAAGATGTGAAAGTTCTACATCAAATG
CCCCTACTCAACCTCTCTTTAGATTTGTCCTTGAACCCTTTAGACTTTATTGAACCAGGTACCTTTAAAGAAATTA

Cluster Differentiation 14 (CD14), accession number, 41386759

, size 230 bp

ATGGTGTGCGTGCCCTACCTGCTGCTGCTGCTGCTGCCGTCACTGCTGCGTGTGTCTGCGGACACAACAGAAC
CCTGCGAGCTGGACGACGACGATTTCCGTTGTGTCTGCAACTTCACGGATCC<mark>GAAGCCTGACTGGTCTAGCG</mark>C
CGTTCAGTGTATGGTTGCCGTCGAGGTGGAGATCA<mark>GTGCCGGCGGCCGCAGCCTGGAACAGTTTCTCAAGGG</mark>A
GCCGACACCAACCCGAAGCAGTATGCTGACACAATCAAGGCTCTGCGCGTTCGGCGACTCAAGCTGGGCGCTG

222

CACAGGTTCCTGCTCAGCTTCTGGTCGCCGTTCTGCGCGCGCT<mark style="background:green">CGGGTACTCTCGTCTCAAGG</mark>AACTGACGCTT
GAGGACCTGGAGGTAACCGGCCCAACGCCCCCGACGCCTCTGGAAGCCGCTGGGCCTGCGCTCACCACCCTC
AGTCTCCGTAACGTATCGTGGACAACAGGAGGTGCCTGGCTCGGCGAACTGCAGCAGTGGCTCAAGCCTGGGC
TCAGGGTGCTGAACATTGCCCAAGCACACTCGCTTGCCTTTCCGTGCGCAGGGCTCTCCACCTTCGAGGCGCTC
ACCACCCTAGACCTGTCTGACAATCCCAGTCTCGGCGACACGGGGCTGATGGCAGCTCTCTGTCCGAACAAGTT
CCCGGCCCTCCAATATCTAGCGCTACGCAACGCGGGGATGGAGACGCCGAGCGGCGTGTGCGCGGCGCTGGC
GGCAGCGAGGGTGCAGCCCCAAAGCCTGGACCTCAGCCACAACTCGCTGCGCGTCACCGCCCCGGGTGCTAC
CCGATGTGTCTGGCCCAGTGCACTAAGGTCTCTCAATTTGTCGTTCGCTGGGCTGGAGCAAGTGCCTAAGGGA
CTGCCCCCTAAGCTCAGCGTGCTTGATCTCAGCTGCAACAAGCTAAGCAGGGAGCCGCGGCGAGACGAGCTGC
CCGAGGTAAATGACCTGACTCTGGACGGAAATCCCTTTCTGGACCCTGGAGCCCTCCAGCACCAAAATGACCCG
ATGATCTCCGGCGTGGTCCCAGCCTGTGCGCGTTCTGCCTTGACCATGGGGGTGTCAGGAGCCCTGGCGCTGC
TTCAAGGAGCCCGAGGCTTCGCGTA

Lymphocyte antigen 96, (MD-2) accession number: AB072456.1, size 125 bp

GTTTTGGAGAGATTGAATCATGTTTCCATTTGTGCTTTTTTCCACCCTGTTTTCTTCCATATTTACTGAACCTAGG
GA<mark style="background:red">GAATCGTTGGGTCTGCAACT</mark>CCTCTGACGCCACCGTTTGGTACGACTACTGTGATAA<mark style="background:yellow">CTTGAAATTTCCTGTT</mark>
<mark style="background:yellow">TCAATACGTTCT</mark>GAGCCCTGTATAACATTGAA<mark style="background:green">GGGAAGCCGTGGAATACTCT</mark>ATCTCTACTACATTCCAAGAAGA
GATATAAGGAGCTTGTATTTCAATATCTATCTATCTACTAAGTCCATGAATTTCCCATTGCGCAAAGAATTTATTT
GCCGAGGATATGATGATGATTTTTCCTTTTGCAGAGCTCTGAAGGGAGAGACTGTGAACACAACAATACAATTC
TCCTTCAGGGGAATACGATTTTCTAAGGGACAGTACAACTGTATTACAGAAGCCATCGAGGGGAACACTGAAGA
AAAGCTCTTTTGCTTGAACTTTACTATCATACATTACCCTGATTTCAATTAG

SUZ12, used as a reference gene, accession number NM_001205587.1, size 286bp

ATGGCGCCTCAGAAGCACGGCGGTGGGGGAGGGGGCGGCTCGGGGCCCAGCGCGGGGTCCGGGGGAGGCG
GTTTCGGGGGGTTCGGCGGCGGTGGCGGCGGCGACGGCGTCGGGCGGCAAATCCGGTGGCGGGGGCTGTGGA
GGCGGCGGCAGTTACTCGGCCTCCTCCTCCTCCTCCGCGGCGGCAGCGGCGGGGGCCGCGGTGTTACCGGTG
AAGAAGCCGAAAATGGAGCACGTCCAGGCTGACCACGAGCTTTTCCTCCAGGCCTTTGAGAAACCAACGCAGA
TCTATAGATTTCTTCGAACACGAAATCTTATAGCGCCAATATTTTTGCATAGAACTCTTACTTACATGTCTCATCG
AAACTCCAGAACAAACATCAAAAGGAAAACATTTAAAGTTGATGATATGTTATCAAAAGTAGAGAAAATGAAAGG
AGAGCAAGAATCTCATAGCTTGTCAGCTCATCTGCAGCTTACATTTACTGGTTTCTTCCACAAAAATGATAAACC
ATCACAAAATTCAGAAAATGAACAAAATTCTGTCACCCTGGAAGTCCTGCTTGTGAAAGTTTGCCACAAAAAAAG
AAAGGATGTAAGTTGTCCGATAAGGCAAGTTCCTACAGGTAAAAAGCAGGTGCCTTTGAATCCTGACCTCAATC
AAACAAAACCTGGAAACTTCCCATCCCTTGCAGTTTCCAGTAATGAATTTGAACCTAGTAACAGCCATATGGTGA
AGTCTTACTCATTGCTATTTAGAGTGACTCGTCCAGGAAGAAGAGAGTATAATGGGATGATTAATGGAGAAACC
AATGAAAATATTGATGTCAATGAAGAACTTCCAGCTAGAAGAA<mark style="background:red">AACGAAATCGTGAAGATGGG</mark>GAAAAAACGTT
TGTTGCACAAATGACAGTATTTGATAAAAA<mark style="background:yellow">CAGGCGCTTACAGCTTTTAGATGGGGAATATGAA</mark>GTAGCCATGC
AGGAAATGGAAGAATGTCCAATAAGCAAGAAAAGAGCAACATGGGAGACTATTCTTGATGGGAAGAGACTGCC
TCCATTTGAAACATTTTCTCAGGGACCTACATTGCAGTTCACTCTTCGTTGGACAGGAGAGACCAATGATAAATC
TACAGCTCCTATA<mark style="background:green">GCCAAACCTCTTGCCACTAG</mark>AAATTCAGAGAGTCTTCATCAAGAAAATAAGCCTGGTTCAGT
AAAACCTACTCAAACTATTGCTGTTAAAGAATCATTGACTACAGATCTGCAAACAAGAAAAGAAAAAGATACTTC

AAATGAAAACCGACAAAAATTAAGAATATTTTATCAGTTCCTTTATAACAACAATACGAGACAACAAACTGAAGC
AAGAGATGACCTACATTGCCCTTGGTGCACCCTGAACTGCCGCAAACTTTATAGCTTACTTAAACATCTTAAACT
CTGCCATAGCAGATTTATCTTCAATTATGTTTATCATCCAAAAGGTGCTAGGATAGATGTTTCTATCAACGAGTGT
TATGATGGCTCCTATGCAGGAAATCCTCAGGATATTCATCGCCAGCCCGGATTTGCTTTTAGTCGCAATGGACCA
GTAAAGAGAACACCTATCACACACATTCTTGTGTGCAGGCCAAAAAGAACAAAAGCAAGTATGTCTGAATTTCTT
GAATCCGAAGATGGAGAAGTGGAACAGCAGCGAACATACAGTAGTGGACACAACCGCCTCTATTTCCATAGTGA
TACTTGTTTACCTCTCCGTCCACAAGAAATGGAAGTAGATAGTGAAGATGAAAAAGACCCCGAATGGCTAAGAG
AAAAAACCATTACTCAAATTGAGGAATTTTCTGATGTTAATGAAGGAGAGAAAGAAGTGATGAAACTATGGAATC
TCCATGTCATGAAGCATGGGTTTATTGCTGACAATCAAATGAATCATGCCTGTATGCTGTTTGTAGAAAACTATG
GACAGAAAATAATTAAGAAGAATTTATGTCGAAACTTCATGCTTCATCTAGTCAGCATGCATGACTTTAATCTTAT
TAGCATAATGTCAATAGATAAAGCTGTTACCAAGCTCCGTGAAATGCAACAAAAATTAGAAAAAGGAGAATCTGC
TTCCCCTGCAAATGAAGAAGTCACTGAAGAACAAAATGGGACAGCAAATGGATTTAGTGAAATTAACTCAAAAG
AGAAAGCTTTGGAAACAGATGGTGTCTCAGGGGTTTCAAAACAGAGCAAAAAACAAAAACTCTGA

Appendix III

Reagents

Reagent	Supplier
100 bp DNA ladder	New England BioLabs,
ABC Elite Kit	Vector Laboratories Ltd Orton Southgate, Peterborough, UK
Acetone	Fisher-Scientific Ltd Loughborough, Leics, UK
Agarose, powder	Sigma-Aldrich (Poole, Dorset, UK)
Amersham ECL Prime Western Blotting Detection Reagent	GE Health care (UK)
Ammonia solution	Fisher-Scientific Ltd Loughborough, Leics, UK
Ammonium chloride	Sigma-Aldrich (Poole, Dorset, UK)
Amphotericin-B solution	Sigma-Aldrich (Poole, Dorset, UK)
Anti–mouse IgG antibody, HRP–linked (for western blotting)	Sigma-Aldrich (Poole, Dorset, UK)
Bio-Rad solution (Protein determinant)	Bio-Rad laboratories Ltd Hemel Hempstead, UK
Bouins	VWR (Lutterworth, Leics, UK)
Bovine serum albumin	Sigma-Aldrich (Poole, Dorset, UK)
Calcium chloride	Fisher-Scientific Ltd Loughborough, Leics, UK
Citric acid	VWR (Lutterworth, Leics, UK)

DAB	Vector Laboratories Ltd Orton Southgate, Peterborough, UK
DNase type IV	Sigma-Aldrich (Poole,Dorset, UK)
DPX mounting medium	VWR (Lutterworth, Leics, UK)
EBM-2 medium	Lonza Wokingham Ltd Loughborough Leics, UK
Epidermal growth factor	Lonza, Wheldon Rd, Castleford, UK
Ethanol	Fisher-Scientific Ltd Loughborough, Leics, UK
Ethidium bromide solution	Fisher Scientific
Fetal bovine serum	Invitrogen (Paisley, UK)
Fibronectin	Sigma-Aldrich (Poole, Dorset, UK)
Gel Loading Dye, Blue (6X)	New England BioLabs
Haematoxylin	VWR (Lutterworth, Leics, UK)
Hydrogen peroxide	Fisher-scientific Ltd Loughborough, Leics, UK
Insulin transferrin selenium	Sigma-Aldrich (Poole, Dorset, UK)
E.coli O111:B4, LPS	Alpha diagnostic international, Cat.No. LPS13-1
Methanol	Fisher-Scientific Ltd Loughborough, Leics, UK
Mouse IgG	Sigma-Aldrich (Poole, Dorset, UK)
Sodium chloride	Fisher-Scientific Ltd Loughborough, Leics, UK
Normal goat serum	Sigma-Aldrich (Poole, Dorset, UK)
Nuclease–free water	Invitrogen,
Paraffin wax	Fisher-Scientific Ltd Loughborough, Leics, UK
PBS	Sigma-Aldrich (Poole, Dorset, UK)
Penicillin-streptomycin	Sigma-Aldrich (Poole, Dorset, UK)
Phosphate buffered saline (PBS), 1X liquid	Invitrogen (Paisley, UK)
Potassium bicarbonate	Sigma-Aldrich (Poole, Dorset, UK)
Potassium dihydrogen phosphate	Fisher-Scientific Ltd Loughborough, Leics, UK
Progesterone ELISA	Ridgeway Science (St Briavels, Glos, UK)
Protease inhibitor cocktail	Roche Products Ltd (Welwyn Garden City)

Rabbit IgG	Sigma-Aldrich (Poole, Dorset, UK)
Sodium bicarbonate	Sigma-Aldrich (Poole, Dorset, UK)
Sodium Citrate	VWR (Lutterworth, Leics, UK)
Sodium phosphate dibasic di hydrate	Fisher-Scientific Ltd Loughborough, Leics, UK
Texas Red avidin and FITC anti-rabbit IgG	Vector Laboratories Ltd (Orton Southgate, Peterborough, UK)
Thermo scientific microscope slides	Fisher-Scientific Ltd Loughborough, Leics, UK
Toluene	Fisher-Scientific Ltd Loughborough, Leics, UK
Tris Glycine SDS sample buffer (2X)	Sigma-Aldrich (Poole, Dorset, UK)
Triton-x100	Fisher-Scientific Ltd (Loughborough, Leics, UK)
Trypan blue solution	Sigma-Aldrich (Poole, Dorset, UK)
Trypsin from bovine pancreas TPCK treated	Sigma-Aldrich (Poole, Dorset, UK)
Tween 20	Fisher Scientific
Vectashield mounting medium with DAPI	Vector Laboratories Ltd (Orton Southgate, Peterborough, UK)
Xylene	Fisher-Scientific Ltd Loughborough, Leics, UK
Antibody	Source
VWF	Dako (Ely, Cambs, UK)
ACTA2	Sigma-Aldrich (Poole, Dorset, UK)
STAR	Abcam, (Cambridge Science Park, UK)
HSD3B	Dr Richard Parker, University of Alabama
Histone	Abcam, (Cambridge Science Park, UK)
CYP11A	Abcam, (Cambridge, UK)
Caspase3	Cell signaling technology (New England Biolabs Ltd, UK)
Goat anti rabbit IgG HRP	Sigma-Aldrich (Poole, Dorset, UK)
Sheep anti-mouse IgG HRP	Sigma-Aldrich (Poole, Dorset,

UK)

Product	Source
12 well tissue culture plates (NUNC)	Fisher-scientific Ltd(Loughborough, Leics, UK)
70µm mesh filter	Scientific Laboratory Supplies (Nottingham, UK)
Coverslips for tissue culture	Scientific Laboratory Supplies (Nottingham, UK)
Ministar sterile srynge filter, 0.2 micron	VWR(Lutterworth, Leics, UK)
Nalgene bottle top filter units, 0.2 micron	VWR(Lutterworth, Leics, UK)
Sterile scalpel blades (swann-morton)	Fisher-scientific Ltd(Loughborough, Leics, UK)