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Prostaglandin Transport in the Bovine Uterus

Aileen Fitzgerald

Ph.D 2011
Abstract

In the present study, the effect of bovine oestrous cycle stages and rat oestrous cycle stages were investigated for the expression of PGT protein in uterine tissue, while isolated BUECs were also examined to detect the PGT protein under different conditions. In addition, BUECs were studied to measure $[^3H]$ PG uptake, cytosolic accumulation of $[^3H]$ PG, membrane incorporation of $[^3H]$ PG, vectorial transport of $[^3H]$ PG and expression of PGT mRNA under different conditions.

PGT protein is localized in uterine luminal epithelial cells and is species and oestrous cycle stage specific, where PGT protein is confined to uterine tissue from the luteal phase (progesterone stage) in cows but is expressed in an estradiol rich environment in rat uterine tissue. The differential expression of PGT is most likely due to different lengths of each cycle and the duration in which the tissue is exposed to hormones. Furthermore, expression of PGT protein in BUECs is similar to expression in rat uterine tissue, where estradiol increases PGT, while progesterone maintains a basal level of expression of the protein.

Transport studies indicate a phenol stimulatory effect on the uptake of $[^3H]$ PGF$_{2\alpha}$ into BUECs. In addition, progesterone inhibited $[^3H]$ PGF$_{2\alpha}$ uptake, an effect reversed and exacerbated when BUECs were co-cultured with progesterone and mifepristone for 72 hours in media supplemented with 10% FCS. Conversely, $[^3H]$ PGF$_{2\alpha}$ transport was not altered by estradiol stimulation. PUFA treatment or PUFA co-culture with estradiol did not reveal conclusive results. Increasing glucose concentration promoted the uptake of $[^3H]$ PGE$_2$ and $[^3H]$ PGF$_{2\alpha}$ in a dose dependent manner. In addition, uptake of $[^3H]$ PGE$_2$ and $[^3H]$ PGF$_{2\alpha}$ into BUECs, treated with progesterone or estradiol increased at 2mins compared to 10mins, while the reverse is true for unstimulated cells. Neutral PGs (pH 3.0) were transported more efficiently than anionic PGs (pH 7.4) ($p<0.05$). Efflux studies revealed that elution of the neutral PG was higher than elution of the anionic PG. Interestingly, efflux of the neutral PG into equimolar PG was higher than efflux into saline at pH 3.0. However, efflux of the anionic compound was higher into saline than efflux into equimolar PG. In addition, efflux of $[^3H]$ PGE$_2$ and $[^3H]$ PGF$_{2\alpha}$ was time-dependent that increased linearly overtime. Vectorial flux was higher from the basolateral to the apical surfaces compared to apical to basolateral flux, suggesting greater permeability of the basolateral surface to PGs. PGT mRNA expression in BUECs was maintained at a basal level after all treatments with subtle differences, indicating that these cells are capable of producing PGT protein at any time under these conditions.

The components and transport mechanisms of PGs are undoubtedly regimentally controlled and rely heavily on hormonal milieu, fatty acid content, energy source and is largely dependent on the ionic nature of the PG.
Acknowledgements

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<tr>
<td>15-PGDH</td>
<td>15 prostaglandin dehydrogenase</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3-beta-hydroxysteroid dehydrogenase</td>
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<tr>
<td>GLC</td>
<td>gas liquid chromatography</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
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<tr>
<td>bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCM</td>
<td>basic culture medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
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<td>BSS</td>
<td>basic saline solution</td>
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<td>BUEC</td>
<td>bovine uterine epithelial cell</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CCM</td>
<td>complete culture medium</td>
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<td>CL</td>
<td>corpus luteum</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>cPGES</td>
<td>cytosolic prostaglandin E synthase</td>
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<td>cPLA₂</td>
<td>cytosolic PLA₂</td>
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<td>CS-FCS</td>
<td>charcoal stripped fetal calf serum</td>
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<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
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<td>DHA</td>
<td>docoshexaenoic acid</td>
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<td>DIDS</td>
<td>diisothiocyno-2,3-stilbene-disulfonic acid</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
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<td>E2</td>
<td>estradiol</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EP</td>
<td>PGE$_2$-receptor</td>
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<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<td>ER</td>
<td>estradiol receptor</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>PGF$_{2\alpha}$-receptor</td>
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<td>G-protein coupled receptor</td>
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<td>HBSS</td>
<td>Hanks balanced saline solution</td>
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<td>hCG</td>
<td>human chorionic gonadotropin</td>
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<td>HGNC</td>
<td>HUGO Gene Nomenclature Committee</td>
</tr>
<tr>
<td>HPG Axis</td>
<td>hypothalamic-pituitary-gonadal axis</td>
</tr>
<tr>
<td>HUVECs</td>
<td>human umbilical cord vein endothelial cells</td>
</tr>
<tr>
<td>ICM</td>
<td>initial culture medium</td>
</tr>
<tr>
<td>IFN-τ</td>
<td>interferon-tau</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGF-binding protein</td>
</tr>
<tr>
<td>iPLA$_2$</td>
<td>calcium independent PLA$_2$</td>
</tr>
<tr>
<td>ITS</td>
<td>insulin transferring selenium</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>long chain fatty acid</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>MAPKs</td>
<td>mitogen activated protein kinases</td>
</tr>
<tr>
<td>MBD</td>
<td>membrane binding domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>mGC</td>
<td>multi-nucleated giant cell</td>
</tr>
<tr>
<td>mPGES</td>
<td>microsomal prostaglandin E synthase</td>
</tr>
<tr>
<td>n-3</td>
<td>omega-3</td>
</tr>
<tr>
<td>n-6</td>
<td>omega-6</td>
</tr>
<tr>
<td>NEB</td>
<td>negative energy balance</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>oatp</td>
<td>organic anion transporting polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>organic cation transporter</td>
</tr>
<tr>
<td>OTR</td>
<td>oxytocin receptor</td>
</tr>
<tr>
<td>P4</td>
<td>progesterone</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PBDu</td>
<td>Phorbol 12,13-dibutyrate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>prostaglandin F2 alpha</td>
</tr>
<tr>
<td>PGT</td>
<td>prostaglandin transporter</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>phospholipase A$_2$</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>rbIFN-τ</td>
<td>recombinant interferon-tau</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RUP</td>
<td>rumen undegradable protein</td>
</tr>
<tr>
<td>SLC</td>
<td>solute carrier</td>
</tr>
<tr>
<td>SLCO</td>
<td>solute carrier organic transporter</td>
</tr>
<tr>
<td>sPLA₂</td>
<td>secretory PLA₂</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
</tbody>
</table>
Literature Review
1.1. Introduction

The practice of selecting dairy cows of high milk yield and merit has impacted negatively on bovine reproductive performance. Essentially, cows selected for high milk production have developed an antagonistic relationship that leads to inefficient reproductive performance and poor fertility (Berger et al., 1981; Laben et al., 1982; Faust et al., 1988; Hageman et al., 1991; Nebel and McGilliard, 1993; Ouweltjes et al., 1996; Lucy, 2001; McCarthy et al., 2007; Dobson et al., 2007).

Extensive studies of dairy cow pregnancy rates through the years have highlighted a substantial steady decline in the region of 0.45-1% annually (Nebel and McGilliard, 1993). In response to several studies related to embryo transfer and subsequent survival rates in cattle (Avery et al., 1962; Rowson and Moor., 1966; Rowson et al., 1972; Christie et al., 1979; Newcomb et al., 1978; Newcomb et al., 1980), embryonic mortality in cattle was summarized (Sreenan and Diskin, 1980). Sreenan and Diskin (1980) calculated an embryonic and foetal mortality rate of approximately 40% for moderate-milk producing cows based on a fertilization rate of 90%, with an estimated 70-80% of the loss occurring between day 8 and 16 post-insemination. These early stages of pregnancy (days 8-16), therefore represent a sensitive period of reproductive failure and features as a key juncture linked to poor bovine fertility (Sreenan and Diskin, 1980).

Reproductive failures result in economic losses in the dairy sector that directly affect total milk yield and number of calves born per annum (Santos et al., 2004; Dobson et al., 2007; Lee and Kim, 2007). Furthermore, the reproductive wastage associated with infertility is the primary reason for involuntary culling (Kelly and Whitaker, 2001; Mohammadi and Sedighi, 2009) and threatens the financial efficacy of dairy herds (Esslemont et al., 2001). Thus, the causative grounds of bovine infertility need to be addressed to enable modern dairy farmers to select reproductively viable cows with high milk yield ensuring respectable profit margins without compromising animal welfare (Roche, 2006; Dobson et al., 2007).
1.2. Farm Management Strategies and Reproductive Efficiency

Implementation of stringent farm management techniques is the primary route to protect against bovine fertility problems, while ensuring animal welfare (De Kruif., 1978; Stevenson., 2001). Farm management techniques involve the monitoring of bovine environmental factors (Section 1.2.1), prompt treatment of pathologies (Section 1.2.2), assessing hormone concentrations (Section 1.2.3), accurate detection of estrous and maintenance of general animal welfare (Section 1.2.4), eliminating genetic defects (Section 1.2.5) and introducing novel feeding regimes (Section 1.2.6). A collective balance between these farm management techniques may potentially reverse the trend of poor fertility rates.

1.2.1. Environmental Factors and Infertility

Temperature and humidity are important regulators of bovine fertility, where conditions of high climatic temperature and humidity reduce the fertilization rate and increase embryonic loss (Stott and Williams., 1962). Also, cows bred in autumn and winter, have poorer fertilization rates compared to animals bred in spring, and this is most likely due to reduced hours of daylight and impaired signs of estrous behaviour during the winter months (Mercier and Salisbury., 1947; Hussain Shah et al., 1989). Analysis of these studies (Stott and Williams., 1962; Mercier and Salisbury., 1947; Hussain Shah et al., 1989), reveal that springtime is the most desirable season for improved fertility. Bovine housing conditions can also affect fertility rates (De Kruif., 1978), and a housing system that enables freedom of movement which allows the animal to show increased signs of estrous is necessary for breeding (Kiddy, 1977). Collick et al., (1989) evaluated the effects of lameness on cow fertility and concluded that the interval between calving and conception was 14 days longer for lame cows compared to control cows (cows without lameness) and the pregnancy rate for lame cows was 46% while control animals displayed a higher pregnancy rate of 56%. In addition, lame cows required on average 2.14 services for conception compared to 1.72 services for control cows (Collick et al., 1989). The poor fertility arises mainly from poor signs of estrous behaviour in lame cows (Walker et al., 2008).
The higher incidence of lameness is correlated with a higher incidence of infertility, and correct housing conditions and farm management practice must be employed to prevent post-partum lameness. Another environmental aspect is hygiene quality at calving, where poor hygiene conditions result in a 5-10% decline in pregnancy rate compared to control, as well as the possible introduction of reproductive tract abnormalities that can impair fertility (Bell and Roberts., 2007).

1.2.2 Pathological Factors and Infertility

Several pathologies have been implicated in bovine infertility. These include bacterial, viral, fungal mycoplasmal and protozoan infection. Early embryonic loss (EEL) encompasses embryonic loss from the period from conception to the end of the differentiation stage (approx. 45 days post fertilization) (Ayalon., 1978). Campylobacter fetus is the causative bacterial agent of vibrio fetus, and the bacterium can invade uterine tissue resulting in endometritis, which leads to early embryonic loss, while abortion after 4 months is sometimes observed (Hoffer., 1981). Bovine viral diarrhoea virus (BVDV), transmitted transplacentally to the developing embryo, can also induce EEL (Kelling, 2007). Trichomonas fetus is a venereally transmitted flagellated protozoan (Bicknell et al., 1994), and infection has also been implicated in EEL (Morgan., 1947). Interestingly, infection with trichomonas fetus or BVDV can precede both EEL and abortion (Morgan., 1947; Graham et al., 2009). Fungal infections can also introduce infertility into dairy herds. Aspergillus fumigate is fungal infection responsible for bovine aspergillosis and spreads in the blood to the placenta and causes placentitis through necrosis and inflammation of the transient organ. Aspergillus fumigate can go on to infect the developing fetus via natural fungal expansion from placenta into amniotic fluid (Miller and Quinn., 1975). Neospora caninum is a unicellular parasite that is transmitted transplacentally from infected females to developing foetus (Dubey and Lindsay, 1996). Infected foetuses may die in utero, be resorbed, autolyzed, mummified, stillborn or born alive but with developmental defects (Dubey and Lindsay, 1996).
Mycoplasma isolated from semen, vaginal and endometrial discharge, oviduct, aborted foetuses, inflammatory udder secretions, nasal discharges and pneumonic lungs can lead to poor conception rates and abortion (Ashfar et al., 1967). A 10 year study by Kirkbride (1992) demonstrated that bacteria caused 14.49% of abortions, viruses preceded 10.57% of abortions, while fungi caused 5.31% abortions, thus highlighting the prevalence of infection associated abortions.

1.2.3. Hormonal Imbalance and Infertility

Hormones of reproduction are regimentally controlled via feedback loops that results in normal estrous cycles and fertilization. Asynchrony between progesterone, luteinizing hormone and estrogen levels is another factor of cow infertility. In non-fertile cows, progesterone levels are lower after ovulation, the estrogen:progesterone ratio is elevated (approx. day 3 and 6), and the LH surge prior to ovulation is lower compared to normal fertile cows (Erb et al., 1976; Maurer and Echternkamp., 1982). There is a strong correlation between reduced fertility in dairy cows with lower progesterone concentration in systemic circulation during the preceding oestrous than animals that conceived successfully after one insemination (Folman et al., 1972; Meisterling and Dailey., 1987).
1.2.4. Accurate Estrous Detection and General Animal Welfare

Accurate detection of estrous is an important factor of proper farm management to help limit bovine infertility and serves to limit artificial insemination (AI) wastage, support successful fertilization and subsequent pregnancy rates (Heersche and Nebel., 1994) as well as providing the synchronization of a compact herd calving pattern (Diskin and Sreenan., 2000). Timing of insemination after parturition is also an important factor of bovine infertility and cows inseminated less than 50 days after parturition show reduced fertilization rates compared to cows inseminated ~100 days after calving (Shannon et al., 1952; Trimberger., 1954). Additionally the age, breed and herd size are contributory factors of bovine reproductive performance (Kidder et al., 1954; Hare et al., 2006), and must be carefully assessed by the producer. Pregnancy failure increases with each parity (Lee and Kim., 2007) and Hare et al., (2006) demonstrated that pregnancy rates drop after first calving, while animals more than 7 years old will have a lower fertilization rate compared to younger cows. Breed of sire does not affect fertilization rates significantly, where rates remain in the region of 80-90% for Guernseys and Holstein-friesians (Kidder et al., 1954).

1.2.5. Genetic Anomalies and Infertility

Chromosomal anomalies, individual gene mutations errors at meiosis or fertilization or environmental factors directly affecting developing embryos can all impact negatively on embryo survival (Wilmut et al., 1986; Van Raden and Miller., 2006). The process of natural selection favours spontaneous abortion of defective embryos, so females can devote more resources to producing healthy offspring. Deficiency of uridine monophosphate synthase (DUMPS) is an example of a recessive defect that causes embryonic death between d40 and d50 post fertilization (Shanks and Robinson., 1989). It has been estimated that chromosomal abnormalities occur at a rate of 7.5% in cattle leading to embryonic loss, abortions or stillbirths (Wilmut et al., 1986). Eradication of these genetic abnormalities through selective breeding, can reduce embryonic mortality and help improve fertility (Van Raden and Miller., 2006).
1.2.6. Nutritional Management and Infertility

Appropriate nutritional management is a critical component of normal bovine oestrous cyclicity and successful pregnancy rates (Hess et al., 2005; Roche., 2006). Body Conditioning Score (BCS) is a standard management tool used by dairy professionals to quantify the energy reserves of cows (Santos., 2001). BCS of scoring system applied that gives an indication of bovine fat disposition and ranges from a score of 1.00 to 5.00. Animals with a BCS of 1.00 are severely under conditioned, whilst animals with a BCS of 5.00 are over conditioned (Wildman et al., 1982). A BCS of 3.00 is desirable for calving, but during the breeding season, early lactation and drying off a BCS of 2.75 is optimal (Mulligan et al., 2006). As a consequence, proper farm management techniques that regimentally control bovine BCS is a major contributory factor to improved fertility. Following parturition and during early lactation, there is a shift in the energy requirements of high producing cows, where the energy necessary for milk production and normal physiological function exceeds the available energy intake from feed (Butler and Smith., 1987; Jorritsma et al., 2003). The resulting relationship between increased energy utilization and dietary energy intake culminates to a state of negative energy balance (NEB) that may persist for several weeks postpartum (Butler and Smith., 1989; Jorritsma et al., 2004). Prolonged NEB predisposes the dairy cow to fertility problems (Mayne et al., 2002), where it can have adverse effects on ovarian activity and subsequent conception rates, by delaying the interval between parturition and first ovulation (O’Callaghan and Boland., 1999; de Vries and Veerkamp., 2000).
A feeding regime that contains supplemental fats is the preferred route for increasing dietary energy density, thereby alleviating NEB in the early postpartum cow and helps maintain efficient reproductive performance. Mattos et al., (2000), have illustrated that polyunsaturated fatty acids (PUFAs) supplementation in bovine meal (Menhaden meal) can improve reproductive efficiency, independent of its role at alleviating NEB, by regulating PG synthesis during the oestrous cycle and pregnancy to maintain efficient reproductive performance. One possible reason for this is that PUFAs can increase the bioavailability of fatty acid precursors for eicosanoid synthesis. These local lipid molecules are important mediators in many aspects of ruminant reproduction and can have luteolytic or luteotropic effects, thus are key components of bovine reproductive efficiency (see section 1.12). Mineral supplementation may also be necessary to ensure efficient reproductive farm management. For example, selenium deficiency is associated with an increased incidence of retained foetal membranes at parturition (Julien et al., 1976).
1.3. Bovine Oestrous Cycle

The bovine oestrous cycle consists of an orderly sequence of events, occurring over a period of 17 – 24 days, unless interrupted by pregnancy (Olds and Seath., 1951; Rajakoski., 1960; Noseir., 2003; Lucy., 2007). Cows are spontaneous polyestrous ovulators that ovulate at a precise time during the oestrous cycle, regardless of mating (Frandsen et al., 2003). The coordinated interplay of hormones and reproductive tissue ensures the generation of one fertilizable oocyte per cycle. In the event of a successful pregnancy, specific modifications to the oestrous cycle predominate to maintain a viable conceptus until term. This cycle is rigidly controlled through synchronized communication between the hypothalamus, pituitary, ovary and uterus and is composed of 4 stages (Fig. 1): estrous (day 0), followed by the post-ovulatory or metestrous phase (day 1 to 3), that gives rise to the luteal or diestrous phase (day 4 to 17) and finally the follicular or proestrous phase (day 18 to 20).

Figure 1: Hormonal profile of bovine oestrous cycle
1.3.1. Proestrous

During proestrous (days 18-20), estradiol levels begin to increase and this in combination with declining levels of progesterone promotes FSH secretion from the anterior pituitary (Fig. 2). (Wettemann et al., 1972; Rajamahendran et al., 1979; Nanda et al., 1988). In addition, elevated activin and IGF secretion from the granulosa cells of the dominant follicle act in an autocrine or paracrine manner to increase FSH secretion and upregulate FSH-receptors in granulosa cells to drive follicular maturation (Findlay and Drummond., 1999; Zhou et al., 1997). The binding of FSH to its receptor promotes aromatase activity to increase estrogen production by follicular cells (Section 1.5.1.3). Increasing levels of estradiol also triggers the release of PGF$_{2\alpha}$ from uterine epithelial cells which in cattle help regulate oestrous cycle length, (Section 1.13.3). PGF$_{2\alpha}$ synthesized by uterine epithelial cells is transported from the uterine vein into the ovarian artery, and back to the ovary to induce CL regression (McCracken et al., 1999). As a consequence of CL regression, progesterone levels fall and the oestrous cycle recommences. At this initial stage of follicular development, a cohort of about 24 follicles are recruited for folliculogenesis and begin to grow in a wave like pattern (Ireland et al., 2000).
1.3.2. Estrous

The estrous stage of the cycle lasts 12-18 hours and is defined as day 0 of the bovine oestrous cycle (Dransfield et al., 1998; Xu et al., 1998) with ovulation occurring approximately 14 hours after estrous (Nalbondov and Casida., 1942). Estrous can be identified initially by inspection where the cow exhibits ‘heat’ or sexual desire and acceptance of the male. Thin vaginal and cervical secretions are also apparent at this stage (Diskin and Sreenan., 2000). Steroid concentrations in ovarian venous blood and peripheral circulation are altered during estrous, with estrogen concentrations rising to > 5 pg/mL and progesterone levels dropping to < 1ng/mL (Nalbondov and Casida., 1942; Nosier, 2003). The dominant follicle selected for ovulation at estrous reaches a diameter of ~10mm and is identifiable on the ovary only 48 hours before estrous (DuFour et al., 1972). The high estradiol concentration (Bassett and Zeleznik., 1990), coupled with an increase in intrafollicular inhibin and follistatin prevents further FSH-secretion, ensuring the maturation of only one follicle (Findlay., 1993). Follicle growth after selection switches from FSH to LH dependence (Campbell et al., 1999) and the increased estradiol levels initiate the preovulatory anterior pituitary LH surge, which bind to LH-sensitive receptors on thecal and granulosa cells of the dominant follicle (Rao., 2001). LH luteinizes these cells and increases PG production to induce follicle wall rupture for ovulation (Section 1.12.1). The elevated levels of estradiol during estrous characterize the proliferative phase of the endometrial cycle and stimulate hypertrophy and hyperplasia of endometrial stromal, epithelial and endothelial cells (Reynolds et al., 1998; Gambino et al., 2002). Luminal epithelial cells change from a thin, narrow and short morphology (1-2mm thick) to columnar and grow outward towards the lumen (3-4mm) (Haféz and Haféz., 2000). Glandular epithelial cells become mitotic and tortuous and migrate towards the luminal epithelium in preparation for the secretory phase. Stromal cells proliferate and actively secrete collagen which acts as a provisional extracellular matrix for luminal epithelium (Williams et al., 1995).
1.3.3. Metestrous

Metestrous (day 1-3), occurring after estrous, is characterized by luteinization of the thecal and granulosa cells of the ovulated dominant follicle as evidenced by cellular hyperplasia, hypertrophy and a return to progesterone secretion (Henderson and Franchimont., 1983). The luteinized follicle forms the corpus luteum (CL) and there is a shift in steroid hormone synthesis from estradiol towards progesterone (Rekawiecki et al., 2008). Progesterone is the main secretory hormone of the CL and there is a direct correlation between size of CL and progesterone production, where the small CL observed at metestrous produces less progesterone compared to the larger CL in the latter stages of the cycle (Wise et al., 1982). During metestrous the CL can also synthesize estradiol in the region of 15pg/mL, and since estradiol levels in systemic circulation remain low during the luteal phase, the estradiol produced by the CL presumably acts locally (Pitzel et al., 1990) and is implicated in maintenance of the CL during the luteal phase (Shibaya et al., 2007).

1.3.4. Diestrous

Metestrous progresses to the diestrous stage (days 4-17), which is the longest phase of the cycle and constitutes the ovarian luteal phase. Estrogen levels remain relatively low, while progesterone secretion increases as the CL enlarges. As the diestrous phase continues, the peripheral plasma concentration of progesterone levels off (4.0 ± 0.3ng/mL) between days 12-15 days post estrous (Noseir., 2003). Progesterone suppresses further release of hypothalamic GnRH (Skinner et al., 1998) and although follicular waves still occur during the diestrous stage, the low levels of GnRH suppress LH secretion from the anterior pituitary, thus preventing ovulation of another oocyte (Skinner et al., 1998; Burger et al., 2002; Burger et al., 2004). The non-ciliated, secretory cells of the endometrium are slender, columnar and tall with numerous microvilli and contain many secretory vacuoles, some of which empty their contents into the glandular epithelium (Kojima and Selander., 1970).
In the early secretory phase glycogen vacuoles and some lipid droplets emerge at the basolateral cytoplasm of glandular epithelium and nuclei are displaced to the centre of the cell (Guyton and Hall., 1996). Giant mitochondria, rough endoplasmic reticulae, Golgi bodies and secretory vesicles develop in the glandular epithelia (Williams et al., 1995). In the stromal cells, nuclei enlarge and the packing density of the fibroblasts increases due mainly to the enlarged glandular volume (Williams et al., 1995). Glycogen, mucin and other glycoproteins are released into the lumen of the uterus by apocrine and exocrine secretion at diestrous. The changes occurring in the endometrium during the diestrous stage generates the desirable conditions for implantation in the event of a successful fertilization.

1.3.5. Establishment of Pregnancy

After a successful fertilization, communication between the uterus and the conceptus, at days 16-18, is necessary for the establishment and maintenance of pregnancy (Thatcher et al., 1984). The maternal endometrial surface is composed of tall, pseudostratified columnar cells at day 17 post-fertilization (King et al., 1981). As pregnancy continues (days 20 and 23), maternal giant cells appear that are larger than adjacent cells, with a large cytoplasm volume and are usually multi-nucleated (King et al., 1981). By days 19-20 of pregnancy trophoblastic attachment begins and there is a definite site of association between the trophoblast and the endometrial epithelium (King et al., 1981). Two cell-types emerge on the surface of the trophoblast; mononuclear columnar epithelial cells containing smooth endoplasmic reticulae with large mitochondria and large, pale, multi-nucleated giant cells (mGC) scattered between the columnar cells that arise from the trophoblast. mGCs possess many endoplasmic reticulae and extensive Golgi bodies and synthesize and secrete placental lactogens into the maternal and foetal circulation (Igwebuie., 2004). Placental lactogens have lactogenic and somatogenic properties in the maternal circulation and are involved in the regulation of foetal growth (Flint et a., 1979; Byatt et al., 1992).
The migration of mGCs across the junction and fusion with the maternal epithelium form a non-invasive point of conceptus attachment (Wooding and Wathes, 1980; King et al., 1981). Each discrete area of attachment is known as a placentome, and collectively gives rise to the synepitheliochorial placenta in ruminants (MacIntyre et al., 2002). The foetal portion is known as the cotyledon and the maternal portion is called the caruncle. Bovine interferon-tau (bIFN-τ) secreted by the developing embryo around day 15-24 of pregnancy (Thatcher et al., 1984; Roberts et al., 1990) reduces uterine secretion of luteolytic PGF$_{2α}$ (Meyer et al., 1995) and promotes synthesis of luteoprotective PGE$_2$ by endometrial stromal cells (Asselin et al., 1997; Asselin et al., 1998; Parent et al., 2002) to maintain pregnancy.
1.4. Hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is a highly complex neuroendocrine system that regulates the reproductive process (Fig. 2). The axis is regimentally controlled by a series of feedback mechanisms that maintain hormonal balance and ensure production of viable gametes and subsequent survival of the species (Haféz and Haféz., 2000; Frandson et al., 2003; Reece., 2009).

Figure 2: Hypothalamic-Pituitary-Gonadal Axis; adapted from Downey., (1980).
1.4.1. Hypothalamus and Anterior Pituitary

The hypothalamus, located inferior to the thalamus at the centre of the brain, is the main orchestrator of the female reproductive cycle. Specialized cells of the preoptic area and adjacent sites in the rostral region of the hypothalamus synthesize and secrete gonadotropin releasing hormone (GnRH), a decapeptide that promotes the secretion of gonadotropins from the anterior pituitary and is thus the main orchestrator of the reproductive cascade (Haféz and Haféz., 2000; Frandson et al., 2009; Reece., 2009). GnRH is synthesized as a preprohormone that is enzymatically cleaved to generate the active hormone. The intrinsic pulsatile secretion of GnRH from nerve terminals into the hypophyseal portal system stimulates basophilic gonadotrope cells in the medullary area of the anterior pituitary (Haféz and Haféz., 2000; Frandson et al., 2009; Reece., 2009). The basophilic gonadotrope cells produce and secrete the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Factors known to activate GnRH secretion include pheromones (Ichimaru et al., 1999), steroid hormones (Scott et al., 2000), stress (Dobson et al., 2003), light (Chappell et al., 2003) and glucose (Ohkura et al., 2004). Estrogen is a potent regulator of GnRH secretion than can exert either positive or negative feedback responses that are time and dose dependent, as reviewed by Herbison., (1998). Low levels of estrogen, obvious during the luteal phase, inhibits GnRH mRNA expression, while elevated estrogen levels, observed during the follicular phase, increase GnRH mRNA expression and promote a positive feedback loop on the hypothalamic secretion of GnRH (Gore and Roberts., 1995; Petersen et al., 1996).
1.4.2. Gonadotropin Hormones

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropin hormones that belong to a family of glycoproteins, which also includes thyroid stimulating hormone (TSH) and hCG. These are heterodimeric proteins that share a common α-subunit but differ in their β-subunits (Davis, 1994). FSH and LH are synthesized in response to pulsatile hypothalamic GnRH stimulation (see Section 1.4.1) and in vitro, GnRH upregulates the common α-subunit and FSHβ- and LHβ-subunit mRNA levels as well as increasing transcription rate and protein synthesis (Burger et al., 2002; Burger et al., 2004). The anterior pituitary responds to low frequency pulses of GnRH by increasing FSH synthesis and release, directed primarily via GnRH-induced upregulation of FSHβ mRNA (Burger et al., 2002; Burger et al., 2004). Serum FSH levels peak twice or three times during an estrous cycle, resulting in a corresponding number of follicular waves (Section 1.5.1.1); firstly, two days prior to the preovulatory LH surge, approaching the end of the luteal phase (Akbar et al., 1974), and again one day before the emergence of the growth of a cohort of follicles at the onset of a follicular wave (Adams et al., 1992) when estradiol levels begin to increase (Fig. 1). High frequency hypothalamic GnRH pulses promote LH secretion from anterior pituitary gonadotropes (Burger et al., 2002; Burger et al., 2004). Levels of LH are highest two days before ovulation in the cow, with approximately 90% of pituitary LH released during estrous, ovulation and early luteal growth (Hackett and Hafs., 1969). Estradiol increases the number of LH-receptors on the follicular cells and promotes a positive feedback loop responsible for the elevated GnRH-dependent LH secretion that precedes ovulation (Richards et al., 1976).
1.4.3. Gonadotropin Receptors

Gonadotropin hormones secreted from the anterior pituitary travel in the blood and bind to specific receptors. Both FSH and LH receptors are G-protein coupled receptors (GPCRs) with 7 transmembrane domains (Simoni et al., 1997). The activated hormone-receptor complex binds to the intracellular G-protein causing the Gα-subunit to dissociate from the Gβγ dimer. The liberated Gα-subunit and Gβγ-subunits are then able to initiate intracellular responses (Deupi and Koblika., 2007). The FSH-receptor complex works exclusively via the cAMP-dependent protein kinase A (PKA) signalling system (Simoni et al., 1997), while LH acts via cAMP-dependent PKA or PKC to induce its effects (Dufau., 1998). Although, LH and FSH are gonadotropic hormones, their receptors have also been identified in extragonadal bovine reproductive. LH receptors are abundant in the endometrium during the luteal phase (Freidman et al., 1995; Fields and Shemesh., 2004) and FSH receptor number increases in the cervix at estrous (Mizrachi and Shemesh., 1999).
1.5. Bovine Reproductive Tract

The reproductive tract of the cow lies in the pelvic cavity and is made up of the primary reproductive organs (ovaries) and secondary reproductive organs, which are a series of tubes that are essential for ovum and sperm transport, fertilization, early embryo development and foetal growth (Haféz and Haféz., 2000).

![Figure 3: Bovine Reproductive Tract, adapted from Roberts., (1986)](image)
1.5.1 The Ovary

The primary reproductive organs consist of a pair of ovaries located at either side of the abdominal cavity and have two central roles; to produce viable oocytes for fertilization and synthesize steroid hormones to maintain reproductive performance (Guyton and Hall., 1996). Each ovary is between 1 and 4cm in length with a diameter of 1 to 3cm, but the size and shape can vary depending on stage of the estrous cycle (Haféz and Haféz., 2000; Borisenkov and Vakhnina., 2004; Frandson et al., 2003; Reece., 2009). Primordial follicles established in the ovary during foetal development consist of a single oocyte surrounded by pre-granulosa cells (Erickson., 1966) arrested in prophase I of meiosis (Fortune., 1994). Follicular growth recommences at puberty and involves the maturation of a sequence of primordial follicles, ovulation of one follicle and subsequent regression of the other subordinate follicles (Fortune., 1994). Follicular maturation, is not however a continuous process and follows a pattern of recruitment of primordial follicles, selection of follicles to continue growing and the eventual dominance of one follicle that ovulates, releasing the mature oocyte (Section 1.5.1.1.). Less than ~0.1% of follicles are destined to ovulate, while greater than 99% undergo atresia at different phases of development (Lucy., 2007). Atresia of follicles usually begins with apoptosis of granulosa cells (van Wezel et al., 1999), that progresses to complete abolition of the follicle. The mature dominant follicle selected for ovulation, contains a primary oocyte, surrounded by a layer of granulosa cells containing a fluid-filled antrum and a layer of a richly vascularized thecal cells (Fig. 4) (Reece., 2009). The details of the fate of a developing follicle are discussed in the next section.
Ovarian follicular growth and development is a highly selective, delicate process that occurs in a series of waves in cattle and ensures the ovulation of one oocyte per oestrous cycle (Lucy., 2007). Rajakoski (1960) was the first to outline a 2-wave concept of follicular turnover for each bovine oestrous cycle. Later studies (Sirosis and Fortune, 1988; Savio et al., 1988; Ginther et al., 1989; Lucy et al., 1992; Noseir, 2003) have reported that follicular growth can occur in a 2 or 3 wave pattern per cycle. A surge of follicular growth occurs on day 0, (24 hours prior to ovulation) and on day 10 of the oestrous cycle in a 2-wave pattern of maturation and on days 0, 9 and 16 during a 3-wave pattern (Ginther et al., 1989). At the onset of a follicular wave, a cohort of about 24 follicles (2-5mm in diameter) is recruited to proceed with maturation and continue growing (Ireland et al., 2000). One follicle is selected as the dominant follicle at approximately 3 days after the beginning of a wave, and switches its dependence from FSH towards LH-dependence (Campbell et al., 1999). Follicles that are recruited at the beginning of a follicular wave are FSH-dependent, but as the wave propagates, the dominant follicle acquires LH-receptors in the granulosa and thecal cells and becomes sensitive to LH stimulation (reviewed by Bao and Garverick., 1998; Mihm et al., 2006).
In cattle, numerous factors are involved in the selection of the future dominant ovulatory follicle, including increased concentrations of intrafollicular IGF-1 and estradiol and upregulation of LH receptors (Fortune et al., 2001; Beg and Ginther., 2006). As the dominant follicle matures, the high estradiol concentration (Bassett and Zeleznik., 1990), coupled with a concomitant increase in intrafollicular inhibin and follistatin (Section 1.5.1.4.) initiate a feedback loop that prevents further FSH-secretion, ensuring maturation of only one follicle (Findlay., 1993). The dominant follicle selected for ovulation has a diameter 1-2mm greater than the next largest follicle and begins to grow until it reaches ~10mm when it is identifiable on the ovary 48 hours before estrous (DuFour et al., 1972). At its maximum size when estradiol plasma concentrations are >5pg/mL and progesterone plasma concentrations are <1ng/mL, ovulation occurs (Nalbondov and Casida, 1942; Nosier, 2003).

1.5.1.2. Prostaglandins and Luteal Function

After ovulation, a transient endocrine gland, the corpus luteum (CL) gland formed from the ovulated follicle that displays active angiogenesis and steroidogenesis (Niswender et al., 1976; Niswender et al., 2000). The CL is active for 17-18 days in the non-pregnant cow (Miyamoto et al., 2009) and its primary function is to secrete progesterone, which is essential for preparing the endometrium for implantation and subsequent maintenance of pregnancy (Haféz and Haféz., 2000; Frandson et al., 2009; Reece., 2009). The CL is composed of luteinized granulosa and thecal cells as evidenced by cellular hyperplasia and hypertrophy and a consequential increase in progesterone secretion (Henderson and Franchimont., 1983; Frandson et al., 2009) (Fig. 5).
A mechanism for CL regression evolved to enable the development of a new ovulatory follicle and subsequent resumption of the oestrous cycle within a short period of 2-4 weeks (Poyser., 1995) in the absence of a viable embryo. In the event of a successful fertilization the CL persists for approximately 200 days, producing sufficient progesterone to maintain desirable uterine conditions for embryonic/foetal development (Estergreen et al., 1967; Chew et al., 1979; Ball and Peters., 2004). As pregnancy progresses in the cow, the secretory capacity of the CL diminishes and by mid-gestation the bovine placental cells (foetal cotyledon and maternal caruncle cells) begin to synthesize progesterone thus maintaining the foetus until term (Shemesh et al., 1983). The ruminant luteolytic process is tightly regulated and ensures the demise of the CL at the end of the normal non-pregnant oestrous cycle and its maintenance at the establishment of pregnancy (Okuda et al., 2002). Prostaglandins (PGs) of uterine/foetal origin are key regulators in the fate of the CL; essentially PGE₂ is a luteotropic factor that prevents CL regression while PGF₂α is a luteolytic factor that promotes regression (Section 1.12.2), however, failure of the system may lead to early embryonic loss which is preceded by luteal regression (Kastelic et al., 1991). Untimely, CL regression can occur in infertile cows, due to the inability of the embryo to prevent the secretion of luteolytic PGF₂α in response to an oxytocin challenge (LaFrance et al., 1989). In heifers bearing normal viable embryos, an oxytocin challenge does not elevate PGF₂α secretion (LaFrance and Goff., 1985).
1.5.1.3. Ovarian Steroidogenesis

Ovarian follicles at different stages of development can produce progesterone, estrogens and small quantities of androgens in specific follicular cells (Ball and Peters., 2004). Cholesterol is the precursor to all steroids and once mobilized to the mitochondria can continue along the steroidogenic pathway (Hu et al., 2010) (Fig. 6).

![Diagram of Ovarian Steroidogenesis in Thecal and Granulosa Follicular Cells](image)

Figure 6: Schematic of Ovarian Steroidogenesis in Thecal and Granulosa Follicular Cells
The initial step in ovarian steroidogenesis is the conversion of cholesterol to pregnenolone via the action of cholesterol side chain cleavage enzyme, P450\textsubscript{scC}, which is the rate limiting step in steroid hormone synthesis (Irving-Rodgers et al., 2003). P450\textsubscript{scC} mRNA is localized in both thecal and granulosa cells, with the level of its expression increasing as the follicle matures (Irving-Rodgers et al., 2003). Once synthesized, pregnenolone is converted to progesterone under the influence of 3\(\beta\)-HSD (Bao et al., 1998; Hu et al., 2010). 3\(\beta\)-HSD is a non-P450 enzyme located on mitochondria and smooth endoplasmic reticulum, of which there are 2 isomers, type 1 and type 2 (Sanderson, 2006). As a follicular wave develops, 3\(\beta\)-HSD mRNA expression increases in follicular thecal and granulosa cells, with the dominant follicle displaying increased 3\(\beta\)-HSD mRNA expression compared to subordinate, atretic follicles (Bao et al., 1998). Progesterone and pregnenolone act as precursor molecules for the synthesis of all the other steroid hormones. Elevated LH stimulation, near estrous, induces increased cytochrome 17\(\alpha\)-hydroxylase P450 (P450\textsubscript{17\(\alpha\)}) activity exclusively in thecal cells (Arlotto et al., 1996; Bao et al., 1998) which governs the third step of ovarian steroid synthesis, and converts progesterone to androstenedione (Miller, 1988). Androstenedione can then be reduced by 17-\(\beta\)-HSD yielding testosterone. Androgen production is the rate-limiting step regulating the initial increase in estradiol biosynthesis in small follicles (Bogovich and Richards., 1984). Androgens and testosterone are transferred to granulosa cells where they are rapidly converted to estradiol by the actions of types 1 and 7 and aromatase (P450\textsubscript{arom}) (Mindnich et al., 2004). P450\textsubscript{arom} mRNA is found exclusively in granulosa cells (Bao et al., 1998). Follicle stimulating hormone (FSH), acting via the cAMP second messenger system, and low quantities of androgens maintain aromatase activity in granulosa cells and subsequent estradiol production at estrous (Stocco., 2008). After ovulation and a subsequent drop in gonadotropin concentration, 17\(\beta\)-HSD activity declines and estradiol biosynthesis is reduced (Wahawisan and Gorell., 1980). The ovulated follicle is now luteinized to form the corpus luteum (see Section 1.5.1.2) which predominately synthesizes progesterone due to increased P450\textsubscript{scC} expression and 3\(\beta\)-HSD activity. Progesterone and estradiol are central to the synthesis and secretion of endometrial PGs and are discussed in Section 1.13.
1.5.1.4. Ovarian Regulatory Peptides

Proteins synthesized and secreted by ovarian follicles at different stages of follicular growth initiate feedback loops that are important in the control of reproductive hormone synthesis and release. Inhibin, follistatin and activin are three unique proteins synthesized by ovarian granulosa cells that help maintain reproductive function (Bicsak et al., 1986; Findlay, 1993; DePaolo et al., 1991). IGFs also play critical roles in follicular cell proliferation and steroid hormone synthesis (Spicer et al., 1993). Inhibin and follistatin are anti-FSH proteins secreted by granulosa cells into the follicular fluid and the bloodstream that act at the pituitary level to prevent FSH secretion (Ling et al., 1985; Miyamoto et al., 1985; Robertson et al., 1985; Robertson et al., 1987; Ueno et al., 1987). Inhibin secretion by granulosa cells increases as the dominant follicle emerges and the effective signalling between inhibin (Henderson and Franchimont, 1983) and estradiol (Bassett and Zeleznik, 1990) establishes a negative feedback loop on GnRH-induced FSH secretion that abolishes the growth and maturation of subordinate follicles (Findlay, 1993). Progesterone prevents inhibin secretion from granulosa cells (Henderson and Franchimont, 1983), while the initial trigger for inhibin synthesis has been characterized as FSH, as inhibin production coincides with the first pulse of FSH secretion (McNeilly et al., 1989). Follistatin was first identified due to its ability to suppress FSH (Robertson et al., 1987; Ueno et al., 1987) and expression of the follistatin protein in bovine ovarian follicles is highest in follicular granulosa cells (Singh and Adams., 1998). Follistatin favours luteinization because it suppresses FSH secretion thereby inhibiting estradiol synthesis in granulosa cells and maintains progesterone synthesis in luteinized cells (Findlay, 1993).
Activin was first isolated and characterized by Vale et al., (1986) from porcine follicular fluid and has been shown to stimulate pituitary FSH secretion and overcome the inhibitory effects of inhibin (Vale et al., 1986). Activin is involved in the promotion and maintenance of folliculogenesis and in the presence of FSH, triggers aromatase activity to increase estradiol synthesis (Findlay, 1993) and upregulates LH-binding sites in granulosa cells (Sugino et al., 1988) important for ovulation. Insulin-like growth factors (IGFs) are peptides structurally related to proinsulin, which in the ovary are produced by granulosa cells under the control of FSH and estradiol (Spicer et al., 1993). IGFs work in synergy with FSH to drive proliferation and steroidogenesis of follicular cells (Spicer et al., 1993) by upregulating P450scc mRNA levels (Spicer and Aad., 2007; Paul et al., 2010). Insulin-like growth factor binding proteins (IGFBPs) are 25-45kDa high affinity carrier proteins that bind IGFs, thereby prolonging their half-life, while blocking their biological action (Spicer and Echternkamp, 1995). There are six members of the IGFBP family that are structurally related, with high but different binding affinities for IGF-I and IGF-II (Webb et al., 1999). IGFBP-2, -4 and -5 levels are low in estradiol-active follicles compared to atretic follicles (Spicer and Echternkamp, 1995; Gérard and Monget, 1998). This confirms the dual role of IGFBPs to reduce free IGF concentration in non-ovulatory follicle while promoting increased free IGF concentration in ovulatory follicles, enabling IGF to carry out its topic actions.

1.5.2. The Oviduct

The oviduct is in close apposition to the ovary and is involved in ovum ‘pick-up’; transport and nourishment of the mature oocytes; aiding in sperm transport and providing the correct environment for fertilization (Pauerstein and Eddy, 1979). The oviduct is connected to the uterus at the utero-tubal junction.
1.5.3. The Uterus

The bovine uterus is a hollow, thick-walled, muscular reproductive organ composed of a uterine body of approximately 2.5cm that bifurcates to give rise to two specialized uterine horns each about 23cm in length that are separated from each other by a septum (Haféz and Haféz., 2000). The uterine body connects the cervix to the uterine horns and is suspended by the broad ligament in a coiled manner (Drennan and MacPhearson., 1966). Uterine horns contain 3 muscle layers (Section 1.5.3.1) and a heavy network of blood vessels (Section 1.5.3.3). The uterine horns are bound caudally by the intercornual ligament and the organ itself is attached to the abdominal and pelvic walls by the broad ligament (Hafez and Hafez, 2000). After fertilization in the oviduct, the embryo travels to one of the uterine horns, where it matures during pregnancy (Melton et al., 1951). At the end of gestation, the uterus is active in the expulsion of the foetus at parturition (Haféz and Haféz., 2000).
1.5.3.1. The Uterine Wall

The uterine wall is composed of 3 layers (Fig. 7); the outer perimetrium, the middle myometrium and inner endometrium (Burrow and Humphrey., 1994). The uterus is covered externally by the perimetrium which arises from the peritoneum and is made up of simple squamous epithelium and areolar connective tissue that wraps around the uterus anteriorly and posteriorly (Ross et al., 1995). The myometrium is the middle fibromuscular layer and forms most of the uterine wall. It is divided into 3 distinctive strata, a middle muscle layer that contains numerous large blood vessels, nerves and lymphatics with smooth muscle cells arranged in a circular pattern (Frandsen et al., 2003). The inner and outer strata smooth muscle cells are orientated in a longitudinal manner (Lambert et al., 1990). The specialized smooth muscle cells contain actin and myosin filaments (Webb., 2003) and are coupled to each other via gap junctions that enable immediate electrical communication (Sims et al., 1982). These properties of smooth muscle cells prove vital for uterine contractility necessary for gamete transport and parturition. Uterine contractility is under hormonal control, where increased contractility is observed at estrous and parturition and uterine quiescence occurs during the luteal phase of the bovine oestrous cycle and pregnancy (Frandsen et al., 2003).

Figure 7: Uterine Wall
1.5.3.2. The Endometrium

The endometrium comprises the innermost layer of the uterine wall and contains a layer of connective tissue (endometrial stroma) that is lined by a simple columnar epithelium of secretory and ciliated cells. It is a highly responsive glandular structure and following hormonal and biochemical stimuli, secretes luminal fluid and undergoes morphological changes that give rise to a suitable environment for gamete transport (Aguilar and Reyley., 2005) and neonatal development after a successful fertilization (Moffatt et al., 1987; Burrows and Humphrey., 1994; Gray et al., 2001). Fibroblastic stromal cells make up the majority of the endometrium, offer a rich blood supply, contain lymphatic spaces and also provide anchorage for luminal and glandular epithelium (Williams et al., 1995). Raised ridges of the endometrial epithelium contain aglandular caruncles that provide a site of attachment between the maternal and foetal membranes during placentation (Reece., 2009). Intercaruncular regions between the caruncles are glandular and contain numerous branched and coiled endometrial glands that are lined with columnar epithelium and open into the uterine lumen (Haféz and Haféz., 2000). The glands of the endometrium arise from columnar epithelial cells with shallow glands located towards the luminal epithelium and surface deep glands located towards the myometrium (Haféz and Haféz., 2000; Frandson et al., 2003; Reece., 2009). The endometrial stratum basilis acts as a boundary between the endometrium and the myometrium, while the stratum functionalis is located towards the uterine lumen and has 4 distinct zones (Kaiserman-Abramof and Padykula., 1989). The ovarian steroid hormones, estradiol and progesterone, regulate endometrial morphology and induces structural modifications to luminal epithelium, glandular secretions and stromal cytology (Section 1.3.).
1.5.3.3. General structure of epithelia

Epithelia are specialized cells that line the mucosal surfaces of the body and consist of cells usually arranged in sheets that are attached to an underlying basement membrane (Fig. 8). The architecture of a simple, homogenous epithelium consists of two membrane domains, an apical (luminal) and basolateral (serosal), separated by a tight junction. The space between the cells is the lateral intercellular space (Lewis, 1996). The apical membrane faces the lumen of a tissue, while the basolateral membrane faces the interstitium (e.g. basal cells, connective tissue, basement membrane, blood supply and nerve innervation) (Schwiebert and Zsembery, 2003). The movement of substances across the epithelium is limited to two pathways; paracellular pathway (across the tight junction and lateral intercellular space) and; transcellular pathway (across the apical and basolateral cell membranes). Traditionally, epithelial cells are known as barrier cells that line the fluid-filled cavities of many organs of the body, separating the external environment from the interstitium. They also function as asymmetric transporters of salt, non-electrolytes and water, thereby influencing the volume and composition of the fluid compartments they define.

Epithelia can be divided into two functional categories based on the resistive properties of the tight junction. Epithelia with low resistance tight junction are termed ‘leaky epithelia’ and those with high resistance tight junctions are termed ‘tight epithelia’. Having a low resistance effectively means that the tight junctions are highly permeable to ions and therefore leaky epithelia are known to be very efficient in the movement of electrolytes and water. The uterine epithelium is considered a ‘tight epithelium’, due to its high resistance (Matthews et al., 1998).
Figure 8: Basic structure and transport pathways of an epithelium
1.5.3.4. Uterine Vasculature

The middle uterine artery is the principle artery supplying the uterus and it arises from the ventral surface of the internal iliac artery and passes through the broad ligament. As the middle uterine artery approaches the uterus it divides into several Rami uterini branches that penetrate the vascular layer of the uterine wall (Yamauchi and Sasaki., 1968). The uteroovarian artery is highly tortuous and supplies the cranial portion of the uterine horns, while the posterior uterine artery nourishes the uterine horns laterally. Rami uterini from the middle uterine artery are involved in the formation of several anastomoses with uteroovarian and posterior uterine arteries (Fig. 9).

Figure 9: Arterial blood supply to bovine uterine horn

Uterine blood flow is positively correlated with systemic estradiol concentrations, where blood flow increases during the early follicular phase, declines during early to mid-luteal phase and begins to increase at the end of the luteal phase (Ford et al., 1979). The fluctuations observed in uterine blood flow are similar for both uterine horns in the non-pregnant animal (Ford et al., 1979). In the pregnant cow, blood flow to the gravid uterine horn is comparatively higher than blood flow to the non-gravid uterine horn, thereby indicating a role for the early conceptus on the control of blood supply to the uterus during pregnancy (Ford et al., 1979; Reece., 2009).
The close apposition of the uterine vein and utero-ovarian artery (Fig. 10) facilitates counter exchange between veins and arteries, which allows substances, such as PGs produced in uterine tissue to be transported to the ovary to induce their luteal effects (Section 1.12). The overall functional importance of the close connection of the ovarian artery and the utero-ovarian vein was neglected until the 1970s, when separation of the two blood vessels resulted in prolonged lifespan of the CL in sheep and cows (Ginther., 1974 and 1976). Secretions of uterine origin are collected by the uterine vein, and can empty into the uteroovarian artery that is wrapped around the vein in a coiled manner, thus covering a large surface area and creating a countercurrent transport mechanism. This anatomical feature enables the local transfer of uterine substances into the uteroovarian artery for delivery into ovarian tissue (McCracken et al., 1999; Stefańczyk-Krzymska et al., 2005).

Figure 10: Utero-ovarian blood supply, adapted from Ireland et al., 1984.
1.5.3.5. Uterine Steroid Hormone Receptors

Uterine expression and activation of steroid hormone receptors elicit responses that can affect uterine architecture and secretion. Estradiol receptor (ER) and progesterone receptor (PR) localization in uterine tissue is dependent on the hormonal profile of the steroid hormones (Boos et al., 1996). ER and PR expression has been shown to be at its highest at estrous and reaches maximum expression at metestrous (day 1-6) when circulating progesterone concentrations are at their lowest (Robinson et al., 2001). ERs are localized in uterine luminal epithelium, glandular epithelium and stromal cells throughout the estrous cycle with maximum expression observed at estrous and metestrous (Boos et al., 1996; Robinson et al., 2001) showing a transient increase in the luminal epithelium on days 16-18 (Robinson et al., 2001). PR protein is expressed predominately in stromal cells and follows a similar pattern to ER expression, where PR peaks at estrous and metestrous, and declines during the diestrous stage (Boos et al., 1996; Robinson et al., 2001). The stimulatory effect of estradiol and inhibitory effects of progesterone on receptor expression were assessed in vitro by Xiao and Goff (1999). In this study, short-term culture of bovine endometrial epithelial cells with estradiol resulted in an increase in ERs and PRs in stromal and epithelial cells and long term culture displayed a further increase in ER and P4 protein (Xiao and Goff., 1999). Comparatively, although progesterone did not inhibit ER and PR activity in endometrial epithelial cells it did inhibit the stimulatory effects of estradiol in a co-incubation system. ER and PR concentration in uterine tissue intensifies in response to estradiol and at the onset of luteolysis, introducing a novel regulatory role for these uterine receptors in ovarian follicle development and regression (Xiao and Goff., 1999).
1.5.3.6. Uterine Oxytocin Receptors

Activation of oxytocin receptors in uterine tissue can mediate uterine contractility (Fuchs et al., 1992) uterine prostaglandin synthesis (Section 1.13.1). Oxytocin is a neuropeptide hormone and in ruminants is secreted by the posterior pituitary gland and the CL (Moore et al., 1986; Higuchi., 1995). Oxytocin receptors (OTRs) are located on myometrial and endometrial uterine tissue (Fuchs et al., 1992). In endometrial tissue, expression is confined to luminal and glandular epithelium. OTR mRNA and protein are upregulated at estrous and at term of pregnancy, which establishes a role for progesterone priming and introduction of estradiol for increasing uterine sensitivity to oxytocin and promote uterine contractility (Bathgate et al., 1995).

1.5.3.7. Uterus as a Transporting Epithelium

The composition of the uterine lumen is ultimately determined by the transport properties of the epithelial cells lining the lumen (Cox and Leese., 2010). Uterine secretions are necessary for spermatozoal activity, growth and development of fertilized ova and in the control of uterine infections and is under hormonal control (Heap., 1962). Although the uterus is largely secretory and displays elevated K⁺, N and carbohydrate secretion into luminal fluid during the luteal phase (Heap., 1962), the uterus can also uptake substances such as PGs in uterine fluid via a re-direction of transport that is dependent on an electrochemical gradient (Thatcher and Bazer., 1977). This bi-directional transport of PG suggests diffusional release of PGs down their concentration gradient into the luminal fluid and followed by the concentrative uptake of PGs via exchange with lactate using PGT, where PGT can regulate PG levels via uptake (Schuster., 2002).
1.6. Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are the direct precursors of PG synthesis, which are important regulators of reproductive function. Fatty acids are aliphatic carboxylic acids that contain a hydrocarbon backbone chain which consists of between 12 and 24 carbon atoms (Horton et al., 2006). Monosaturated fatty acids (MUFAs) and saturated fatty acids remain relatively stable in lipid membranes because they are not essential fatty acids and can therefore be synthesized from acetyl Co-A, while the PUFA content is subject to fluctuations and is regimentally controlled by dietary intake (Hulbert et al., 2005). Phosphoglycerides are the predominant phospholipid of the cell membrane and are composed of a glycerol molecule esterified to 2 fatty acids and a phosphorylated alcohol (Murray et al., 2000). The unbranched hydrophobic fatty acid chains contain 16 to 24 carbon atoms; one fatty acid is saturated and the other is typically unsaturated and contains 1-4 cis bonds. The unsaturated fatty acid is usually AA but can be displaced by members of the n-3 PUFAs, if they are present in the diet. The carbon atoms are numbered from the carboxyl carbon (Carbon -1), where the carbon adjacent to the carboxyl carbon is the α-carbon and the carbon furthest away from the carboxyl carbon is the ω-carbon (Murray et al., 2000). Polyunsaturated fatty acids (PUFAs) have two or more double bonds between carbons along the hydrocarbon backbone that are usually in the cis configuration that introduces a 120° ‘kink’ (Fig. 11) which prevents close packing and generates flexible and fluid aggregates (Murray et al., 2000; Garrett and Grisham., 2005).

![Chemical Structure of the PUFA, arachidonic acid](image)

Figure 11: Chemical Structure of the PUFA, arachidonic acid
1.6.1. Characterization of Omega-6 and Omega-3 PUFAs

PUFAs are essential fatty acids that have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels (Gurr et al., 1989), cardiovascular system (Kahn et al., 2003; Wijendran and Hayes., 2004; Radaelli et al., 2006), immune function (Calder., 1999; Simopoulos., 2002; Yaqoob and Calder., 2007), insulin action (Clandinin et al., 1993; Storlien et al., 2000) neuronal development (Uauy et al., 2001; Horrocks and Farooqui., 2004), visual function (Uauy et al., 2001; Connor et al., 2007) and reproduction (Wathes et al., 2007). PUFAs can be classified into two distinct families of essential fatty acids with respect to the location of the first double carbon bond in relation to the omega (ω)-end of the hydrocarbon backbone, generating either the omega-6 or omega-3 families (Insel et al., 2009).

Linoleic acid (LA), abbreviated as 18:2 n-6, is the parent compound of the omega-6 fatty acid family. Linoleic acid contains 18-carbon atoms with two unsaturated fatty acids, where the first double carbon bond occurs between the 6th and 7th carbons from the ω-end. (McDonald et al., 2002; Sizer and Whitney., 2007). The major derivative of LA is arachidonic acid (AA), a major constituent of phospholipid membranes. Vegetable oils such as safflower oil, sunflower oil, corn oil, sesame oil, hemp oil, pumpkin oil, soybean oil, walnut oil, wheatgerm oil, evening primrose oil and legumes are rich sources of linoleic acid (LA) the parent compound of the omega-6 fatty acids (Hedelin et al., 2010).

In contrast, α-linoleic acid (ALA) is the parent compound of the omega-3 fatty acid family and contains 18-carbon atoms with three unsaturated fatty acids. The first double bond in ALA occurs between the 3rd and 4th carbons from the ω-end, and ALA can be abbreviated to 18:3 n-3 (McDonald et al., 2002; Sizer and Whitney., 2007). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the two major derivatives of ALA. Canola seeds, flaxseed and green leafy vegetables provide a rich source of the primary omega-3 fatty acid, ALA (Kris-Etherton et al., 2000). The preferred source of omega-3 is eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), which are found abundantly in fish oil.
LA and ALA are deemed essential PUFAs because they must be obtained in the diet, as mammals do not possess desaturase enzymes that introduce a double bond beyond Carbon-9 (Murray et al., 2000; Horton et al., 2006). Therefore, adequate mammalian LA and ALA levels depend on sufficient dietary intake and absorption (Werner et al., 2003). Omega-3 and omega-6 fatty acids are produced in plants via enzymatic desaturation of oleic acid at the C-12 position generating LA or at both the 12- and 15-position producing ALA (Murray et al., 2000; Garrett and Grisham, 2005).

1.6.2. PUFA Metabolism

Once ingested, LA and ALA are converted to their respective metabolites in order for them to elicit their full range of biological activity (Horrobin, 1993). Metabolism of the parent PUFA involves insertion of a carbon double bond by desaturation enzymes and addition of carbon atoms by desaturase and chain elongation enzymes (Fig. 12). The initial step of LA metabolism, involves desaturation of LA yielding γ-linolenic acid (GLA) by the Δ-6 desaturase enzyme, which is the rate limiting step of LA metabolism (Horrobin, 1993). GLA is then elongated to dihommo γ-linolenic acid (DGLA) (Mostofsky et al., 2001; Fragakis and Thomson, 2007) before Δ-6 desaturation of DGLA producing AA (Mostofsky et al., 2001; Fragakis and Thomson, 2007).
ALA is metabolized to EPA or DHA, however, the efficiency of ALA conversion is low in the region of 0-21%, displays considerable variability between studies (Burdge et al., 2002; Burdge and Wootton., 2002) and relies on vitamin B₆, magnesium, zinc, niacin and vitamin C levels (Kidd., 2007). Phytoplankton and algae are rich sources of ALA (Sargent., 1997; Burtin., 2003), and following ingestion by marine life, Δ6 and Δ5 desaturase convert ALA to EPA (Fig. 12) by insertion of a carbon double bond and elongation of the hydrocarbon backbone. Sequential elongation of EPA yields docosapentaenoic acid (DPA), that is desaturated and elongated to a 24 carbon PUFA before it is oxidized in the liver via peroxisomal β-oxidation in the liver generating DHA (Fig. 12) (Sprecher., 2000). Direct dietary intake of fish oil containing omega-3 fatty acids (EPA and DHA), is the preferred route of omega-3 fatty acid supplementation because EPA and DHA do not need to be metabolized and can be incorporated readily into the lipid membranes (Katan et al., 1997; Cao et al., 2006).

Figure 12: Metabolism of Dietary PUFAs adapted from Horrobin., 1993
Omega-6 and omega-3 PUFAs cannot be interconverted (Mattos et al., 2000), so the dietary intake of individual PUFAs affects physiological concentrations. One major effect of PUFAs is to alter PG production, which can occur at three levels. Firstly, PUFAs compete for metabolic enzymes. The affinity of PUFAs for metabolic enzymes, decreases from the n-3 series to the n-9 series and when n-3 PUFAs are present in sufficient amounts, n-6 metabolism is reduced, thereby lowering 2-series PG production (Wathes et al., 2007). Secondly, n-6 and n-3 PUFAs compete for the COX enzyme, which will regulate the synthesis of 2-series and 3-series PGs (Mattos et al, 2000). Thirdly, n-3 PUFAs can inhibit PG production via downregulation of COX-2 expression in various systems, as observed in human umbilical cord vein endothelial cells (HUVECs) (Lee et al., 2009) and in the MDA-MB-231 human breast cancer cell line (Horia and Watkins., 2007). N-3 PUFAs reduce PGF$_{2\alpha}$ secretion in a variety of systems, including peritoneal macrophages (Lokesh et al., 1986), skeletal muscle (Sohal et al., 1992), immortalized bovine endometrial cells (Caldari-Torres et al., 2006) and blood mononuclear leukocytes (Vedin et al., 2009).

1.6.3. PUFA Consumption in the Diet

Humans are thought to have evolved on a diet high in fibre, fruit, vegetables, lean meat and fish, that led to an n-6:n-3 ratio of 1:1 (Kris-Etherton et al., 2000; Sanders., 2000). Nowadays, due to the lack of dietary fish and the consumption of a diet rich in n-6 PUFAs, this ratio has risen to 20:1 in some Westernized countries (Simopoulos., 2000). This elevated n-6:n-3 ratio has been implicated in the increased incidence of cardiovascular disease, cancer, anti-inflammatory diseases and reproductive disorders to name but a few (reviewed by Simopoulos., 2000). Inuits, Mediterraneans and Japanese populations have a an n-6:n-3 ratio that approaches 1:1, that is primarily due to increased fish, fruit and vegetable consumption typical of their diets. The effects of this elevated intake of n-3 has been examined and is strongly associated with reductions in the incidence of prostate cancer (Dewailly et al., 2003), delaying atherogenesis (Sekikawa et al., 2008) and possessing anti-arthritis properties (Kjeldsen-Kraugh., 2003). The return of the satisfactory PUFA diet may therefore be able to reduce certain diseases and help maintain physiological functions.
1.6.4. Introduction of Supplemental PUFAs to Dairy Cow Diet

The practice of adding fishmeal to dairy cow feedstuffs stemmed from its ability to abolish NEB after parturition. NEB occurs in the early postpartum dairy cow when the energy demand exceeds intake required to meet the needs of early lactation. Supplemental fats were initially added to the diet of the early postpartum dairy cow in order to increase energy density and relieve the effects of NEB (Grummer and Carroll., 1991; Staples et al., 1998). Interestingly, an additional role for supplemental fats has been attributed to enhanced reproductive performance, independent to its role at alleviating NEB (Mattos et al., 2000).

The impact of PUFA supplementation on reproduction has been inconsistent and relies heavily on condition of the animal, the amount and type of PUFA and duration and time (pre-and postpartum) of feeding (Staples et al., 1998). Unsupplemented feed intake contains approx. 2% long chain fatty acids (LCFAs) that are predominately unsaturated (PUFAs) (Staples et al., 1998). N-6 LA is the main fatty acid in corn and soybean meal, while the n-3 ALA, predominates in forage (Palmquist and Jenkins., 1980). Although, essential fat can be derived in the diet, additional supplementation of feedstuffs with fats enhances bovine reproductive performance (Staples et al., 1998; Mattos et al., 2000). Fishmeal is a light brown flour produced by cooking, pressing, drying and milling of fresh raw fish and provides a natural balanced feed ingredient and a rich source (65%) of rumen undegradable protein (RUP), that contributes to a desirable amino acid intake, especially the essential amino acids lysine, methionine and tryptophan. In general, the fat content of fishmeal is ~8%, 66% of which is composed of PUFAs, including 10.8% EPA and 11.1% DHA (Burke et al., 1997).

Manipulation of the n-3 PUFA content in animal products via specific feeding regimes offers one method of reducing the n-6:n-3 ratio (Kris-Etherton et al., 2000). The fatty acid content of meat and milk producing animals has received considerable interest in light of its implications on fatty acid content in the human diet (de Smet et al., 2004). PUFAs, especially LA (n-6) and ALA (n-3) are abundant in grass and other ruminant feedstuffs, yet are comparatively low in meat and milk (Maia et al., 2010).
1.6.5. PUFA Biohydrogenation

Ruminants possess a unique mechanism of lipid digestion compared to monogastric animals. Once ingested, the chemical structure of PUFA undergoes rapid conformational changes in the bovine rumen transforming the PUFA to saturated fatty acids (Jenkins., 1993; Doreau and Chilliard., 1997; Kim et al., 2008). This conformational change involves release of fatty acids from the glycerol backbone via lipases and fatty acid biohydrogenation by anaerobic metabolic activity of ruminal bacteria, especially those of the Butyrivibrio phylogenetic tree (Polan et al., 1964; Maia et al., 2010). Biohydrogenation of PUFAs is extensive in the ruminal environment and multiple studies have calculated between 70-90% biohydrogenation of LA (18:2) and ALA (18:3) to saturated fatty acids (Murphy et al., 1987; Wu and Palmquist., 1991; Reddy et al., 1994). PUFA metabolites do not escape ruminal degradation, where biohydrogenation of EPA was 85% and 75% for DHA (Castañeda-Guitiérrez et al., 2007). In order to overcome extensive biohydrogenation to increase PUFA bioavailability for incorporation into milk and other physiological systems, rumen-protected PUFA can be added as a supplement to ruminant feed. Encapsulation of PUFAs inside a microbial resistant shell or alterations of fatty acid structure are two mechanisms of protecting the PUFA against ruminal biohydrogenation. Encapsulation of PUFAs with formaldehyde treated protein resists ruminal biohydrogenation by shielding the internal fatty acid from the microbial flora (Scollan et al., 2001). As the formaldehyde encapsulated PUFA passes on to the acidic environment of the abomasum, the formaldehyde complex is hydrolysed releasing the PUFA for absorption (Scollan et al., 2001). Formaldehyde treated PUFAs can resist ruminal biohydrogenation, whereby only 38% is degraded and 62% passes onto the abomasum (Hood., 1977). Calcium soaps of PUFAs partially protect against biohydrogenation and increase LA content in milk by 16% after a 14 day treatment compared to control (Theurer et al., 2009), while biohydrogenation of calcium salts of PUFA was less (47.1%) than untreated PUFAs (71%) (Wu and Palmquist., 1991).
The ruminally-derived saturated fatty acids then pass through the abomasum and onto the small intestine, where they are readily absorbed (Doreau and Chilliard., 1997). Some studies provide evidence supporting an inhibitory role for EPA and DHA on biohydrogenation as these fatty acid levels increase in vitro (Dohme et al., 2003; Abu-Ghazaleh and Jenkins., 2004). Interestingly, Wąsowska et al., (2006) concluded that EPA and DHA suppress growth and isomerase activity of the B. fibrisolvens bacterium responsible for ruminant biohydrogenation, thereby supplying the intestine with a greater amount of PUFA for absorption.
1.6.6. PUFAs and Bovine Reproduction

Appropriate nutrition is essential for proper reproductive performance (Funston., 2004). A dairy cow feeding regime that guarantees the maintenance of a successful pregnancy until term, followed by prompt uterine involution and return to estrous is desirable to ensure herd reproductive performance and producer profitability (Mattos et al., 2000). Diets supplemented with PUFAs, can affect reproductive efficiency by influencing PG production and steroidogenesis (Mattos et al., 2000). A diet rich in n-3 PUFAs could potentially decrease the number of services per conception, abolish early embryonic loss (day 8 to 16 post insemination) and ensure maintenance of pregnancy until term. In contrast, a diet rich in n-6 PUFAs (LA) could be necessary at parturition and for early lactation cows, by reducing NEB and promoting PGF$_{2\alpha}$ synthesis required for uterine involution, complete regression of CL and a return to estrous. Dairy cows fed diets rich in LA (n-6) and ALA (n-3) PUFAs produce a plethora of effects on reproductive function. LA and ALA diets reduce plasma progesterone particularly in the early luteal phase, while a diet rich in ALA increases plasma estradiol during the follicular phase. Ovarian follicles respond to dietary LA, with increases in intrafollicular fluid IGF-1 and peripheral HDL-cholesterol concentration, thereby increasing medium-sized follicle diameter (Talavera et al., 1985; Thomas et al., 1997; Robinson et al., 2002), which has a positive effect on fertilization rates and maintenance of pregnancy (Perry et al., 2005). An intensive study carried out by Burns et al., (2002), involving 82 lactating cows over a 2 year period demonstrated improved first service conception rates in cows fed a diet supplemented with fishmeal (EPA & DHA), compared to a corn gluten meal (LA) (76% vs. 62%, respectively). A similar study demonstrated first service conception rates of 72.6% for cows fed ALA-enriched diet and 47.5% for cows fed predominately LA (Ambrose et al., 2006). Interestingly, Cullens et al., (2004) demonstrated that a diet enriched with the n-6 LA, increased return to estrous and subsequent first service conception rates, regardless of the time of commencement of the feeding regime. It was also concluded that PGF2aa metabolite (PGFM) concentration after parturition was highest in animals fed LA for 28 days prior to calving (Cullens et al., 2004).
The incorporation of PUFAs into the ruminant diet appears to improve fertility. Lactating cows fed tallow (4.3% LA) at 3% of dry matter intake have an improved conception rate by day 98 post insemination than cows not fed tallow (Son et al., 1996). Beef heifers following a high LA dietary intake, 65 days prepartum, experienced greater pregnancy rates at first service compared to unsupplemented diets (Bellows., 1999). The maintenance of pregnancy which is regulated by foetal suppression of uterine PGF$_{2\alpha}$ by IFN- $\tau$ can be further inhibited by the n-3 PUFAs EPA and DHA. The mechanism of n-3 PUFA inhibition is most likely due to competition with AA for COX-2 (Mattos et al., 2003). Therefore, a feeding regime that includes n-3 PUFAs prior to insemination and during early pregnancy should reduce early embryonic losses, where early embryonic loss accounts for the majority of reproductive failures (Diskin and Sreenan., 1980).

1.6.7. PUFAs and Bovine Endometrial Cells

The effects of PUFA diet supplementation on bovine endometrial PG synthesis was examined by Cheng et al., (2001). Dietary LA (n-6) reduces in vitro basal and oxytocin or calcium-stimulated PG production from bovine endometrial explants whereas supplementation with ALA (n-3) did not affect basal or stimulated PG production (Cheng et al., 2001). In addition, alterations in PG synthesis were examined in ovine endometrial cells (epithelial and stromal) from late gestation cultured and treated with different n-6 PUFAs. All n-6 treated cultures showed an elevated E2:F2$\alpha$ ratio, but LA decreased PGF$_{2\alpha}$ production while GLA and AA treatments increased PGF$_{2\alpha}$ and PGE$_2$ secretion. The conflicting effects of the n-6 PUFAs is most likely due to the poor conversion of LA to GLA and AA in the in vitro model system employed. The consumption of diets rich in LA during gestation increases maternal estradiol and PGF$_{2\alpha}$ and elevates foetal PGE$_2$. The additive effects of these PGs on reproductive function may increase the risk of preterm birth by stimulating cervical ripening, uterine contraction and CL regression (Elmes et al., 2005). N-3 fatty acids can also alter PG production in bovine endometrial cells, where EPA reduces the capacity of these cells to synthesize PGF$_{2\alpha}$ in response to phorbol ester stimulation, a phenomenon that was reversed when the n-6:n-3 ratio increased (Caldari-Torres et al., 2006).
1.7. Prostaglandins

Enzymatic cleavage of membrane PUFAs gives rise to local signalling molecules known as eicosanoids. The eicosanoid family include PGs, leukotrienes and thromboxanes (Nelson and Cox., 2000). These fatty acid metabolites contain a cyclopentane ring in a 20 carbon chain backbone and are synthesized by virtually all cells of the body (Lands., 1979). PGs were initially characterized by von Euler in 1934 as lipid soluble, smooth muscle stimulating compounds, of seminal fluid origin and were so called because of the original assumption that the active compound originated from the prostate gland (Baskett., 2003). In a series of experiments in the 1960s Bergstrom determined the chemical structure of individual PGs (Bergstrom and Sjovall., 1960; Bergstrom et al., 1962; Bergstrom et al., 1963). The actions of PGs can be difficult to study because they are synthesized in response to a plethora of stimuli in many tissues, where they elicit regulatory and pathophysiological effects at a local cellular or tissue level. In addition, PGs offer a short window of time to initiate their potent autocrine or paracrine effects (Anderson et al., 1976; Piper., 1977) because they are synthesized rapidly on demand, are not stored in tissue (Funk., 2001) and promptly metabolized to less bioactive mediators (see Section 1.15.6).
1.7.1. Arachidonic Acid Cascade

Metabolism of AA is a tightly controlled process that is activated by numerous factors and involves the activity of several enzymes to generate 2-series PGs (Fig. 13).

Figure 13: Biosynthesis of 2-series PGs, adapted from Smith., 1989
The initial rate-limiting step of PG synthesis involves the catalytic release of esterified arachidonic acid (AA) from the sn-2 position of biological membrane phospholipids (Leaver and Poyser., 1981). Arachidonic acid concentration in cell membrane phospholipids can vary between cell types and is in the region of 14-25% of total fatty acid content (Neufield and Majeurs., 1983; Cocchi et al., 2010). The hydrolytic cleavage of the sn-2 ester acyl bond is mediated by a family of phospholipase A2 (PLA2) enzymes that hydrolyze the fatty acid acyl group generating free AA and lysophospholipid by-products (Irvine., 1982; Capper and Marshall, 2001; Burke and Dennis., 2009). Free AA has several distinct fates and is involved in the regulation of calcium flux (Damron et al., 1993 Shuttleworth., 1996; Mottola et al., 2005), transcription factors (Stuhlmeier et al., 1997; Monjazelo et al., 2006), glutamate transporter (Zerrangue et al., 1995; Trotti et al., 1995), H+ conductance (Kapus et al., 1994; Cavallini et al., 1996) and G-proteins (Glick et al., 1996; Marinero et al., 2000). Arachidonic acid can also be re-incorporated into membrane phospholipids or be further metabolized generating the eicosanoids (Al-Ubaidi and Bakhle., 1979). Four different families of PLA2 enzymes have been identified in mammalian cells; low molecular weight secretory PLA2 (sPLA2) enzymes, platelet activating factor (PAF) acid hydrolase, cytosolic PLA2G4 (cPLA2) and calcium-independent PLA2G6 (iPLA2) (Tithof et al., 2007; Godkin et al., 2008). The classification of several distinct PLA2 enzymes in mammalian cells, confirms a complex mechanism of controlling AA turnover (Leslie., 1997). cPLA2, is expressed ubiquitously in mammalian tissues (reviewed by Gijón and Leslie., 1999) and requires an increase in intracellular calcium and phosphorylation for its activation (reviewed by Leslie., 1997; Gijón and Leslie., 1999). The amino terminus of cPLA2 contains a calcium ligand binding (CaLB) domain, C2 domain, (Nalefski and Falke., 1996) necessary for calcium-mediated translocation of the enzyme from the cytosol to the nuclear and endoplasmic reticulae membranes (Schievalla et al., 1995) and calcium-dependent binding of the enzyme to membrane phospholipid membranes (Nalefski and Falke., 1996). cPLA2 can be fully activated following phosphorylation of Ser505, Ser515 and Ser727 of the enzyme by mitogen activated protein kinases (MAPKs) (Börsch-Haubold et al., 1998; Hefner et al., 2000; Pavicevic et al., 2008).
1.7.2. Formation of the PGH$_2$ Intermediate

The conversion of free AA to PGH$_2$ is the first committed step of PG biosynthesis and is catalyzed by cyclooxygenase (COX) enzymes (Smith et al., 1996; Vane et al., 1998; Smith et al., 2000). COX is a bi-functional, glycosylated membrane-bound enzyme, of which there are 2 isoforms, COX-1 and COX-2 (Smith et al., 1996; Vane et al., 1998; Smith et al., 2000), are localized in the endoplasmic reticulum and nuclear envelope (Otto and Smith, 1994). The homodimeric COX enzyme consists of 2 monomers joined via hydrophobic interactions, H-bonding and salt bridges creating the homodimeric protein (Simmons et al., 2004), with a molecular mass of 67-72 kDa and sequence homology of 60-65% between the enzymes (Smith et al., 1996). Each COX monomer is composed of 3 distinct domains; an N-terminal epidermal growth factor (EGF)-like module, a membrane binding domain (MBD) with a spiral of 4 amphipathic helices and a globular catalytic region that contains the cyclooxygenase and peroxidase active sites (Garavito and DeWitt, 1999; Marnett et al., 1999). The COX enzyme is anchored to one leaflet of the endoplasmic reticulum or nuclear membrane via the MBD and this arrangement exposes the COX active site (Otto and Smith, 1994). Sequence alignment of COX-1 and COX-2 reveals a number of differences between the two enzymes. The most notable difference is the substitution of isoleucine (COX-1) with valine (COX-2) at the 523 position, which is located near the cyclooxygenase active site. This substitution does not affect the catalytic activity of the enzyme but is sufficient to confer COX specificity (Gierse et al., 1996). COX-1 is constitutively expressed in most tissue while COX-2 is generally induced by a plethora of stimuli in various physiological and pathophysiological conditions. COX-1 is involved in cellular housekeeping functions such as gastric cytoprotection (Jackson et al., 2000), vascular homeostasis (Caughey et al., 2001; Hong et al., 2008) and its constitutive expression in renal tissue identifies a role for COX 1 in renal function (Câmpean et al., 2003).
COX catalyzes AA by via a two-step reaction; firstly, by cyclooxygenase activity that catalyzes the oxygenation and cyclicization of AA generating PGG$_2$ and a second peroxidase reaction that reduces PGG$_2$ to PGH$_2$ (Fig. 14). In the presence of two molecules of O$_2$, AA enters the COX active site and is catalyzed forming the bicyclic peroxide intermediate, PGG$_2$ (Marnett et al. 1999). Arg$^{120}$ present on the hydrophobic COX active site enables attachment of the carboxylate moiety of AA thereby facilitating the enzymatic reaction (Bhattachryya et al., 1996; Rieke et al., 1999). Tyr$^{385}$ in the COX active site is activated through a single electron reduction with a heme group generating a tyrosyl radical (Needleman et al., 1986; Chandrasekharan and Simmons., 2004). The tyrosyl radical initiates the removal 13-pro(S)-hydrogen of AA at the COX active site (Shimokawa et al.,1990), generating the arachidonyl radical, that bind two molecules of oxygen forming the endoperoxide bridge that joins C-9 and C-11 (Chandrasekharan and Simmons., 2004). Cyclicizat occurs when the ring closes at C8 to C12 to give PGG$_2$ (Fig. 14). PGG$_2$ diffuses to the peroxidase active site located on the luminal side of the enzyme where it is reduced yielding the more stable PGH$_2$ intermediate of AA metabolism (Needleman et al., 1986; Chandrasekharan and Simmons., 2004).
Figure 14: COX Catalytic Pathway, adapted from Smith et al., 2000
1.7.3. End Stage PG Synthesis

PGH$_2$ is an intermediate endoperoxide that can be converted to one of several PGs via specific terminal prostaglandin synthases (Helliwell et al., 2004).

1.7.3.1. Prostaglandin E Synthase

Prostaglandin E synthase (PGES), of which there are 2 isoforms, cytosolic PGES (cPGES) and microsomal PGES (mPGES), catalyzes the isomerization of PGH$_2$ to PGE$_2$ (Ogino et al., 1977; Tanioka et al., 2000). cPGES requires glutathione as a cofactor and directs the isomerization of COX-1 derived PGH$_2$ in the endoplasmic reticulum (Tanioka et al., 2000). mPGES is closely associated with COX-2 (Jakobsson et al., 1999; Murakami et al., 2000; Soler et al., 2000; Mancini et al., 2001) and directs PGE$_2$ synthesis in the nuclear envelope from derived from COX-2 derived PGH$_2$ (Murakami et al., 2000; Yamagata et al., 2001).

1.7.3.2. Prostaglandin F Synthase

Prostaglandin F synthase (PGFS) is the enzyme responsible for the production of PGF$_{2\alpha}$ and has a dual capacity (Komoto et al., 2006). Firstly, PGFS catalyzes the reduction of the PGH$_2$ intermediate of AA metabolism to PGF$_{2\alpha}$ via 9,11-endoperoxide reductase activity and secondly, PGFS stimulates the conversion of PGD$_2$, to the PGF$_{2\alpha\beta}$ epimer via 11-ketoreductase activity in the presence of NADPH (Watanabe et al., 1985; Komoto et al., 2006).

1.7.3.3 Reversible Enzymatic PG Interconversion

PGE$_2$ 9-keoreducatase catalyzes the reversible conversion of PGE$_2$ to PGF$_{2\alpha}$ in the presence of the coenzymes NADH or NADPH and is responsible for the generation of small quantities of PGE$_2$ or PGF$_{2\alpha}$ (Lee and Levine., 1974).
1.8. PG Mechanism of Action

PGs elicit their biological effects via a specific family of G-protein coupled receptors (GPCRs), with each GPCR containing 7 transmembrane domains that are linked to a heterotrimeric G-protein (Narumiya et al., 1999; Narumiya and FitzGerald., 2001). When the PG binds to a specific GPCR, the PG signal is transduced to an intracellular response, and stimulates the dissociation of the G-protein subunits (α and βγ) leading to the activation of intracellular second messengers (Coleman et al., 1994). Four different GPCRs, designated EP1, EP2, EP3 and EP4 mediate the actions of type E prostaglandins, while one PGF$_{2\alpha}$-receptor, FP, has been identified (Coleman et al., 1994; Narumiya et al., 1999).

Figure 15: Prostaglandin GPCR, adapted from Dorsam and Gutkind., 2007
1.8.1. Prostaglandin E Receptor Subtypes

EP1 and EP3 are the so called “contractile” PGE receptors and their activation promotes smooth muscle contraction in the gut (Botella et al., 1993), respiratory system (Tilley et al., 2003), uterus (Ma et al., 1999), bladder (Lee et al., 2007; Wang et al., 2008; Su et al., 2008), kidney (Morath et al., 1999) and vasculature (Norel et al., 2004). Activation of EP1 causes dissociation of the G-protein and liberation of a Gq protein. The G-protein stimulates phospholipase C (PLC) which ultimately elevates intracellular Ca\(^{2+}\) concentration and activates protein kinase C (PKC). EP1 effects can also be mediated via the Gi protein which inhibits cyclic adenosine monophosphate (cAMP) to induce contraction (Narumiya et al., 1999; Coleman et al., 1994; Breyer et al., 2001; Bos et al., 2004). The main signalling mechanism of EP3 is via Gi, where it reduces cAMP concentration and may elevate phosphatidyl inositol (Narumiya et al., 1999; Coleman et al, 1994; Breyer et al., 2001; Bos et al., 2004; Sugimoto and Narumiya et al., 2007).

EP2 and EP4 are the “relaxant” PGE receptors since their activation is linked to adenylyl cyclase that mediates smooth muscle relaxation (Botella et al., 1993; Angulo et al., 2002) and although the receptors are structurally dissimilar, both are coupled to a Gs protein (Nishigaki et al., 1996; Neuschäfer-Rube et al., 1999). The EP2 and EP4 receptors are located in smooth muscle, renal tissue, inflammatory cells and sensory afferent neurons (Narumiya et al., 1999; Coleman et al, 1994; Breyer et al., 2001; Bos et al., 2004; Sugimoto and Narumiya et al., 2007).
1.8.2. Prostaglandin F Receptor Subtype

The contractile PGF\textsubscript{2α}-receptor FP is located in reproductive (Abramovitz \textit{et al.}, 1994; Arosh \textit{et al.}, 2004), gastric (Okada \textit{et al.}, 2000), cardiovascular (Ponicke \textit{et al.}, 2000) and ocular tissues (Mukhopadhyay \textit{et al.}, 2001) as well as the kidney (Hébert \textit{et al.}, 2005). FP is coupled to a Gq protein and once stimulated, activates phospholipase C (PLC) to increase intracellular Ca\textsuperscript{2+} and PKC, leading to smooth muscle contraction (Narumiya \textit{et al.}, 1999; Coleman \textit{et al.}, 1994; Breyer \textit{et al.}, 2001; Bos \textit{et al.}, 2004; Sugimoto and Narumiya \textit{et al.}, 2007).
1.9. Prostaglandins and Physiological Processes

PGs are involved in a range of physiological functions. PGs synthesized in renal tissue can act as a regulator of renal function to prevent excessive changes in plasma osmolarity (Danon et al., 1978; Craven et al., 1981). Activation of EP1 and EP3 promotes excess salt and water secretion by inhibiting sodium and water reabsorption (Breyer and Breyer, 2000). The relaxant EP2 and EP4 receptors regulate renal blood flow and renin release (Breyer and Breyer, 2000). PGs, especially of the E2 series secreted at the onset of inflammation are important immunomodulatory mediators. PGE_1 and PGE_2 induce the “wheal and flare response” associated with inflammation by increasing vascular permeability and vasodilation (Crunkhorn and Willis, 1971). Stimulation of EP1 receptors in the preoptic area of the hypothalamus induces the fever response (Stock et al., 2001), while PGE_2 is involved in pain perception by sensitizing the peripheral nociceptors at multiple sites along the neuroaxis (Lin et al., 2006). PGF_2α is released by the endothelium of hypoxic or ischemic tissues, and acts as chemoattractant molecule that might be involved in the early recruitment of neutrophils in ischemic organs (Arnould et al., 2001). PGs also mediate secretions in the gastrointestinal tract. PGE_2 binding to EP3 receptors reduce gastric acid secretion, while binding to EP4 receptors promotes acid secretion from parietal cells (Kato et al., 2005) thereby playing a central role in the maintenance of optimal gastric pH. PGE_2 activation of the EP4 receptor can augment gastric mucus secretion, providing a protective epithelial film (Takahashi et al., 1999). Pulmonary tissue can respond to PG eicosanoids by inducing smooth muscle relaxation, while those of the F series cause bronchial muscle contraction (Smith and Cuthbert, 1972). PGE_1 and PGE_2 cause bronchodilatation in asthmatic subjects when given by metered aerosol (Cuthbert, 1969), however PGE_1 is not used clinically as it can induce upper respiratory tract irritation.
The effective interplay between PGE and PGF eicosanoids help maintain smooth muscle tone. In the guinea pig gastrointestinal tract, prostaglandins help regulate muscle tone. Both PGEs and PGFs initiate contraction of longitudinal smooth muscle cells in the ileum and colon, while only PGF compounds promote circular smooth muscle cell contraction (Bennett et al., 1975). In the human urinary system, PGFs induce contraction of bladder and ureteric smooth muscle, while PGEs only induce contraction in the bladder smooth muscle (Abrams and Feneley., 1976).
1.10. PG Synthesis in Reproductive Tissue

The synthesis of PGF$_{2\alpha}$ and PGE$_2$ in bovine uterine tissue is largely dependent on hormone concentrations and bovine oestrous cycle stage (Pratt et al., 1977; Okuda et al., 2002). PGF$_{2\alpha}$, synthesized chiefly by endometrial epithelial cells, displays a cyclical pattern of release unlike PGE$_2$, which is synthesized primarily by endometrial stromal cells and relies heavily on maternal recognition of pregnancy for its production (Asselin et al., 1997; Asselin et al., 1998; Parent et al., 2002) but is also comparatively higher during the mid- to late-luteal phases of the oestrous cycle (Miyamoto et al., 2000).

1.10.1. cPLA$_2$ Expression in Bovine Endometrium

Estradiol upregulates PLA2 activity, while progesterone maintains PLA2 at a low basal level of activity in the rat endometrium (Dey et al., 1982; Pakrasi et al., 1983). In bovine endometrial cells (BEND cells), cPLA$_2$, subtype G4A, mediates PGE$_2$ and PGF$_{2\alpha}$ synthesis (Godkin et al., 2008; Tithof et al., 2008), interestingly, IFN-τ can reduce expression of cPLA$_2$, subtype G4A to suppress PGE$_2$ and PGF$_{2\alpha}$ synthesis, while oxytocin directed PG production is mediated by iPLA$_2$ (Tithof et al., 2008).
1.10.2. COX Expression in the Bovine Endometrium

The COX-1 enzyme is undetectable across the estrous cycle, while COX-2 displays differential levels of expression. Arosh et al., (2002) examined COX expression in bovine endometrial tissue at different stages of the estrous cycle. COX-2 mRNA and protein are positively correlated and are expressed at low concentrations between days 1-12 with significantly higher concentrations from days 13-21, peaking at day 18. The biphasic upregulation of COX-2 mRNA and protein at days 16-18 coincides with the luteolytic cascade. Hormonal stimulation of bovine endometrial cells alters COX-2 expression in vitro (Xiao et al., 1998). COX-2 mRNA expression in bovine endometrial epithelial cells is downregulated after estradiol and recombinant IFN-τ (rbIFN-τ) treatment, which ultimately suppresses PG synthesis. The behaviour of rbIFN-τ is cell specific and upregulates COX-2 mRNA in endometrial stromal cells, to promote PGE₂ synthesis (Xiao et al., 1998). Progesterone treatment of cultured bovine endometrial epithelial cells has no effect on COX-2 protein but PGF₂α secretion is augmented (Xiao et al., 1998), possibly due to its ability to elevate endometrial accumulation of arachidonic acid necessary for PG synthesis (Silvia et al., 1991). Exogenous AA (Parent et al., 2003), oxytocin (Asselin et al., 1997) and activation of protein kinase C (PKC) by PBDu (Binelli et al., 2000) are also capable of elevating COX-2 expression and promoting PGF₂α synthesis.

1.10.3. PG Synthases in the Endometrium

Studies reveal that PGES is highest at ovulation and luteolysis in the bovine endometrium when estradiol levels are dominant, but that there is great variability between animals (Arosh et al., 2002; Parent et al., 2005). The expression of PGFS is maintained at a basal level in the endometrium across the bovine oestrous cycle (Xiao et al., 1998).
1.11. PGs and Uterine Contractility

Uterine contractility is necessary for metestrous bleeding in cows, gamete transport (Kissler et al., 2004), successful implantation (Bulletti and de Ziegler., 2005) and parturition (Mesiano and Welsh., 2007). Failures in contractility can lead to ectopic pregnancies, early embryonic losses, miscarriages and retrograde bleeding (Bulletti and de Ziegler., 2005). PGs participate in uterine contraction and relaxation, contributing to efficient reproductive performance. PGF$_{2\alpha}$ perfusion into non-pregnant swine uteri causes a dose-dependent increase in uterine contractility as measured by elevated intrauterine pressure (IUP) that levels off at higher doses, while PGE$_1$ and PGE$_2$ perfusion results in a distinct pattern of contraction that progresses from the uterine isthmus towards the body of the uterus (Mueller et al., 2006). PGE from semen may also be the stimulus for sperm transport towards the oviduct for fertilization (Mueller et al., 2006). PGE$_2$ induces cervical ripening at term (Kelly., 2002) via activation of the relaxant EP4 receptors and subsequent cAMP-dependent PKA signalling (Chien and MacGregor., 2003). Expression of the contractile EP3 receptor subtype and FP receptor increases in myometrial circular and longitudinal smooth muscle cells at the onset of parturition (Ma et al., 1999; Al-Matubsi et al., 2001) compared to gestational stages (Matsumoto et al., 1997) and their activation is involved in myometrial contraction and successful parturition via PLC signalling and calcium mobilization.
1.12. PGs and Ovarian Function

Once synthesized PGs can be transferred from the endometrium to the ovary via a 2-step mechanism that involves lymphatic vessels and a local vascular system to enable the eicosanoids induce their endocrine effects. Heap et al., (1985) proposed a 2 way countercurrent exchange mechanism of PGF$_{2\alpha}$ transfer from the endometrium to the ipsilateral ovary in ruminants (Section 1.5.3.4). Firstly, newly synthesized PGF$_{2\alpha}$ drains into the afferent uterine lymphatic vessels before emptying into the utero-ovarian vein. The close apposition of the utero-ovarian vein and ovarian artery facilitates PGF$_{2\alpha}$ counter exchange and enables PGF$_{2\alpha}$ transport back to the ovary to induce its effects. Nowadays, it is agreed that PGF$_{2\alpha}$ can be transferred back to the ovary via the utero-venous system or the lymphatic vessels and bypass the systemic circulation in cows (McCracken et al., 1999; Stefańczyk-Krzymowska et al., 2005), where 65-99% of the prostaglandin would otherwise be metabolized in one passage through the lungs (Anderson et al., 1976; Piper., 1977; McCracken et al., 1999). A specific, carriermediate prostaglandin transporter (PGT) has recently been identified that mediates the transport of prostaglandins from their endometrial site of synthesis back towards the ovary (see next Section).

1.12.1. PGs and Ovulation

Cows fail to ovulate after intraovarian treatment with the COX inhibitors, although luteinization of the follicle progresses, demonstrating the necessity of PGs for ovulation (De Silva and Reeves., 1985). Activation of the LH-receptor of bovine preovulatory follicles increases COX-2 (Sirois., 1994) and PGES expression in bovine granulosa and thecal cells after ovulation (Filion et al., 2001). The subsequent elevation in PGE$_2$ initiates an acute inflammatory reaction, involving hyperaemia, oedema, leukocyte extravasation, and induction of proteolytic and collagenolytic activities to induce follicle wall rupture and expulsion of the mature oocyte (Espey., 1980). PGF$_{2\alpha}$ contributes to ovulation in the cow by initiating luteal regression, which in turn reduces progesterone concentration thereby increasing estradiol and promoting the ovulatory LH surge (Louis et al., 1974).
1.12.2. Regulation of the Corpus Luteum lifespan by PGs

At the end of the luteal phase, two events can occur depending on whether or not a successful pregnancy has occurred. In the event of a successful pregnancy, the CL will persist to secrete progesterone to support a developing embryo (see Section 1.5.1.2). However, in the absence of a viable embryo, the CL regresses to allow the resumption of the oestrous cycle (Haféz and Haféz., 2000; Frandson et al., 2003; Reece., 2009). Prostaglandins of uterine origin mediate the fate of the CL at this juncture, approx. d17-19 post-estrous.

1.12.2.1. PGE₂ and Luteoprotection

PGE₂ is luteoprotective and promotes CL maturation during the normal bovine oestrous cycle and CL maintenance at the establishment of pregnancy (Pratt et al., 1977). PGE₂ directs angiogenesis in newly formed corpora lutea (Sakurai et al., 2005) which is essential for normal tissue growth, development and function as well as providing steroidogenic cells with oxygen, nutrients and precursors for progesterone synthesis and subsequent delivery of newly synthesized progesterone to the circulation during the luteal phase or pregnancy (Fraser and Wulff., 2003). PGE₂ promotes the synthesis of progesterone in luteinized granulosa cells via the EP2-cAMP second messenger pathway (Richards et al., 1994; Elvin et al., 2000) and the progesterone produced acts to suppress luteal cell apoptosis (Rueda et al., 2000; Okuda et al., 2004). In the bovine endometrium PGE₂ is produced at a basal level throughout the estrous cycle but is comparatively higher at the mid- to late luteal phases (Miyamoto et al., 2000), a period of luteolysis in the absence of a viable embryo or maternal recognition of pregnancy if fertilization occurs.
The EP2 receptor subtype is the primary route of PGE$_2$ paracrine action at the establishment of pregnancy and works via cAMP-dependent PKA activation (Arosh et al., 2003; Arosh et al., 2004). PGE$_2$ signalling via the EP2 receptor acts as an immunomodulatory mediator (Emond et al., 2000), promoting uterine quiescence (Price and López-Bernal, 2001; Arosh et al., 2003), increasing uterine vascular permeability (Hamilton and Kennedy, 1994) and inducing luminal epithelial differentiation at implantation (Lim and Dey, 1997). PGE$_2$ is the dominant PG during early pregnancy and its synthesis by endometrial stromal cells is stimulated by interferon-τ of embryonic origin (Asselin et al., 1997; Asselin et al., 1998; Parent et al., 2002). PGE$_2$ synthesis after exposure to bIFN-τ is associated with upregulation of mPGES and COX-2 mRNA in bovine endometrial cells. TNFα or oxytocin do not alter mPGES gene expression in bovine uterine epithelial cells but stimulate an increase in mPGES in stromal cells (Parent et al., 2002). Steroid hormones have no effect on PGFS mRNA levels in bovine endometrial epithelial cells, while rbIFN-τ downregulates the enzyme in a dose-dependent manner (Xiao et al., 1998).

1.12.2.2. PGF$_{2\alpha}$ and Luteolysis

Suppression of progesterone synthesis and cellular alteration of the CL to produce the corpus albinacs are characteristic of luteal regression (McCracken et al., 1999). PGF$_{2\alpha}$ of endometrial origin has been identified as a luteolytic mediator in cattle, (Louis et al., 1974; Hansel et al., 1975; Hansel et al., 1975; McCracken et al., 1999), sheep (Douglas and Ginther, 1973; Nett et al., 1976; Pratt et al., 1977), pigs (Diehl and Day, 1974; Kotwica, 1980) and rats (Kenny and Robinson, 1976). A luteolytic mediator from bovine endometrial extracts identified as an AA derivative was identified as PGF$_{2\alpha}$ using thin layer chromatography (TLC), gas liquid chromatography (GLC) and mass spectrometry techniques (Hansel et al., 1975).
Furthermore, Hansel et al., (1975) identified elevated levels of PGF$_{2\alpha}$ in the bovine endometrium and uterine vein in the late luteal phase that were able to induce luteolysis as measured by a drop in systemic progesterone concentration after 24 hours and an initial fall in estradiol that began to rise again after 9 hours. Silvia et al., (1991) demonstrated that PGF$_{2\alpha}$ was elevated in a series of pulses in uterine venous blood during luteolysis, while levels of PGFM, the PGF$_{2\alpha}$ metabolite, in the peripheral blood of cows showed pulsatile peaks during luteolysis (Milvae and Hansel., 1983; Ginther et al., 2007) supporting a role of PGF$_{2\alpha}$ as a luteolytic mediator. Also, exogenous intrauterine PGF$_{2\alpha}$ administration during the diestrous phase of the cycle caused dramatic luteolysis in cows, followed by increased estrogen secretion and an ovulatory surge of LH coincidental with onset of estrous, and ovulation within 72 hours (Louis et al., 1974).

1.12.2.3. Mechanisms of PGF$_{2\alpha}$ induced Luteolysis

Endometrial PGF$_{2\alpha}$ induces luteolysis by initiating a sharp drop in vascularisation and angiogenesis and termination of progesterone synthesis (Niswender et al., 1976; Acosta et al., 2002). This leads to a drop in luteal size and ultimate abolition of the CL (Kayacik et al. 2005). PGF$_{2\alpha}$ is a potent venoconstrictor (Hyman., 1969) and initiates the first stages of luteolysis by decreasing ovarian blood flow (Acosta et al., 2002). Injection of PGF$_{2\alpha}$ into normally cycling cows triggers an initial rapid increase in ovarian blood flow followed by a swift reduction that decreases significantly 8 hours post injection (Acosta et al., 2002), thereby inhibiting nutrient and oxygen delivery to the CL. PGF$_{2\alpha}$ prevents progesterone production synthesis at a site subsequent to cholesterol entry into luteal cells (Pate and Condon., 1989; Grusenmeyer and Pate., 1992). The most plausible site for PGF$_{2\alpha}$-induced inhibition of progesterone is at the point of the conversion of pregnenolone to progesterone via the 3β-HSD enzyme (Tian et al., 1994).
PGF$_2\alpha$ causes a significant decline in 3β-HSD mRNA expression in luteal cells of Holstein heifers that correlates strongly with a decline in systemic progesterone concentration (Tian et al., 1994). 3β-HSD profile was assessed across the oestrous cycle of sheep, pigs and cows and its expression increased with respect to progression of the luteal phase and declined gradually with luteal regression (Conley et al., 1995). Furthermore, PGF$_2\alpha$ and PGE$_2$, although to a lesser extent, block the LH-dependent production of cAMP which results in a decrease in progesterone secretion in cultured luteal cells (Thomas et al., 1978). PGF$_2\alpha$ reduced LH-stimulated progesterone secretion in bovine luteal cells from mid- to late luteal phase but did not affect early luteal phase luteal cells (Girsh et al., 1995). PGF$_2\alpha$ also mediated apoptosis of luteal cells by inducing internucleosomal DNA fragmentation indicative of apoptosis (Rueda et al., 1995) and elevating apoptotic proteins such as bax and interleukin-1β-converting enzyme mRNA during structural luteolysis (Rueda et al., 1997). Administration of PGF$_2\alpha$ during the luteal phase of the estrous cycle initiates luteinization of granulosa and thecal cells that are shrunken, contain irregular nuclei with condensed chromatin, abundant lipid droplets and swollen mitochondria (Nett et al., 1976).
1.13. Hormonal Regulation of PG Synthesis in the Endometrium

The female sex steroids (Silvia et al., 1991), oxytocin (Fairclough et al., 1984) and interferon-τ (Asselin et al., 1997) are important regulators of PG production in the bovine uterus, substantiating a role for the stage of the bovine oestrous cycle stage and hormonal milieu on PG synthesis.

1.13.1. Oxytocin

Oxytocin is a major stimulus for uterine PGF$_{2\alpha}$ secretion, although luteolysis can still occur when animals are treated with oxytocin antagonists, suggesting a role for other factors in the luteolytic cascade (Kotwica et al., 1997). In ruminants, pulses of PGF$_{2\alpha}$ occur concurrently with pulses of oxytocin during luteolysis (Fairclough et al., 1980; Flint and Sheldrick, 1983). Several research groups have concluded a common mechanism of oxytocin-induced PGF$_{2\alpha}$ secretion in bovine uterine epithelial cells, which involves a positive feedback loop (see Section 1.13.1.1.). Oxytocin administration induces a rapid increase (30 minutes) in PG synthesis in bovine endometrial tissue (Burns et al., 1997) and elevated PG secretion in cultured endometrial epithelial cells after 24 hours treatment (Xiao et al., 1998). Oxytocin regulates PG secretion by activating cPLA$_2$ to provide the AA necessary for prostanoid synthesis. Recent studies reveal that oxytocin can phosphorylate cPLA$_2$ via 2 mechanisms; (i) via activation of PKC (Kim and Fortier, 1995; Skarzynski et al., 2000; Burns et al., 1997) and (ii) activation of ERK1/2 MAPK (Burns et al., 2001). Both of these complex intracellular signalling cascades ultimately phosphorylate and activate cPLA$_2$, the rate-limiting enzyme of PG synthesis.
1.13.1.1. Oxytocin-PGF$_{2\alpha}$ Positive Feedback Loop

Pituitary oxytocin, released in a pulsatile pattern serves as a pulse generator, for PGF$_{2\alpha}$ secretion (McCracken et al., 1999). Oxytocin binds to oxytocin receptors (OTRs) in a responsive endometrium and via activation of cPLA$_2$ activates the AA metabolic cascade stimulating PGF$_{2\alpha}$ release (Lee and Silvia., 1994). The initial pulse of PGF$_{2\alpha}$ triggers the synthesis of additional oxytocin from the CL (Moore et al., 1986), which in turn induces additional PGF$_{2\alpha}$ secretion from the uterus (McCracken et al., 1999). This method of PGF$_{2\alpha}$ secretion results in several pulses of short duration that are more effective at initiating luteolysis rather than a pro-longed increase in oxytocin concentration (Flint et al., 1994; McCracken et al., 1999).

1.13.2. Progesterone

Progesterone has a potent effect on uterine pulsatile secretion of PGF$_{2\alpha}$ and luteolysis. Exogenous administration of progesterone to cows from days 1 to 4 of the oestrous cycle advances PGF$_{2\alpha}$ release and reduces cycle length from an average of 21.6 days to 16.7 days (Garrett et al., 1988). Other studies (Woody and Ginther, 1968; Ottobre et al., 1980) have shown that treatment with progesterone early in the oestrous cycle, before the CL normally reaches biologically active levels, induces premature luteolysis and shortening of interestrous intervals, presumably via stimulation of PGF$_{2\alpha}$ secretion (Ottobre et al., 1980). *In vitro* studies confirm a stimulatory effect of progesterone on PGF$_{2\alpha}$ production. Bovine endometrial epithelial cells treated with progesterone show significantly higher PGF$_{2\alpha}$ secretion into culture media (Asselin et al., 1996; Skarzynski et al., 1999), while incubation with the progesterone inhibitor, onapristone, reduces PGF$_{2\alpha}$ secretion (Skarzynski et al., 1999). Interestingly, co-incubation of bovine endometrial epithelial cells with progesterone and oxytocin results in a dramatic elevation in PGF$_{2\alpha}$ compared to cells treated with progesterone alone, indicating a synergistic effect of these hormones (Skarzynski et al., 1999).
The most likely mechanism by which progesterone-induces PGF$_{2\alpha}$ secretion in vitro is its ability to augment the number of lipid droplets in uterine epithelial cells, thereby providing sufficient esterified arachidonic acid for prostanoid synthesis (Brinsfield and Hawk, 1973). Progesterone appears to elevate PGF$_{2\alpha}$ secretion in vitro but in vivo this phenomenon only occurs after tissue has been primed with progesterone for 10-14 days (Thorburn et al., 1977; Ottobre et al., 1980; Silvia et al., 1991).

1.13.3. Estradiol

The role of estradiol in PGF$_{2\alpha}$ secretion is dependent on progesterone-priming (LaFrance and Goff., 1988). Estradiol regulates oxytocin at a posterior pituitary (Walker et al., 1997; Bossmar et al., 1995) and endometrial level (Hixon and Flint, 1987). Infusion of estradiol into ovariectomized ewes results in a pulsatile secretion of posterior pituitary oxytocin similar to that observed in intact ewes during the follicular phase (Walker et al., 1997) a phenomenon that is also observed in primates (Bossmar et al., 1995). The pulsatile secretion of oxytocin can increase PGF$_{2\alpha}$ to induce luteolysis (Section 1.13.1.1.). Concentrations of PGFM (PGF$_{2\alpha}$ metabolite) were elevated after estradiol administration to cows in the mid-luteal phase and luteolysis ensued (Thatcher et al., 1986) presumably by upregulation of oxytocin receptors (Hixon and Flint, 1987). In the endometrium, estradiol can enhance cPLA$_2$ and may elevate PGF$_{2\alpha}$ as discussed in Section 1.10.1. In contrast, in vitro studies demonstrated that estradiol suppresses PG secretion in bovine endometrial epithelial cells (Asselin et al., 1996; Xiao et al., 1998), while extensive studies reveal a role for progesterone pre-treatment on estradiol-induced luteolysis via PGF$_{2\alpha}$ (Loy et al., 1960; Hawk and Bolt, 1970; Thatcher et al., 1986).
1.14. PG Carrier Mediated Transport

Chemically, PGs are organic anions and their transport across cellular membranes is mediated by members of the organic anion transporting polypeptide (oatp) family. With a molecular weight of >350 and their prevalence as the charged organic anion at physiological pH (Schuster., 1998; Nelson and Cox., 2000), PGs are virtually impermeable to biological membranes (Bito and Baroody., 1975). PGs are known to cross membranes via simple diffusion but the diffusional rate is very low and thought to be insufficient to initiate a biological response and thus a transport mechanism is required (Schuster., 1998; Nelson and Cox., 2000; Schuster., 2002). Early studies revealed that PG transport was saturable and carrier-mediated in many diverse tissues, including the lung (Anderson et al., 1976), choroid plexus (Bito and Salvador., 1972; Bito et al., 1976; DiBenedetto and Bito., 1986), liver (Dawson et al., 1970), anterior chamber of the eye (Bito and Salvador., 1973; Bito et al., 1976), vagina and uterus (Bito 1972; Jones and Harper., 1983; Cao et al., 1984). Furthermore, it has been shown that the extent of PG uptake does not follow a strict pattern and variation between cells and disparate tissues is common (Bito., 1972). Recently, a novel prostaglandin transporter (PGT) has been identified in rat (Kanai et al., 1995), human (Lu et al., 1996), mouse (Pucci et al., 1999), cow (Banu et al., 2003), sheep (Banu et al., 2008) and pig (Van Poucke et al., 2009). PGT is a member of the ‘oatp’ superfamily of transporters. Oatps are expressed in multiple tissues, including the liver, kidney, small intestine, lung, heart, blood brain barrier (Tamai et al., 2000), and their substrates are anionic, amphipathic compounds with high molecular weight that are bound to albumin in plasma (Hagenbuch and Meier., 2003). Most oatps are polyspecific and mediate the transport of a broad range of organic solutes such as bile salts, organic dyes, steroid conjugates, anionic polypeptides, numerous drugs and other xenobiotics (Meier et al., 1997; Kullak-Ublick., 2001; Meier and Stieger., 2002; König and Seithel., 2006).
Transport via oatp is sodium independent (Jacquemin et al., 1994; Noé et al., 1997) and mediated through anion exchange, where the cellular uptake of an anion is coupled to the efflux of another anionic compound e.g. chloride, bicarbonate, glutathione, lactate (Shi et al., 1995; Satlin et al., 1997; Li et al., 2000; Chan et al., 1998). Transport via oatp is bidirectional, where the overall direction of substrate transport is governed by substrate gradients across the plasma membrane (Hagenbuch and Meier., 2004). In 1994, the first oatp was identified by Jacquemin et al., (1994) in rat liver. Today, members of the transporter family continue to grow with the identification of diverse oatps in tissues of different species (Hagenbuch and Meier., 2004). Oatps are particularly abundant in tissues involved in the excretion of xenobiotics or harmful endogenous compounds (Diaz., 2000; Hagenbuch and Gui., 2008). The expression of PGT in a broad range of tissues denotes the importance of PG transport in physiological and pathophysiological conditions (Lu et al., 1996; Itoh et al., 1996; Schuster., 1998; Schuster., 2002). Furthermore, the increased levels of PGT protein in pulmonary, hepatic and renal tissues confers its role in the inactivation of PGs (Lu et al., 1996; Pucci et al., 1999; Banu et al., 2003; Van Poucke et al., 2009).

1.14.1. Oatp Nomenclature

The rapid identification of new oatps by independent research groups has led to chaotic and erroneous classification of oatps, where different names have been allocated to the same transporter or similar names given to non-orthologous gene products (Hagenbuch and Meier., 2003). In 2004, Hagenbuch and Meier proposed a new species independent classification and nomenclature system that has since been approved by the HUGO Gene Nomenclature Committee (HGNC). The new nomenclature system classifies oatps based on divergent evolutionary sequences, which define an umbrella oatp-gene superfamily, composed of 6 individual but related families (OATP1/ OATP2/ OATP3/ OATP4/ OATP5/ OATP6) that share 40% homology. Within the families a plethora of related subfamilies have been elucidated that share 60% homology.
1.14.2. Regulation of Oatps

The regulation of oatp expression and function is under the control of a multitude of signals that confer species and tissue specificity. The functional regulation of rat oatp-1 and oatp-2 is susceptible to short term control by protein kinase C (PKC), whereas protein kinase A (PKA) is without effect (Gou and Klaassen, 2001). Oatps contain multiple PKC phosphorylation sites and their apparent activation by PKC activators suppresses oatp-mediated uptake after short term treatment in rat hepatocytes (Guo and Klaassen, 2001). Köck et al., (2010) observed rapid internalization of the oatp subtype, OATP2B1, following PKC stimulation, in MDCK II cells transfected with the transporting protein. Cellular stimulation with phorbol ester PMA activates intracellular PKC and initiates sequestration of OATP2B1 from the plasma membrane to the intracellular compartment and on towards the lysosome for degradation by acidic hydrolases (Köck et al., 2010). Interestingly, in murine hepatic tissues, activation of the cAMP-PKA pathway seems to be the underlying mechanism for the induction of oatp2 expression (Chen et al., 2007). Furthermore, steroid hormones can influence oatp expression, which is also species and tissue specific. Expression of renal rat oatp is under strong stimulatory control by testosterone (Lu et al., 1996), while testosterone inhibits expression of the ubiquitous OATP2B1 in MDCK II cells over-expressing OATP2B1 (Grube et al., 2006). Estrogens are generally inhibitory modulators that reduce oatp-mediated transport (Lu et al., 1996; Grube et al., 2006). Progesterone has a strong influence on oatp regulation, the progesterone antagonist, mifepristone, and pregnenolone sulphate confer an inhibitory role, while progesterone, hydroxyprogesterone and pregnenolone stimulate oatp expression (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006). Additionally, due to the elevated levels of progesterone in placentomes during gestation (Conley and Ford, 1987), the hormone may influence the transport of organic anions like PGs to maintain pregnancy. Furthermore, the oatp glycosylation sites appear to be involved in oatp activity and inhibition of the N-glycosylation sites with tunicamycin or by mutating the glycosylation sites reduces the activity of rat oatp1 (Lee et al., 2003).
1.14.3. Prostaglandin Transporter Structure

Banu et al., (2003) characterized a bovine prostaglandin transporter (bPGT), with a molecular weight of ≈70kDa, that contains 644 amino acids. bPGT has a net 3.8% positive charge that facilitates anion transport (Banu et al., 2003). Like other oatp members, bPGT contains 12 transmembrane domains (12 TMDs), a “signature oatp sequence” at the border between extracellular loop 3 and transmembrane domain 6 and four N-glycosylation sites in extracellular loops 2 and 5 (Fig. 16). bPGT also contains 3 cytoplasmic consensus sites for serine phosphorylation by cAMP-dependent protein kinase A (PKA) and 1 cytoplasmic site for threonine phosphorylation by protein kinase C (PKC) (Schuster et al., 1997). The predicted sequences share 90%, 82% and 81% homology with human, mouse and rat PGTs respectively. Sequence homology lies in the transmembrane domains while divergence occurs in the extracellular and cytoplasmic loops. The oatp “signature” sequence at the border between extracellular loop 3 and TMD 6 and of the transporters identified, shares 53 conserved amino acid residues (Hagenbuch and Meier., 2003). Unlike other members of the oatp family, which display broad tissue distribution, species divergence and multispecificity, PGT is highly conserved, exhibits a narrow spectrum of specificity and is involved in distinct physiological functions in many species (Hagenbuch and Meier., 2004). The bovine PGT transports eicosanoids in the affinity order PGE2 = PGF2α = PGD2 >> Arachidonic acid (Banu et al., 2003).
1.14.4. PG transport via PGT

PGs bind to PGT via transient electrostatic forces, which permit the rapid transfer of the substrate (Schuster et al., 2000). PGT binding sites contain positively charged amino acids that form weak bonds with the carboxyl moiety located at the 1-position of the substrate (Schuster et al., 2000). The binding site also contains a more hydrophobic region that confers specificity to the remaining regions of the substrate (Chan et al., 2002). Arginine at position 560 of PGT and lysine at position 614 have been identified as necessary amino acids within the protein required for binding and transportation of PGs (Itoh et al., 1996; Banu et al., 2003).
The precise structural requirements for PG uptake in rat lung comprise a substrate that contains a COO⁻ moiety at position-1, 15-(S)-OH group, an oxygen group at position-11 and a double bond between C13 and C14 (Eling and Ally., 1984). PG binding is impaired when the carboxyl group at position-1 is neutralized or the 15-(S)-OH group is metabolized yielding 15-(R)-OH or the keto group (Itoh et al., 1996). Increasingly, evidence suggests that PGT-mediated transport is Na⁺, H⁺ and Cl⁻ independent in liver tissue (Kanai et al., 1995), in Xenopus oocytes and cultured mammalian cells (Chan et al., 1998).

1.14.5. Cellular Inactivation of PGs

As individual PGs have the capacity to mount multiple diverse biological actions throughout the body, the signal is generally terminated locally to prevent any undesirable effects (Schuster., 2002). After entering the general circulation PGs are inactivated, degraded and ultimately eliminated from the body by the lungs, liver and kidney (Vane., 1969; Bito et al., 1972; Anderson et al., 1976; Piper., 1977; Irish., 1979; Hankin et al., 1997). The most common mechanism of PG metabolism requires cellular uptake of the parent compound, followed by its intracellular oxidation and reduction to biologically inactive derivatives (Kankofer., 1999; Backlund et al., 2005). Uptake of the parent PG involves carrier-mediated transport across the plasma membrane via PGT (Nomura et al., 2004). After successful uptake, intracellular 15-prostaglandin dehydrogenase (15-PGDH) oxidizes the active prostaglandin at the 15-hydroxyl position yielding the 15-keto prostaglandin (Fig. 17). Two 15-PGDH enzymes have been described, with the NAD⁺-dependent enzyme displaying greater affinity for eicosanoids compared to the NADP⁺-dependent enzyme (Lee and Levine., 1975; Parent et al., 2006). 13-Δ reductase catalyzes the removal of the double bond at C13-C14, and converts the 15-keto prostaglandin to the more biologically inactive metabolite, 15-keto-13,14-dihydroprostaglandin (Tai et al., 2002). 15-keto-13,14-dihydroprostaglandins are useful markers for monitoring prostaglandin levels in blood, where PGEM is the stable metabolite of PGE₂ and PGFM is the more stable metabolite of PGF₂α (Kankofer., 1999).
The classical mechanism of PG metabolism and elimination is via pulmonary uptake from the venous blood, followed by intracellular oxidation and reduction to less biologically active derivatives (Crutchley and Piper, 1974). Pulmonary PG metabolites can be further broken down in the liver and kidney via β- (Hamberg, 1968; Diczfakusy and Alexson, 1990) or ω-oxidation (Israelsson et al., 1969; Kupfer et al., 1978) yielding dinor- (18 carbon) or tetranor (16 carbon) PGs before excretion in the urine.

Figure 17: Metabolism of PGs
1.14.6. Counter-Current Exchange

The functional role of PGT acting as an anion exchange transporter was confirmed by Chan et al., (1998) and Banu et al., (2003). Chan et al., (1998) treated PGT-transfected HeLa cells with anion exchange inhibitors (disulfonic stilbenes, nifumic acid and thiol reactive anion MTSES) and observed a substantial inhibition of tracer PGE$_2$ uptake. Reversible inhibition was achieved when the cells were washed after pre-incubation with disulfonic stilbenes, 4,4'-diisothiocyanato-2,2'-stilbene-disulfonic acid (DIDS) or 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid (H$_2$DIDS), for 15 minutes, and PGE$_2$ uptake was restored to $\approx80\%$ in DIDS pre-incubated cells and $\approx65\%$ for H$_2$DIDS pre-incubated cells. However, a 40 minute pre-incubation with the disulfonic stilbenes did not restore PG uptake and irreversible inhibition was suggested. Banu et al., (2003) observed inhibition of bPGT-mediated uptake of PGE$_2$ and PGF$_{2\alpha}$ in a dose-dependent manner by DIDS. In vivo, ewes injected with 100mg DIDS had longer interestrous intervals of 35-42. This dose of DIDS also inhibited oxytocin-induced release of PGF$_{2\alpha}$ from endometrial tissue (Banu et al., 2008). Lactate a derivative of cellular glycolysis was identified as the preferred prostaglandin exchange molecule (Chan et al., 2002). Thus in the case of PGT, the evidence points to a model that transfers lactate for PGT.

Intracellular PG accumulation is time-dependent. Chan et al., (1998) monitored tritiated PGE$_2$ uptake into PGT-transfected HeLa cells and observed rapid PGE$_2$ uptake over 10 minutes that reached a plateau after 20 minutes and returned gradually to baseline by 120 minutes. Banu et al., (2003) observed a similar pattern of PG accumulation in PGT-transfected HeLa cells, with PG influx rate peaking at 20 minutes and decreasing gradually over time. This study also demonstrated a $\approx$20-fold higher influx rate in HeLa cells not transfected with PGT. This pattern of PG accumulation is consistent with the counter-current exchange of a substance down its concentration gradient that dissipates over time. PG mediated intracellular accumulation is coupled to cell glycolysis and the subsequent generation of high intracellular levels of lactate compared to the extracellular environment (Schuster., 2002). This large outwardly directed lactate gradient drives an early, rapid accumulation of PG that shows a distinct peak (Chan et al., 1998). As intracellular lactate levels begin to decline, the outward gradient is removed and the prostaglandin/lactate gradient stops, leading to a gradual return to baseline (Schuster., 2002). Cancerous and rapidly proliferating cells, as observed in an in vitro culture model, have glycolytic rates that are substantially higher than cells from normal tissue, as evidenced by enhanced glucose utilization and lactate formation (Greiner et al., 1994). In the context of bovine female reproduction, estradiol stimulates proliferation of uterine epithelial cells (Reynolds et al., 1998; Gambino et al., 2002) and upregulates the lactate dehydrogenase enzyme responsible for lactate production (Holt and Rhe., 1987). Subsequently, lactate is synthesized in uterine cells in response to estradiol, and it is very likely that lactate is the anion exchanger coupled to PG transport. This biphasic pattern of PG accumulation is directly correlated with intracellular glycolysis (Schuster., 2002). The initial increase in glycolytic activity produces lactate that can be exchanged rapidly with extracellular PG; this transporting system can then subside as a result of the high levels of intracellular anions and transport returns to baseline.
1.14.8. Role for PGT in Physiological Systems

PGT can modulate PG function by regulating the bioavailability of PGs in a particular tissue (Schuster., 1998; Schuster., 2002). Firstly, PGT can deliver PGs to their respective receptors thereby mounting a biological response, or secondly, PGT can terminate the signal by removal of the potent PG from the site. The high level of PGT expression in different regions of the brain (Bito., 1976; Lu et al., 1996; Kis et al., 2006; Scafidi et al., 2007), the vasculature (Topper et al., 1998; Bao et al., 2002; Adachi et al., 2003) and the kidneys (Bao et al., 2002) substantiates a dual role for PGT.
1.15. Prostaglandin Transporter in the Bovine Uterus

In the ruminant uterus, the endometrium is the primary site of PGE\(_2\) and PGF\(_{2\alpha}\) synthesis (Asselin et al., 1996). Endometrial PGs have two distinct fates; transport back to the ovary to induce their endocrine effects or efflux into the uterine lumen (Bazer and Thatcher., 1977). The pattern of bPGT expression during the oestrous cycle and establishment of pregnancy is paramount to understanding the mechanism of PG transport in reproductive tissue to induce its effects.

1.15.1. PGT Expression during the Bovine Oestrous Cycle

PGT was cloned and characterized in bovine endometrium by Banu et al., (2003), and owing to its relatively recent characterization, only limited knowledge is available on the transporter. Banu et al., (2003) monitored bPGT mRNA and protein expression across the bovine oestrous cycle in endometrial and myometrial tissue. Endometrial bPGT mRNA expression was lowest between days 1-9, moderate during days 15 and 19-21 and maximum expression was measured at days 16-18 coinciding with the window of luteal responsiveness to PGs. On day 16, maximum bPGT localization at the basolateral surface of endometrial luminal epithelial cells was observed, with less expression in stromal cells and undetectable levels in myometrial circular and longitudinal smooth muscle cells (Banu et al., 2003). The level of expression is comparatively higher in veins than in arteries. In the myometrial vascular bed on day 16 of the estrous cycle, PGT protein is highly expressed in the tunica intima and smooth cells of tunica media of uterine vein and diffusely expressed in the tunica media of the uterine artery (Banu et al., 2003). Analysis of PGT expression across the human menstrual cycle (Kang et al., 2005) and the ovine estrous cycle (Banu et al., 2008) reveal a similar pattern of expression in endometrial tissue to that observed in bovine endometria. The PGT protein is localized in luminal, glandular, and to a lesser extent, in stromal cells, with maximum expression occurring during estradiol dominance in the human (Kang et al., 2005) and during luteolysis in sheep (Banu et al., 2008).
Elevated levels of bPGT protein during luteolysis at the basolateral surface of luminal epithelial cells and in uterine veins strongly suggests a role for bPGT in the transfer of PGs back towards the ovary (Banu et al., 200; Banu et al., 2008). PGT mRNA and protein are maximally expressed in the bovine corpus luteum in the early luteal phase, 13 days prior to luteolysis and thereafter begin to decline (Arosh et al., 2004).

1.15.2. PGT Expression during Pregnancy

PGs of maternal and foetal origin play critical roles in the maintenance and termination of pregnancy (Banu et al., 2005). In mammals the apparent endometrial transport could prevent accumulation of PGs in the foetal circulation, which would be detrimental to foetal development. In cattle, ineffective PG transport could also induce luteolysis leading to early embryonic loss or abortion (Banu et al., 2005). Caruncular and intercaruncular regions of bovine endometria show elevated PGT mRNA and protein expression at the latter stages of pregnancy compared to the early and mid-stages, in preparation for parturition, whereas the level of expression is constant and low in foetal membranes throughout pregnancy (Banu et al., 2005). PGT mRNA and protein have been shown to be present at a constant basal level of expression in the utero-ovarian plexus both ipsilateral and contralateral to the corpus luteum throughout the course of pregnancy (Banu et al., 2005). The increased bPGT expression in endometria at the end of pregnancy suggests a possible role for bPGT in increasing PG transfer to the ovary where it may contribute to luteolysis and the subsequent steps leading to parturition (Banu et al., 2005).
1.16. Thesis Objective

This study was carried out in order to gain a greater understanding of the components involved in the transport of in bovine uterine tissue. PGs of uterine origin can be relayed back to the CL, where they mediate luteolytic or luteotropic effects, thereby regulating oestrous cycle length and pregnancy. Inappropriate PG synthesis or uncharacteristic PG transport can disrupt bovine reproductive function and may have adverse effects on embryonic survival at the establishment of pregnancy. High embryonic mortality impacts negatively on producer profitability owing to reduced milk yield, fewer calves born and higher veterinary bills to treat infertile cows. By understanding PG transport in uterine tissue more, mechanisms such as addressing dairy cow feeding regimes at different stages of the reproductive cycle may be beneficial to eliminate poor fertility.

The transport of PGs of uterine origin is ultimately determined by total PG synthesis, the uterine hormonal milieu, the presence of a PGT and of exchange anions. Bovine and rat uterine tissue was investigated to identify the PGT protein at different stages of the cycle using immunohistochemistry techniques. In vitro cultured bovine uterine epithelial cells were used as a model to investigate the effect of hormonal stimulation on PGT expression, to assess cytoplasmic accumulation and membrane incorporation of \[^3H\] PGs and to identify bPGT mRNA. Bovine uterine epithelial cells were cultured on SnapWell inserts to form a polarized monolayer and compartmental transport of \[^3H\] PGs between the apical and basolateral membranes was measured. By identifying the components of the PG transport mechanism using different techniques a fuller picture of the determinants of PG transport were obtained. This may ultimately lead to better regulation of the bovine oestrous cycle and prevention of early embryonic loss using enhanced feeding regimes or specially designed therapeutic agents.
Materials and Methods
2.1. Histology

2.1.1. Tissue Samples

Uterine tissue from at least 2 animals (cow or rat) was collected and after the removal of excess material, tissue was cut into transverse sections between 0.5-1cm in length and placed into 4% paraformaldehyde/PBS overnight.

2.1.2. Processing of Tissue for Immunohistochemistry

After overnight incubation in 4% paraformaldehyde, tissue was placed into a processing cassette and dehydrated in different concentrations of ethanol as follows: 70% overnight followed by stepwise incubations in 80%, 90%, 100% and an additional 100% ethanol solution for 2mins each. Dehydrated tissue was placed into 1:1 xylene:ethanol followed by incubation in a pure xylene solution, clearing the tissue of alcohol. Once cleared of ethanol, the cassette containing the tissue was placed into paraffin wax overnight which infiltrated the tissue to help maintain its integrity. After overnight incubation in paraffin wax, the tissue was placed carefully in an upright position into a metal mould and covered with sufficient paraffin wax. This mould was then placed into a fridge at 4°C to allow it to set. Once set, the embedded uterine tissue was cut into ribbons of tissue sections of 7μm in thickness using a microtome. The sections were collected onto a glass microscope slide and allowed dry overnight.
2.1.3. Solutions for Staining Tissue

Tris-buffered saline (TBS) is the preferred rinse buffer for staining tissue sections. TBS was prepared at a stock 10X concentration (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) and stored at 4ºC. TBS was diluted to a 1X concentration for staining tissue. Baths with different concentrations of ethanol (100%; 95%; 90%; 80%; 70%; 50%) and two baths of 100% xylene were prepared prior to staining. An appropriate volume of 3% H₂O₂/MeOH was made directly before use and stored in the dark. Goat serum (G9023) was prepared in 1X TBS at a 10% concentration and was applied as a blocking solution to minimize unspecific background staining. Goat serum (10%) was also the diluent used for the preparation of primary and secondary antibodies. DAPI (Sigma 32670) was supplied as a powder and a stock solution of 1mg/mL was made with distilled water. This 1mg/mL DAPI solution was further diluted 1:50 with water prior to adding to the tissue section.

2.1.4. Tissue Section Staining Procedure

Slides were dewaxed by placing them into two different xylene solutions for 2 minutes each. The sections were rehydrated then by placing into decreasing concentrations of ethanol (100%; 95%; 90%; 80%; 70%; 50%) for 5mins each. Tissue sections were rinsed in distilled water for 5mins and endogenous peroxidase activity was blocked by incubating tissue in 3% H₂O₂/MeOH for 15mins. Sections were washed 3 times in 1X TBS, 5mins per wash. Goat serum (10%) was applied onto the tissue sections and incubated for 90mins in an incubation chamber. After incubation in the blocking solution, sections were incubated overnight at 4ºC with Prostaglandin Transporter Polyclonal Antibody (Cayman Chemicals 160200), which was diluted 1:250 with 10% goat serum. Incubation of the tissue overnight at 4ºC prevents evaporation of the antibody. After overnight incubation, sections were washed in 1X TBS 3 times (5mins per wash). The anti-IgG secondary antibody was then diluted 1:64 in 10% goat serum and applied to the tissue section for 60mins. Sections were washed again three times in 1X TBS before application of the nuclear stain DAPI. After a 20 second incubation with DAPI, slides were washed 3 times in 1X TBS before mounting with Fluoromount and sealing with nail varnish.
2.2. Isolation and Culture of Bovine Uterine Epithelial Cells

2.2.1. Cell Culture Conditions

All cell culture procedures were performed in a sterile laminar flow using strict aseptic techniques. Cells were cultured in an incubator at 37°C in and an atmosphere of 5% CO₂ in air. Milli Q (18MΩ) water was used in the preparation of all media and other solutions. Media and solutions were prepared and filter-sterilized through a 0.2μM minisart filter (Sartorius, Cat No. 16534K) or a 0.22μM millex-GP50 filter (Millipore, Cat No. SLGPB5010), depending on the required volume.

2.2.2. Basic Culture Medium

The basic culture medium (BCM) for bovine uterine epithelial cell (BUEC) culture consisted of a 1:1 mixture of Dulbecco’s Modified Eagle Medium and nutrient mixture Hams F12 with 15mM Hepes (DMEM/F-12 Ham), without phenol red (Sigma D2906). One litre of the medium was prepared in Milli Q water, supplemented with 1.2g NaHCO₃ (Sigma S5761) and the pH adjusted to 7.4. The medium was filter-sterilized and stored at 4°C for no longer than two weeks. Some experiments required the use of culture medium containing phenol red which was obtained as a powder from Gibco (52100-039) and is rich in glucose (4500mg/L). One vial or 13.38g of powdered DMEM was dissolved in 900mL of water which was supplemented with 3.7g NaHCO₃ (Sigma S5761) and 0.11g sodium pyruvate (Sigma P5280) to prepare the phenol red BCM. The solution was made up to 1L, pH adjusted to 7.4, filter sterilised and stored at 4°C.
2.2.3. Supplements for Cell Culture Medium

Stock solutions of 150 IU/mL penicillin G (Sigma P3032); 150IU streptomycin sulfate (Sigma S9137) and 2mM L-glutamine (Sigma G8540) were prepared at 100X in phosphate buffered saline (PBS), Tables 1 and 2 respectively, filter-sterilized and stored at -20ºC. Fungizone (Gibco 15290-026) and foetal bovine serum (Gibco 10270-106) were purchased ready to use, aliquoted into appropriate volumes and stored at -20ºC.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume(g/50mL 1X PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>0.436</td>
</tr>
<tr>
<td>Streptomycin Sulfate</td>
<td>0.724</td>
</tr>
</tbody>
</table>

Table 1: Pen/Strep

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume(g/50mL 1X PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>1.461</td>
</tr>
</tbody>
</table>

Table 2: L-glutamine
2.2.4. Initial Culture Medium

Initial Culture Medium (ICM) consisted of BCM, supplemented as in Table 3, and was used to culture cells directly after their isolation from the bovine reproductive tract. BSA (Sigma A9418) was added to give a final concentration of 1%.

<table>
<thead>
<tr>
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<tr>
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</tr>
<tr>
<td>FCS</td>
<td>0.77</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>3</td>
</tr>
<tr>
<td>Fungizone</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 3: Initial Culture Medium

2.1.5. Complete Culture Medium

Complete culture medium (CCM) for BUEC culture consisted of BCM (Section 2.2.2.) supplemented as in Table 4 and is used routinely in BUEC culture.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume (mL/100mL)</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>FCS</td>
<td>10</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1</td>
</tr>
<tr>
<td>Fungizone</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4: Complete Culture Medium
2.2.6 Serum free Medium

Different cell treatments were prepared in serum free medium (SFM). Serum free medium (Table 5) does not contain FCS, which may influence responsiveness of the cells. FCS is an undefined media supplement that contains components such as growth factors and hormones that can vary even between batches. Serum free media was used to standardize the experiments and eliminate a serum effect. Insulin-transferrin-selenium (ITS) from Sigma (I3145) was added as a supplement to aid cell viability and growth in the absence of serum.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume (mL/100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCM</td>
<td>96</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1</td>
</tr>
<tr>
<td>Fungizone</td>
<td>1</td>
</tr>
<tr>
<td>ITS</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5: Serum-free Culture Medium supplemented with 0.1% BSA
2.2.7. Phosphate Buffered Saline

Cell culture supplements were prepared in 1X phosphate buffered saline (PBS). PBS was made in Milli Q water as in Table 6 and pH adjusted to 7.4 using 1M NaOH or 1M HCl as required. NaCl and KH$_2$PO$_4$ were purchased from Sigma and the other salts were obtained from BDH.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.00</td>
</tr>
<tr>
<td>KCl</td>
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<tr>
<td>Na$_2$HPO$_4$.12H$_2$O</td>
<td>2.89</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 6: Phosphate Buffered Saline
2.2.8. Isolation of Bovine Uterine Epithelial Cells

The method adopted to isolate BUECs is similar to the isolation techniques used by Dickens et al., (1993) with slight modifications. Reproductive tracts obtained at slaughterhouse were transported back to the laboratory where ovaries, oviducts, cervix, vagina, excess connective tissue, major blood vessels and fat tissue were removed. Uterine horns were separated and washed in Ca\(^{2+}\) and Mg\(^{2+}\) free 1X HBSS (Sigma H4891). Isolated uterine horns were brought into the laminar flow and everted before placing into a 50mL centrifuge tube (Sarstedt 62.547.254 PP) containing an enzyme solution of trypsin (Sigma T9201) and pancreatin (Sigma P3292). Everted uterine horns, in enzyme solution, were vortexed and incubated at 37ºC for 60 minutes. After the incubation period, cells were scraped from the luminal surface using a sterile blade to ensure maximum epithelial cell recovery. Cell suspensions were pooled together in Ca\(^{2+}\) and Mg\(^{2+}\) free 1X HBSS and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the pellet contained the epithelial cells. The epithelial cells were combined in Ca\(^{2+}\) and Mg\(^{2+}\) free 1X HBSS and centrifuged in order to wash the epithelial cell suspension before plating onto T75 flasks (Sarstedt 83.1813.300) in ICM gassed with 5% CO\(_2\) and pre-warmed to 37ºC. After 24 hours in culture ICM was replaced with CCM, medium was changed routinely every 48 hours with CCM. In the event of culturing BUECs in well-plates, BUECs were detached from the T75 flask via enzymatic digestion with 5mL 0.05% Trypsin-EDTA (1X) (Gibco 25300) for 5mins. Trypsin-EDTA was inactivated by addition of 5mL FCS and the cell suspension was pooled and centrifuged at 1500rpm for 5mins to form a cell pellet. The supernatant was discarded and cell concentration was obtained using a haemocytometer. A suitable dilution was performed and BUECs were plated at a concentration of 1 x 10\(^5\) cells/mL.
2.2.9. Enzyme Solution for BUEC Isolation

Trypsin and pancreatin are proteolytic enzymes synthesized in the pancreas and can be used to isolate epithelial cells by disrupting cell membrane adhesiveness. Pancreatin was initially added to 100mL 1X HBSS at a concentration of 2.5%, aliquoted in 1mL volumes and stored at -20°C, the enzyme solution for epithelial cell digestion was prepared as in Table 7 directly before use.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.1g</td>
</tr>
<tr>
<td>2.5% Pancreatin</td>
<td>0.54mL</td>
</tr>
<tr>
<td>1X HBSS</td>
<td>20mL</td>
</tr>
</tbody>
</table>

Table 7: Enzyme Solution
2.3. Preparation of Treatments

2.3.1. Steroid Hormone Preparation

Stock solutions 17-β-estradiol (Sigma E8875) and progesterone (Sigma P0130) were prepared at a 50mM concentration, while fulvestrant (Sigma I4409) and mifepristone (Sigma M8046) were prepared at a concentration of 10mM in 5mL ethanol. Hormones and antagonists were stored at -20ºC in glass bottles. Working solutions were prepared by serially diluting the stock solution in culture medium until desired the concentration was obtained. In order to assess the inhibitory effects of the steroid-hormone receptor antagonists, fulvestrant or mifepristone was added 30mins before the addition of 17-β-estradiol or progesterone, respectively.

2.3.2. Oxytocin Hormone Preparation

Syntocinon, a commercially available oxytocin analogue was purchased as a solution at a concentration of 17μM. The solution was diluted in culture medium yielding a treatment concentration of 1μM.

2.3.3. Glucose Preparation

D- (+) - glucose (Sigma 47829) was prepared at a concentration of 0mM, 18mM, 25mM or 42mM, in SFM (Table 5) and added to BUECs for 18hours prior transport study.

2.3.4. Indomethacin and TPA

Indomethacin (Sigma I7378) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma 79346) were dissolved in ethanol and diluted in culture medium to give concentrations of 10μM each.
2.3.5. Polyunsaturated Fatty Acid Preparation

PUFAs were prepared using strict techniques to ensure maximum delivery to the BUECs (Mattos et al., 2003). Arachidonic acid (Sigma A3555), docoshexaenoic acid (Cayman Chemicals 90130) and eicosapentaenoic acid (Cayman Chemicals 90110) were initially dissolved in ethanol to give a stock concentration of 50mg/mL, which was stored at -20°C. On the day of treatment, fatty acid stock solution was diluted in BCM containing 33mg/mL fatty-acid free BSA (Sigma A0281) to give a working concentration of 1mM. The 1mM working solution was incubated for 2 hours in a water bath held at 37°C. After the 2 hour incubation period, fatty acids were serially diluted to generate the treatment concentrations (50µM or 100µM).

2.3.6. Preparation of charcoal stripped FCS (CS-FCS)

Charcoal stripping FCS is a complex process that removes steroids from the serum, thereby permitting the study of the effects of steroid hormones in cell culture systems. In order to charcoal strip FCS, 2.5g washed activated charcoal (Sigma C9157) and 0.25g dextran (Sigma 00268) were added to 1L of 0.01M Tris-base buffer (pH 8.0). This solution was stirred overnight at 4°C and autoclaved the following day. Equal volumes of dextran coated charcoal suspension and FCS were combined and stirred at 45°C for 60 mins. Aliquots were centrifuged at least 3 times at 3,000 rpm for 5 mins to remove excess charcoal. The CS-FCS was filter sterilized, aliquoted in 10mL volumes and stored at -20°C.
2.4. Immunohistochemistry

Immunofluorescence is a technique used to detect antigens in or on cells by using specific antibodies to that antigen. Indirect immunohistochemistry is a 2-step process whereby an unlabelled antibody highly specific to the antigen of interest is incubated with the cell. This antibody is detected via application of a fluorophore-labelled anti-immunoglobulin, specific to the primary antibody applied (Fig. 17). Competent and viable BUECs were immunostained to confirm epithelial cell cytology and to detect the PGT protein in BUECs treated with hormones.

2.4.1. Epithelial Cell Validation

In order to validate the epithelial cell histology, BUECs were positively immunostained for the epithelial cell intermediate filaments cytokeratins (C-11+PCK-26+CY-90+KS-1A3+M20+A53-B/A2), and negatively stained for the fibroblast intermediate filaments, vimentin. Confluent BUECs were washed in pre-warmed PBS (37ºC) and fixed at room temperature in 4% paraformaldehyde/PBS for 25 minutes. BUECs were washed twice with Rinse Buffer and then permeabilised with 0.1% Triton-X/PBS for 10 mins at room temperature, followed by 2 additional washes with Rinse Buffer. A blocking solution consisting of 4% goat serum/PBS was applied for 60 mins at room temperature, followed by 3 washes with Rinse Buffer. The IgG Monoclonal anti-pan cytokeratin (Sigma C9687) and the IgM monoclonal anti-vimentin (V2258) were diluted in blocking solution and added to BUECs for 60 mins at room temperature, which were then washed 3 times with Rinse Buffer. An anti-mouse IgG (whole molecule) -Trit-C secondary antibody (Sigma T5393) was diluted (1:400) in PBS and applied to BUECs to validate the cytokeratin intermediate filaments, while an anti-mouse IgM-Fit C secondary antibody (Sigma F9259) was diluted (1:256) and applied to determine the vimentin intermediate filaments. DAPI is a fluorescent stain that binds to the negatively charged DNA of the nucleus and is applied to cells as a counterstain during immunostaining. BUECs were covered with PBS and were not permitted to dry before visualization with a fluorescent microscope. Fluorescence was detected using the Zeiss Axiovert 200 inverted fluorescent microscope. In line with Planck’s Quantum Theory and the Bohr Theory the fluorophores (Trit-C and Fit-C) absorb light at certain wavelength and emit this energy as a different, discrete wavelength. The excitation wavelength for Trit-C is 540nm and the emission wavelength is 572nm, while Fit-C is excited at 494nm and emits at 518nm.
2.4.2. Localization of PGT in BUECs

The immunostaining technique used to detect PGT is similar to the technique used for determining intermediate filaments (Section 2.4.1.), except different primary and secondary antibodies are employed. Anti-IgG PGT was the primary antibody that was diluted 1:250 with 4% goat serum/PBS (blocking solution) prior to application for 60 mins. The secondary antibody was an anti-IgG antibody with a Fit-C fluorophore. Cells were counterstained with the nuclear stain, DAPI, and visualized using a fluorescent microscope.

Figure 17: Principles of Immunohistochemistry
2.5. Transport Studies

2.5.1. Transport Solutions

The standard solution for all transport studies was a basic saline solution (Table 8), unless otherwise stated. Tritiated PGs purchased from Amersham were prepared at 1nM in BSS and 5% BSA (Sigma A2153) was also prepared in BSS. A cell lysis solution (Table 9) was made up in MilliQ water and the scintillation cocktail solution was purchased from BioChemika (03999). One nM \([3\text{H}]\) PGE\(_2\) was prepared by carrying out a 1:943 dilution in BSS, while 1nM \([3\text{H}]\) PGF\(_{2\alpha}\) was obtained by performing a 1:549 dilution in BSS.

2.5.2. Prostaglandin Uptake Study

BUECs were washed with pre-warmed (37°C) BSS and the study was initiated by addition of 1mL of 1nM \([3\text{H}]\) PG in BSS to each well of a 24-well plate at time zero. PG transport was stopped by removing the \([3\text{H}]\) PG then a rapid wash with 5% BSA/BSS followed by 2 additional rapid washes with BSS. Cells were lysed by adding 200μL of lysis buffer, which was then collected in an eppendorf. The well-plate surface area was further washed with 200μL BSS to ensure maximum recovery of cellular components and this was then added to the cell lysate and mixed thoroughly. A 20μL sample was used to determine protein content, while the remaining solution was measured using a β-scintillation counter.

2.5.2.1. Prostaglandin Uptake Study over Time

For studies that measured PG uptake over time, BUECs were washed with pre-warmed (37°C) BSS and exposed to \([3\text{H}]\) PG for 1; 2; 5; 10; 15 or 20mins. At the end of the exposure time, BUECs were washed with 5% BSA/BSS followed by 2 additional rapid washes with BSS. Cells were lysed by adding 200μL of lysis buffer, which was then collected in an eppendorf. The well-plate surface area was further washed with 200μL BSS to ensure maximum recovery of cellular components. A 20μL sample was used to determine protein content, while the remaining solution was measured using a β-scintillation counter.
2.5.2.2. Prostaglandin Uptake into Cytosolic or Membrane Cellular Compartments

In studies when cytosolic accumulation and membrane incorporation of \[^{3}\text{H}\] PG were measured, the cellular components remaining at the end of the exposure to \[^{3}\text{H}\] PGs were pooled and collected and a 20µL sample was used to determine total protein content. The remaining solution was spun at 15,000 x g for 5mins. After centrifugation, the supernatant, containing the cytosolic compartment and the pellet containing the membranes (reconstituted with 200µL BSS) were placed into separate eppendorfs to localize \[^{3}\text{H}\] PG in BUECs. The BUEC sample solution was combined with 5mL scintillation cocktail and vortexed prior to measurement of radioactivity using a β-scintillation counter.

2.5.2.3. Prostaglandin Uptake into BUECs at different pH levels

\[^{3}\text{H}\] PGs were prepared in BSS at normal physiological pH (pH 7.4) or in an acidic pH (pH 3.0). BUECs were washed with BSS (pH 7.4) and \[^{3}\text{H}\] PGs at either pH were applied for the duration of the experiment. At the end of the experiment, BUECs were washed with BSS (pH 3.0 or pH 7.4) cellular components were recovered and protein content measured before determining radioactivity.

2.5.3. Prostaglandin Efflux Study

In efflux transport studies, \[^{3}\text{H}\] PG was allowed to accumulate in BUECs by incubating cells with the tritiated PG for 20minutes. After the incubation period, \[^{3}\text{H}\] PG was removed and cells were washed rapidly with BSS and the \[^{3}\text{H}\] PG solution was replaced with BSS. Experiment was stopped at 1; 2; 5; 10; 15 and 20mins with the removal of the BSS which was placed into a scintillation vial with 5mL scintillation cocktail, vortexed and radioactivity counted using a β-scintillation counter. A protein content of the BUECs was obtained to standardize the experiments.
2.5.3.1. Prostaglandin Efflux into elution solutions of different pH

\[^3\text{H}\] PGs were prepared in BSS at normal physiological pH (pH 7.4) or in an acidic pH (pH 3.0). BUECs were washed with BSS (pH 7.4) and \[^3\text{H}\] PGs at either pH was applied for 20 minutes in order to pre-load the BUECs with \[^3\text{H}\] PG. Elution solution was replaced with saline or cold PG prepared at pH 3.0 or pH 7.4 and efflux was recorded over time by removing the solution and adding to a scintillation vial for radioactivity measurements. A protein content of the BUECs was obtained to standardize the experiments.

2.5.4. Prostaglandin Vectorial Transport

Polarized monolayers convey cell membrane asymmetry and contain 2 distinct cell membrane surfaces, an apical surface that faces the lumen and a basolateral surface that extends away from the lumen towards the interstitium. Culture of epithelial cells on SnapWell filters facilitates their polarization, thereby providing a physiologically representative cell culture model for experimentation. Asymmetric distribution of proteins between the membranes may impede or enhance vectorial transport of molecules between compartments. For experiments assessing transepithelial PG flux, BUECs were cultured on SnapWell Filters (Costar) to generate a polarized monolayer. 250μL of cell suspension was added onto the filter and 2.4mL CCM was added to the well (Fig. 18). Transepithelial electrical resistance (TEER) was measured daily, using ‘chopstick’ electrode connected to an epithelial voltmeter (EVOM). This device enables TEER measurement by immersing one electrode into the CCM at the bottom of the well-plate and immersing the shorter electrode into the media surrounding the cells cultured on the filter. BUECs with TEER = 2kΩ/cm\(^2\) were used for PG transepithelial flux, whereby either 250μL (apical) or 2.4mL (basolateral) \[^3\text{H}\] PG was added to one surface and the volume on the contralateral surface was recovered at different time points to measure radioactivity. A protein content of the BUECs was obtained to standardize the experiments.
2.5.5. Determination of Protein Content of BUECs

Protein content of BUECs was measured using the Bradford Assay. A sample of the lysed BUECs (6µL) was added to a 96-well plate to which 300µL of Bradford Reagent (Sigma B6916) was added. Protein content was ascertained by measuring the sample in a spectrometer at 595nm against a BSA standard curve.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume(g/L)</th>
</tr>
</thead>
<tbody>
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<td>Na-Hepes</td>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>MgSO₄</td>
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<td>KCl</td>
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</tr>
<tr>
<td>D-glucose</td>
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</table>

Table 8: Basic saline solution
### Materials and Methods

Table 9: Cell lysis buffer

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<thead>
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</tr>
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<td>NaOH</td>
<td>20.00</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
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</tr>
</tbody>
</table>

2.6. Molecular Biology Techniques

Molecular biology techniques were carried out under strict conditions to prevent incorporation of contaminating RNA and DNA. All surfaces, pipettes and equipment were decontaminated with 70% ethanol prior to commencement of molecular work and were frequently decontaminated over the course of the experiment. Tubes were labelled prior to molecular work to ensure coherent continuation of the complex analytical process.

2.6.1. RNA Isolation

Cells used for molecular biology studies were cultured in T25 flasks (Sarstedt 83.1810.300) and treated for 24 hours before RNA was isolated. RNA isolation was achieved using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma RTN70). Cells were lysed in culture flask with 250μL of lysis buffer containing 1% mercaptoethanol. The cell suspension was filtered through a blue filter column, centrifuged at 15,000 x g for 2mins and collected in a collection tube. 250μL of 70% ethanol was added to the lysate and mixed gently by repeat pipetting. The lysate-ethanol suspension was added to a red binding column and centrifuged at 15,000 x g for 15seconds and the flow-through was discarded. The red binding column was washed by addition of 250μL Wash Solution 1 and centrifuged at 15,000 x g for 15seconds. To improve RNA quality, contaminating DNA was removed via DNase treatment of the suspension. This was achieved by the addition of 80μL of DNase and DNA buffer solution to the red binding column for 15mins at room temperature. Following incubation, cells were washed with 250μL Wash Solution 1, centrifuged at 15,000 x g for 15seconds and flow-through was discarded. An additional wash with 500μL Wash Solution 2 was carried out and flow-through was discarded. Pure RNA was finally eluted by the addition of 50μL Elution
Buffer and subsequent centrifugation at 15,000 x g for 1min. RNA quantity and quality was assessed directly after isolation. RNA was used immediately or placed into RNA Later, which preserved RNA for 24 hours at 4ºC or for 4 weeks at -20ºC.

2.6.2. RNA Quantification

RNA was assessed for quantity and quality by measuring the ratio of absorption at 260nm and 280nm. The RNA sample was diluted (1:50) with nuclease free water and placed into a quartz cuvette. Nuclease free water was used to blank the reading before absorption was read. Only RNA samples with ratios in the range of 1.6 and 2.2 were used for future molecular biology analysis.

The RNA concentration was calculated using Beer’s Law, \( C = \frac{A}{el} \), where \( A \) is the measured absorbance at 260nm, \( e \) is the extinction coefficient (25μL/μg/cm) and \( l \) is the pathlength (1cm).

2.6.3. Reverse Transcription – Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was conducted using an enhanced avian HS RT-PCR Kit (Sigma HSRT-100) with specially designed primers. The one-step RT-PCR reaction was performed as per the manufacturer’s instructions and requires the conversion of RNA to cDNA, which is exponentially amplified to enable assessment. Briefly, 20ng/μL of isolated RNA was used per reaction, 1μM of forward and reverse primers, 3.0 mM MgCl₂ (including MgCl₂ in PCR buffer), 0.4 units/μL RNase inhibitor, PCR buffer (100mM Tris-HCl, pH 8.3; 500mM KCl, 15mM MgCl₂; 0.01% gelatin), 200μM dNTPs, 0.4 units/μL eAMV-RT, 0.05 units/μL JumpStart AccuTaq DNA polymerase and PCR reagent water in a total 50μL volume in PCR reaction tubes. The reactions were gently mixed and placed into a Biometra Thermocycler.
2.6.3.1. Thermocycler Conditions

A specific thermo-cycling program was selected, that synthesized first strand cDNA from the RNA template and then exponentially amplified the cDNA. The cycling parameters were as follows: 50°C for 50 minutes; 94 °C for 2 minutes; 35 cycles of (94°C for 15 seconds; 55°C for 30 seconds and 68°C for 1 minute); and 68°C for 5 minutes for the final extension. The PCR products were then analysed using agarose gel electrophoresis and ethidium bromide staining.

2.6.4. RT-PCR Primers

The RT-PCR primers were synthesized by Genosys (Sigma) and prepared at a concentration of 100μM in nuclease-free water. Table 10 summarizes PCR primer sequences and product length.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>18sRNA</td>
<td>DQ222453.1</td>
<td>5’ aagtctttgggtccggg 3’ ggacatctaagggcacca</td>
<td>368</td>
</tr>
<tr>
<td>bPGT</td>
<td>NM_174829.2</td>
<td>5’ cacgctggetgcagtgaatcaac 3’ gagacattgtgtcatagtagac</td>
<td>416</td>
</tr>
</tbody>
</table>

Table 10: RT-PCR Primers
2.6.5. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate and analyze the DNA. The DNA was visualised in the gel by the addition of ethidium bromide, a fluorescent dye that intercalates between the DNA bases. The electrophoresis tray was thoroughly cleaned with 70% ethanol and the well forming comb was inserted. Agarose gel at a concentration of 1.5% was prepared by adding 1.5g to 100mL of 0.5X TBE (Table 11). The solution was heated in a microwave for approximately 1.5 minutes or until the agarose had dissolved. The agarose solution was allowed to cool for about 5minutes, then 2.5μL of ethidium bromide was added (Invitrogen 15585011), giving a 1mg/mL concentration. The gel was slowly and carefully poured into the electrophoresis tray and allowed set for 90minutes. Once the gel had set, the comb was removed and 0.5X TBE was poured into the tray, submerging the gel. The samples were prepared by adding 10μL of the PCR product and 5μL of loading buffer (Table 13) and centrifuged briefly at 15,000 x g. The samples were then carefully added into the wells of the gel and a DNA ladder was added for comparison. The electrophoresis tray was closed and set to run at 60V for 15 minutes, after which the voltage was increased to 75V for 3.5 hours or until the samples had run 90% the length of the gel. The gel was carefully removed from the electrophoresis tray and placed into a UV light box where images of the gel were taken to assess gene expression.

2.6.6. Tris Borate EDTA Buffer

Tris borate EDTA (TBE) buffer, which functions as a solvent that can protect RNA and DNA from degradation, was prepared as 5X stock solution

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>54.00</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>27.50</td>
</tr>
<tr>
<td>Na-EDTA</td>
<td>4.65</td>
</tr>
</tbody>
</table>

Table 11: 5X TBE
2.6.7. Loading Buffer

The loading buffer was used to give a greater density to the samples to guide their direct passage along the gel and since the dye is negatively charged in neutral buffers and moves at the same rate as the DNA.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume(g/10mL Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol Blue</td>
<td>54.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>27.50</td>
</tr>
</tbody>
</table>

Table 12: Loading Buffer

2.7. Statistical Analysis

All transport studies were corrected to dpm per mg protein and results were examined by analysis of variance using the Apple Macintosh software package SuperANOVA.
2.8. Experimental Rationale

2.8.1. Expression of PGT protein in uterine tissue using immunohistochemical techniques
In this section we examined the expression of PGT protein in bovine and rat uterine tissue in order to identify the compartmental localization of PGT within uterine tissue, demonstrate an effect of cycle stage on the expression of PGT and emulate a study carried out by Banu et al., (2003). Immunohistochemistry techniques (Section 2.1) were applied and tissue was visualized via fluorescent microscopy (Section 2.4) to localize PGT expression in uterine tissue.

2.8.2. Characterization of isolated bovine uterine epithelial cells in culture
PGT expression was localized in the epithelial cells of uterine tissue (Section 3.1), therefore a cell culture model using bovine uterine epithelial cells (BUECs) was required to study prostaglandin transport. BUECs were enzymatically isolated (Section 2.2.) and positively stained for cytokeratin, and used to measure PGT expression under different hormonal treatments conditions (Section 3.3.), measure prostaglandin transport (Section 3.4.) and investigate PGT mRNA expression in these cell under different hormonal environments (Section 3.5.).

2.8.3. Effects of hormone treatment on PGT expression in cultured BUECs
In this section we examined the expression of PGT protein in BUECs under different hormonal conditions. We previously demonstrated an effect of stage of oestrous cycle on the expression of PGT in uterine tissue (Section 3.1), and this experiment set out to investigate a role for female sex hormones on the expression of PGT protein in BUECs. Additionally, it has been shown that progesterone can stimulate expression of oatp protein (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006), while oestradiol downregulates oatp protein expression (Lu et al., 1996; Grube et al., 2006). Confluent isolated BUECs (Section 3.2.) were treated with hormones for 24 hours (3.3.1); 48 hours (3.3.2); 72 hours (3.3.3) or with antagonists of the female sex hormones for 24 hours (3.3.4.) in order to investigate a temporal effect of hormones on PGT protein expression as measured by immunohistochemistry techniques (Section 2.1).
2.8.4. Measure PG transport in BUECs under different conditions

Results from immunohistochemistry experiments (Sections 3.1 and 3.3) demonstrate expression of the PGT protein in BUECs that displays differential expression between treatments. Leading on from these experiments, [\(^3\)H] PG uptake into BUECs, [\(^3\)H] PG efflux from BUECs and vectorial transport of [\(^3\)H] PG between apical and basolateral membranes of a polarized monolayer (Section 2.5) was measured to investigate the functional role for PGT. BUECs were grown in different culture media (Sections 3.4.1; 3.4.2; 3.4.3 or 3.4.4) in order to investigate [\(^3\)H] PG uptake in unstimulated BUECs. Some BUECs were treated with female sex steroids and [\(^3\)H] PG uptake was measured to examine an effect of hormones. Immunohistochemistry studies from this thesis demonstrates an effect of hormones on the expression of PGT (Section 3.1. and 3.3.) and measuring [\(^3\)H] PG uptake after hormone treatment was necessary to explore the functional role for PGT. PUFAs are precursors to PG synthesis (Section 1.6) and PGs can directly affect [\(^3\)H] PG transport (Bao et al., 2002), therefore uptake studies investigating the effects of EPA, DHA and AA on [\(^3\)H] PG uptake into BUECs were examined (Section 3.4.6.). In a study conducted by Chan et al., (2002), lactate was shown to be the preferred exchange molecule for PG transport when HeLa cells transfected with PGT were cultured overnight in increasing concentrations of glucose. In line with the Chan et al., (2002), an effect of overnight glucose incubation was carried out to investigate a lactate effect on [\(^3\)H] PG uptake into BUECs (Section 3.4.7). Some BUECs were co-cultured with glucose and hormones to examine a lactate/hormone interaction (Section 3.4.7.). Since PGs predominate as the charged anion at physiological pH (7.4), the effects of different pH levels of the transport of [\(^3\)H] PG in BUECs was examined (Section 2.5.2.3) to investigate the uptake of [\(^3\)H] PGs with different charges (Section 3.4.8.). This study, involving the alteration of the pH in which [\(^3\)H] PGs were prepared and the subsequent uptake into BUECs, was carried out in line with work conducted by Jones and Harper, (1983). Experiments that measured the efflux of [\(^3\)H] PG into saline or equimolar concentration of PGs at different pH levels was conducted to examine whether BUECs were capable of releasing PGs and what conditions favour PG efflux (Section 3.4.9.). Vectorial transport of [\(^3\)H] PG across polarized BUECs gives insight to the apical and basolateral membrane permeability of BUECs to PGs (Section 3.4.10). Furthermore, polarization of BUECs provides a more accurate reflection of the state of epithelial cells in vivo.
2.8.5. PGT mRNA Expression in BUECs

In this section we examined the expression of PGT mRNA in BUECs under different hormone conditions because we wanted to investigate whether BUECs were constitutively expressing PGT mRNA or whether hormones regulated the expression of PGT mRNA in BUECs.
Results
3.1. Expression of PGT protein in uterine tissue using immunohistochemical techniques

3.1.1. Expression of the PGT protein in bovine uterine tissue from the luteal and follicular phases of oestrous cycle.

PG synthesis in bovine endometrial epithelial cells is hormone specific (Asselin et al., 1996; Skarzynski et al., 1999) and immunohistochemical staining of bovine uterine tissue for PGT expression at various times of the cycle was examined to determine if PGT expression is hormone / cycle dependent. Bovine ovaries were assessed to determine estrous cycle stage (Ireland et al., 1980) and were subsequently treated with a saline solution or oxytocin for 15mins prior to fixation, processing and immunostaining (Section 2.1.). These experiments clearly identify PGT in the bovine uterine tubes contralateral and ipsilateral to the CL (Fig. 20).
Results

Figure 20: Representative images from at least 2 animals of uterine tissue from the luteal phase immunostained for PGT (mag. 10X). A) Contralateral uterine tube to CL exposed to oxytocin B) Ipsilateral uterine tube to CL exposed to oxytocin C) Contralateral uterine tube to CL exposed to saline D) Ipsilateral uterine tube to CL exposed to saline. Regions of uterine tissue are labelled as followed: glandular epithelium (GE); luminal epithelium (LE); stroma (ST) and uterine lumen (UL).

Oxytocin treatment increased PGT expression in the ipsilateral uterine tube (Fig. 20B) and decreased protein expression in the contralateral uterine tube (Fig. 20A) to the CL. Furthermore expression was localized primarily in the luminal epithelium (LE) and glandular epithelium (GE) with negligible expression in the stromal tissue (ST) (Fig 19).

In general, PGT protein density was limited in the bovine uterine tract from the luteal phase, where its expression spiked following an oxytocin challenge to the uterine tube ipsilateral to the CL (Fig. 20B). Progesterone priming, a short term pulse of oxytocin or estradiol may therefore be involved in the regulation of PGT in bovine uterine tissue.
PGT expression was absent from the luminal and glandular epithelial layers in bovine uterine tissue isolated from the follicular phase, regardless of oxytocin treatment or proximity to the ovary containing the large follicle (Fig. 21). In these experiments, the follicular phase was assigned to reproductive tissue bearing a large follicle (LF), presumed to be an estrogen-active, dominant preovulatory follicle (Ireland et al., 1980).

Figure 21: Representative images from at least 2 animals of uterine tissue from the follicular phase immunostained for PGT (mag 10X). A) Contralateral uterine tube to LF exposed to oxytocin B) Ipsilateral uterine tube to LF exposed to oxytocin C) Contralateral uterine tube to LF exposed to saline D) Ipsilateral uterine tube to LF exposed to saline. Regions of uterine tissue are labelled as followed: glandular epithelium (GE); luminal epithelium (LE); stroma (ST) and uterine lumen (UL).
3.1.2. Expression of PGT protein in rat uterine tissue from each phase of the oestrous cycle

Rat uterine tissue was assessed for PGT expression at different stages of the estrous cycle and to examine whether oxytocin alters protein expression. The rat was used owing to its short oestrous cycle length and the ease with which detection of the different stages of the cycle achieved. Following dissection, uterine tissue from each stage of the estrous cycle was placed into saline solution or a solution of oxytocin for 15mins prior to PGT identification (Section 2.1.). Expression of PGT was highest in unchallenged tissue during proestrous (Fig. 22C) decreased during diestrous (Fig. 22B) and estrous (Fig. 22D), and was absent at metestrous (Fig. 22A).

Figure 22: Expression of PGT in rat uterine tissue at different stages of the estrous cycle: A) metestrous B) diestrous C) proestrous and D) estrous. Regions of uterine tissue are labelled as followed: glandular epithelium (GE); luminal epithelium (LE); stroma (ST) and uterine lumen (UL).
Results

PGT expression increased in luminal epithelium as proestrous (Fig. 22C) and estrous (Fig. 22D) approached, thereby indicating a role for estradiol or progesterone withdrawal on the transport of PGs in rat uterine tissue, while PGT levels were low in metestrous (Fig. 22A) and diestrous stages (Fig. 22B). PGT expression increased during estradiol dominance, which contrasts to PGT expression in bovine uterine tissue, which may indicate a species effect on the regulation of PGT.
Stimulation of rat uterine tissue with oxytocin delayed PGT expression in rat uterine tissue where highest expression was observed during the estrous stage of the cycle (Fig 23D). This contrasts to elevated PGT expression in unchallenged rat uterine tissue from the proestrous stage (Fig. 22C).

Figure 23: Expression of PGT in rat uterine tissue following an oxytocin challenge at different stages of the oestrous cycle: A) diestrous B) metestrous C) proestrous and D) estrous. Regions of uterine tissue are labelled as followed: glandular epithelium (GE); luminal epithelium (LE); stroma (ST) and uterine lumen (UL).

PGT protein is localized in uterine epithelial cells of rat uterine tissue and displays a differential level of expression that is stage specific with elevated PGT expression during estradiol dominance and oxytocin stimulation in luminal epithelium cells (Fig. 22 and 23).
3.2. Characterization of isolated bovine uterine epithelial cells in culture

The endometrium is composed of an inner stromal connective tissue layer lined by ciliary and secretory epithelial cells (Section 1.5.3.2.). Interspersed between these cells is a myriad of different cell types that can make the *in vitro* isolation of a pure epithelial cell population difficult. Contaminating cells can include endometrial regenerative cells which have been shown to display adult stem cell like activity (Gargett., 2007; Meng *et al.*, 2008; Donofrio *et al.*, 2008), immune cells (Herath *et al.*, 2006) and endometrial endothelial cells (Roman-Ponce *et al.*, 1978; Ford., 1982). Myometrial cells and adipocytes can also contaminate the culture system. In order to achieve an essentially pure cell culture model of bovine uterine epithelial cells (BUECs), cells were mechanically and enzymatically isolated under optimized conditions. In this culture regime, cells formed confluent tightly packed monolayers consistent with an epithelial morphology (Fig. 24). Isolated BUECs were cultured until approximately 90% confluent before immunohistochemical characterizations were performed (Fig. 25).

![Figure 24: Representative image of BUECs in culture for 7 days. (10X mag)](image-url)
BUECs were dual stained for cytokeratins (red) and vimentin (green), which are intermediate filaments predominately found in epithelial cells and connective tissue respectively. Nuclei were counterstained with DAPI (blue, Fig. 25).

Figure 25: Representative image of BUECs in culture for 7 days. (10X mag) Secondary antibodies reveal positive staining for cytokeratins (red) and vimentin (green).

It is clear that under these isolation and culture conditions the majority of cells stain positive for cytokeratins, the epithelial cell marker (Fig. 25). There is some indication for diffuse vimentin staining but it is present in less than 10% of the population and most likely due to minimal stromal cell contamination of the culture. In addition the epithelial cell morphology dominates, these cells are smaller and more uniform in shape compared to the larger spindle-like stromal cells that display a branched cytoplasm (Fig. 25). Morphology and immunostaining confirm that the majority of these cells were epithelial in nature.
3.3 Effects of hormone treatment on PGT expression in cultured BUECs

BUECs were cultured with steroid hormones for different time periods to mimic the uterine environment at different stages of the bovine oestrous cycle and investigate an effect of hormones on the expression of PGT protein.

3.3.1. PGT expression in BUECs treated with hormones for 24 hours measured by immunohistochemistry

Immunohistochemical staining of BUECs for PGT protein after a 24 hour treatment period reveals a regulatory role for these hormones on the expression of the protein (Fig 26).

Figure 26: Representation of BUECs cultured until 70% confluence and treated with hormones for 24 hours and stained for PGT (green) (10x mag).
Incubation with 0.1% BSA revealed PGT expression in approx. 30% of cells (Fig. 26). Immunostaining for PGT in BUECs cultured with 10% FCS showed expression that is located in close proximity to the nuclei, presumably in the nuclear membrane in the majority of BUECs. Treatment with 10nM E2 confirms positive PGT expression in 100% of BUECs, with elevated staining near nuclei and lesser PGT concentrated in the cytosolic domain (Fig. 26). BUECs treated with 1nM E2 and 100nM E2 share similar expression, approx. 50%, which is concentrated near the nuclei with diffuse expression approaching the cell membrane. Low progesterone treatments (1nM and 10nM) inhibit PGT expression seen as reduced staining compared to the BSA control. However, at elevated levels of P4 (100nM), there is an increase in PGT expression in BUECs versus the control. P4 treatment elevates PGT expression in the nuclear membrane with poor localization in cytosolic compartments or cell membranes (Fig. 26).
3.3.2. PGT expression in BUECs treated with hormones for 48 hours measured by immunohistochemistry

After a 48 hour hormone treatment period (Fig. 27), there is a dramatic downregulation in PGT expression in BUECs compared to 24 hour treatment (Fig. 26) and 72 hour treatment (Fig. 28).

![Image of immunohistochemistry results](image)

Figure 27: Representation of BUECs cultured until 70% confluence and treated with hormones for 48 hours and stained for PGT (green) (10x mag).
For all hormone treatments, PGT protein expression remains low after 48 hours (Fig. 27) compared to 24 hours (Fig. 26). Between treatments, P4 stimulated PGT expression after 48 hours, where concentrations of 1nM P4 and 10nM P4 revealed the highest PGT-positive cells, indicative of the stimulatory effect of P4 on oatp expression (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006). Sera and estradiol show low levels of PGT expression in BUECs after 48 hours incubation.
3.3.3. PGT expression in BUECs treated with hormones for 72 hours measured by immunohistochemistry

Hormone incubation for 72 hours reveals a similar pattern of PGT expression as hormone treatment for 24 hours (Fig. 26), while 72 hour treatment upregulates of PGT protein in BUECs (Fig. 28) compared to a 48 hour co-incubation (Fig. 27).

Figure 28: Representation of BUECs cultured until 70% confluence and treated with hormones for 72 hours and stained for PGT (green) (10x mag).
A 72-hour incubation period demonstrated that 1nM E2 and 10nM E2 increased PGT expression, where approx. 50% of BUECs stain positively for PGT. PGT staining is present near the nuclei and also appears at the periphery of the cells, most likely in the cell membrane after E2 treatment. Comparatively treatment with 100nM E2 suppressed PGT expression. P4 also stimulated PGT expression but to a lesser extent than E2 and only at high doses (100nM P4).
3.3.4. PGT expression in BUECs treated with hormones and antagonists for 24 hours measured by immunohistochemistry

BUECs were cultured with steroid hormones and hormone receptor antagonists for 24 hours, prior to immunostaining for PGT (green) and counterstaining with DAPI (blue) (Fig. 29).

Figure 29: Representation of BUECs cultured until 70% confluence and treated with hormones and antagonists for 24 hours and stained for PGT (green) (10x mag).
Fig. 29 illustrated a potent stimulatory effect of 10nM estradiol on PGT expression that is inhibited when co-incubated with the estradiol receptor antagonist, fulvestrant (E2 and Fulv) after a 24hour treatment. The dramatic reduction in PGT protein following fulvestrant treatment highlights a role for estradiol in PGT upregulation in this cell culture model. PGT is expressed in BUECs after 10nM progesterone treatment but the levels are comparatively lower than the estradiol group and appear to be confined to dense colony of cells (orange arrow). Co-incubation of BUECs with progesterone and mifepristone (P4 and Mif) reduces PGT expression compared to progesterone treatment alone after a 24hour treatment (Fig. 29).

In effect, E2 appears to promote PGT in BUECs after a 24hour (Fig. 26 and 29) and 72hour (Fig. 28) treatment period, an effect that is reversed after estradiol co-incubation with fulvestrant for 24hours (Fig. 29). PGT expression is highest after 10nM E2 treatment, but there is also substantial PGT protein content after treatment with 1nM and 100nM E2 for 24hrs (Fig. 26). Treatment with E2 for 48hrs reveals downregulation of PGT expression for all concentrations (Fig. 27). PGT protein concentration is elevated again after 72hr estradiol stimulation, where expression is highest at 1nM and 10nM E2 (Fig. 28. Progesterone has a concentration and temporal effect on PGT expression in BUECs. Following a 24hour treatment with 100nM P4 elevated PGT expression, while 1nM and 10nM P4 only reveals PGT protein in closely packed cells (Fig. 26). Mifepristone abolishes the expression of PGT after 24hours (Fig. 29). Progesterone increases PGT expression again after 72hour treatment with 10nM P4 (Fig. 28).
3.4. Transport Studies

Bovine luteolytic or luteotropic PGs are synthesized in the endometrium and must be transported back to the ovary to mediate their effects. Transport studies were carried out to investigate the transport potential of BUECs under different hormonal conditions and were based on previous work carried out by Chan et al., (1998; 2002) and Banu et al., (2003). Transport studies involved the total uptake of $[^{3}\text{H}]$ PGs into BUECs, cytosolic accumulation of $[^{3}\text{H}]$ PGs, membrane incorporation of $[^{3}\text{H}]$ PGs into BUECs or transepithelial transport of $[^{3}\text{H}]$ PGs across polarized monolayers under different conditions.

3.4.1. Effect of phenol red on $[^{3}\text{H}]$ PGF$_{2\alpha}$ uptake

BUECs cultured in medium containing phenol red accumulated significantly more $[^{3}\text{H}]$ PGF$_{2\alpha}$ compared to BUECs cultured in phenol free media ($p<0.01$) (Fig. 30). The estrogenic component of phenol red (Berthois et al., 1986) may be involved in elevated PGF$_{2\alpha}$ transport. Phenol red is a weak estrogen commonly used as a pH indicator in cell culture studies, however, it is known to bind to responsive cells and elicit an estrogen-like hormonal response (Berthois et al., 1986). In addition, the phenol red DMEM used for these studies had a high glucose content (4500mg/L) and may contribute to the cellular uptake of PGs (Chan et al., 2002).

![Figure 30: Effect of phenol red on $[^{3}\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs. Values shown are mean ± sem, where n = 48.](image-url)
3.4.2. Effect of serum on $[^3]H$ PGF$_{2\alpha}$ uptake

Serum contains different concentrations of fatty acids and hormones that can vary between batches. Fatty acids are precursors to PG synthesis and influence the transport of $[^3]H$ PGs by generating electrochemical gradients. BUECs were cultured in phenol red free or phenol red containing DMEM in the presence or absence of sera to investigate whether different culture conditions affected the transport of $[^3]H$ PGs.

Serum does not significantly alter $[^3]H$ PGF$_{2\alpha}$ uptake into BUECs but it is clear that $[^3]H$ PGF$_{2\alpha}$ transport into BUECs cultured in phenol red medium is consistently higher than transport into BUECs cultured in phenol free media (Fig. 31).

![Figure 31: Effect of phenol red and sera on $[^3]H$ PGF$_{2\alpha}$ uptake into BUECs. Values shown are mean ± sem, where n = 16.](image)

BUECs cultured in phenol red medium supplemented with 0.1% BSA increased $[^3]H$ PGF$_{2\alpha}$ uptake compared to other treatments. BUECs cultured in either phenol free or phenol red DMEM supplemented with 10% FCS decreased the uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs compared to other sera treatments (Fig 31), and this may be attributed to some inhibitory factor of the PG transport system present in the serum.
3.4.3. Uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs over time under different culture conditions

Total $[^3\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs was higher when cells were cultured in phenol red DMEM compared to BUECs cultured in phenol free DMEM over all time points (Fig. 32), although the effect is not statistically significant.

![Graph showing time-dependent uptake of $[^3\text{H}]$ PGF$_{2\alpha}$](image)

Figure 32: Time-dependent uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ in phenol red or phenol red free media. Values shown are mean ± sem, where n =12.

PGF$_{2\alpha}$ uptake into BUECs peaked for both phenol red and phenol free cultured DMEM at 10 minutes and dissipates overtime and declined steadily towards 20 minutes (Fig. 32).
3.4.4. Interaction between phenol and serum over time on the $[^3]$H PGF$_{2\alpha}$ uptake into BUECs

BUECs cultured in either phenol free or phenol red containing DMEM altered the uptake of $[^3]$H PGF$_{2\alpha}$ into cells in different sera conditions (Fig. 33).

Figure 33: Time-dependent uptake of $[^3]$H PGF$_{2\alpha}$ in a) phenol red free media or b) phenol red media supplemented with different sera. Values shown are mean ± sem, where n = 8.

BUECs grown in phenol free DMEM supplemented with 0.1% BSA showed a distinct gradual increase in $[^3]$H PGF$_{2\alpha}$ uptake from 5 to 10 minutes that plateaued from 10 until 20 minutes revealing a constant level of uptake (Fig. 33a). Culturing BUECs in phenol free media supplemented with 10% CS-FCS increased the uptake of $[^3]$H PGF$_{2\alpha}$ that peaked after 10 minutes and gradually dissipated over time. BUECs cultured in phenol free media supplemented with 10% FCS increased $[^3]$H PGF$_{2\alpha}$ at 5 minutes that decreased over time (Fig. 33a).

BUECs supplemented with 0.1% BSA in phenol red DMEM elevated $[^3]$H PGF$_{2\alpha}$ uptake after 5 minutes which then decreased with respect to time (Fig. 33b). BUECs supplemented with 10% CS-FCS in phenol red DMEM (Fig. 33b) showed a similar pattern of $[^3]$H PGF$_{2\alpha}$ uptake as BUECs cultured in phenol free DMEM (Fig. 33a), although uptake was higher in the phenol red cultured DMEM. $[^3]$H PGF$_{2\alpha}$ uptake into BUECs in phenol red DMEM supplemented with 10% FCS demonstrated lowest levels of $[^3]$H PGF$_{2\alpha}$ transport with a modest peak at 10 minutes.
3.4.5. Effect of steroid hormones on the uptake of \([^{3}\text{H}]\text{PGF}_{2\alpha}\) into BUECs

Uptake of \([^{3}\text{H}]\text{PGF}_{2\alpha}\) into BUECs after treatment with steroid hormones and their respective antagonists was carried out to investigate the preferred hormonal conditions that support or impede PG transport in the bovine uterus.

3.4.5.1. Effect of steroid hormones on the uptake of \([^{3}\text{H}]\text{PGF}_{2\alpha}\) into BUECs in phenol free media after 24 hour treatment

Uptake of \([^{3}\text{H}]\text{PGF}_{2\alpha}\) into BUECs following a 24 hour treatment with 10nM E2 or 10nM P4 and their respective inhibitors in phenol free media supplemented with different sera was highly variable even in the absence of serum (Fig. 34).

![Graph showing the effect of steroid hormones on \([^{3}\text{H}]\text{PGF}_{2\alpha}\) uptake into BUECs in phenol free media after 24 hour treatment](image)

Figure 34: Steroid hormone and sera effect on \([^{3}\text{H}]\text{PGF}_{2\alpha}\) uptake into BUECs in phenol red free medium after a 24 hour treatment period. Values shown are mean ± sem, where \(n = 7\).

Transport of \([^{3}\text{H}]\text{PGF}_{2\alpha}\) into BUECs remained similar to the control for BUECs treated with 10nM E2 or co-treated with 10nM fulvestrant and 10nM E2 (Fulv&E2) for 24 hours in either sera supplemented media (Fig. 34). However, treatment with 10nM P4 or co-incubation with 10nM mifepristone and P4 (Mif&P4) in phenol free DMEM containing 10% FCS revealed a P4 effect, whereby P4 reduced \([^{3}\text{H}]\text{PGF}_{2\alpha}\) uptake, while co-incubation with Mif&P4 reversed the role of P4 and elevated \([^{3}\text{H}]\text{PGF}_{2\alpha}\) uptake (Fig. 34).
3.4.5.2. Effect of steroid hormones on the uptake of $[^{3}\text{H}]\text{PGF}_{2\alpha}$ into BUECs in phenol free media after a 72 hour treatment

BUECs hormonally stimulated for 72 hours in medium containing 10% FCS, revealed a significant effect of treatment (p<0.05). P4 treatment in phenol free media containing 10% FCS had an inhibitory effect on $[^{3}\text{H}]\text{PGF}_{2\alpha}$ transport in BUECs which was reversed when cells are co-cultured with Mif&P4 (Fig. 35).

![Graph: Steroid hormone and sera effect on $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs in phenol red free medium after a 72 hour treatment period. Values shown are mean ± sem, where n = 4 for Control and n = 3 for treatments.]

Figure 35: Steroid hormone and sera effect on $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs in phenol red free medium after a 72 hour treatment period. Values shown are mean ± sem, where n = 4 for Control and n = 3 for treatments.
3.4.5.3. Effect of estradiol treatment in serum-free conditions in different phenol-red containing media on the uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs

BUECs cultured in phenol free DMEM treated with E2 showed a distinct pattern of $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake (Fig. 36).

Figure 36: Effect of a 24 hour estradiol treatment period on the uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs in different phenol-red containing media supplemented with 0.1% BSA. Values shown are mean ± sem, where n = 3 for control and n = 6 for treatments.

A 24 hour treatment with 1nM E2 in phenol free DMEM promoted $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake, while increasing concentration levels of E2 (10nM and 100nM), decreased $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake with respect to control (Fig. 36). On the other hand, BUECs cultured in phenol red DMEM treated with E2 displayed an indeterminate level of $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs. Control BUECs in phenol red DMEM increased $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake compared to the control group in the phenol free model (Fig. 36). The effect of E2 treatment in phenol red cultured BUECs may be partially due to the estrogenic component of phenol or the high glucose content of the DMEM, which may exacerbate the E2 effect.
3.4.5.4. Effect of progesterone treatment in serum-free conditions in different phenol-red containing media on the uptake of [³H] PGF$_{2\alpha}$ into BUECs

In the phenol free DMEM, increasing the concentration of P4 elevated [³H] PGF$_{2\alpha}$ uptake in a dose-dependent manner into BUECs (Fig. 37). In contrast, BUECs cultured in phenol red DMEM showed that increasing concentrations of P4 decreased [³H] PGF$_{2\alpha}$ uptake in a dose-dependent manner (Fig. 37).

![Uptake of [³H] PGF$_{2\alpha}$](image)

Figure 37: Effect of a 24 hour progesterone treatment on the uptake of [³H] PGF$_{2\alpha}$ into BUECs in different phenol red containing media supplemented with 0.1% BSA. Values shown are mean ± sem, where n = 3 for control and n = 6 for treatments.
3.4.5.5. Uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs following estradiol priming and oxytocin challenge

A 3 day E2 (1nM; 10nM or 100nM) priming period, without an oxytocin challenge increased $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs compared to the control group, which were cultured only in phenol free DMEM containing 0.1% BSA (Fig. 38). An oxytocin challenge reduced $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake in the control group and BUECs treated with 1nM estradiol dramatically (Fig. 38).

![Bar graph showing uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs with and without oxytocin challenge](image)

Figure 38: Effect of a 3 day estradiol priming and oxytocin challenge on $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs. Values shown are mean ± sem, where $n = 6$ for Control and $n = 3$ for treatments.

E2 priming with 1nM or 10nM estradiol substantially increased $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake, but the effect is not statistically significant, while treatment with 100nM E2 for 3 days elevated $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake only compared to the control (Fig. 38). The inhibitory effect of oxytocin at 0nM or 1nM E2 treatment was overcome following 10nM or 100nM E2 priming, where uptake is similar to BUECs not challenged with oxytocin (Fig. 38).
3.4.5.6. Uptake of $[^3\text{H}] \text{PGF}_{2\alpha}$ into BUECs following progesterone priming and oxytocin challenge

For all P4 treatments (1nM; 10nM or 100nM), uptake of $[^3\text{H}] \text{PGF}_{2\alpha}$ into BUECs (Fig. 39) remained lower than uptake following E2 priming (Fig. 38), indicating an inhibitory role for P4. Oxytocin did not alter the P4 effect, where uptake has a similar pattern without or with an oxytocin challenge.

Figure 39: Effect of a 3 day progesterone priming and oxytocin challenge on $[^3\text{H}] \text{PGF}_{2\alpha}$ uptake into BUECs. Values shown are mean ± sem, where n = 6 for control and n = 3 for treatments.

Uptake of $[^3\text{H}] \text{PGF}_{2\alpha}$ uptake into BUECs remains low after P4 priming regardless of an oxytocin challenge (Fig. 39). There is a discrete increase in $[^3\text{H}] \text{PGF}_{2\alpha}$ uptake following priming with 100nM P4 compared to the control treatment, however, the effect is not significant (Fig. 39).
3.4.6. Time-dependent effect of PUFAs and estradiol treatment on $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs

PUFAs are the direct precursors of PGs synthesis, where omega-6 PUFAs direct 2-series PGs and omega-3 PUFAs direct 3-series PGs. Incubation of BUECs with different levels of PUFAs was carried out to determine whether these PUFAs can alter uterine PG transport.

3.4.6.1. $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs after a 24 hour PUFA incubation

Incubation with PUFAs altered the uptake of $[^{3}\text{H}]\text{PGF}_{2\alpha}$ into BUECs after a 24 hour treatment with either concentration of fatty acid (50µM and 100µM) although the effects were not statistically significant (Fig. 40).

![Graph showing the uptake of $[^{3}\text{H}]\text{PGF}_{2\alpha}$ into BUECs after a 24 hour PUFA incubation.](image)

Figure 40: Effect of a 24 hour PUFA treatment on the uptake of $[^{3}\text{H}]\text{PGF}_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 3.

Incubation with 50µM PUFAs (EPA; DHA or AA) reduced the uptake of $[^{3}\text{H}]\text{PGF}_{2\alpha}$ into BUECs compared to the control after a 24 hour treatment, while incubation with 100µM PUFA reduced $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake in the EPA and DHA treatments (Fig. 39). Incubation with 100µM AA for 24 hours reversed the inhibitory effect of 50µM AA on $[^{3}\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs and achieved a similar uptake pattern as the control (Fig. 40).
3.4.6.2. \(^3\)H PGF\(_{2\alpha}\) uptake into BUECs after a 72 hour PUFA incubation

After a 72 hour PUFA culture period, uptake of \(^3\)H PGF\(_{2\alpha}\) into BUECs (Fig. 41) was less than uptake after a 24 hour PUFA culture period (Fig. 40). Fatty acid treatment (50μM) had a significant effect on \(^3\)H PGF\(_{2\alpha}\) uptake (p<0.05) whereby BUECs cultured with 50μM EPA and 50μM DHA increased the level of \(^3\)H PGF\(_{2\alpha}\) uptake compared to the control and BUECs treated with 50μM AA (Fig. 41). Incubation with 100μM fatty acids did not alter PGF\(_{2\alpha}\) uptake compared to control.

Figure 41: Effect of a 72 hour PUFA treatment on the uptake of \(^3\)H PGF\(_{2\alpha}\) into BUECs. Values shown are mean ± sem, where n = 3.
3.4.6.3. $[^3]$H PGF$_{2a}$ uptake into BUECs after a 168 hour PUFA incubation

Uptake of $[^3]$H PGF$_{2a}$ into BUECs after a 7 day PUFA incubation (Fig. 42) returned to the same level of uptake after a 1 day PUFA incubation (Fig. 40). After 7 days in culture, incubation with 50μM EPA or 50μM DHA, increased $[^3]$H PGF$_{2a}$ uptake into BUECs compared to the control or BUECs treated with 50μM AA (Fig. 42). For all PUFA treatments, 100μM of fatty acid promoted $[^3]$H PGF$_{2a}$ uptake into BUECs compared to the control group. Interestingly, 100μM EPA treatment reduced $[^3]$H PGF$_{2a}$ uptake into BUECs versus 50μM EPA treatment. However, treatment with 100μM DHA or 100μM AA, elevated uptake of $[^3]$H PGF$_{2a}$ compared to their respective 50μM treatments (Fig. 42).

![Figure 42: Effect of a 168 hour PUFA treatment on the uptake of $[^3]$H PGF$_{2a}$ into BUECs. Values shown are mean ± sem, where n = 3.](image)

...
3.4.6.4. $[^{3}H]$ PGF$_{2\alpha}$ uptake into BUECs after a 24 hour PUFA and 1nM E2 incubation

A 24 hour treatment with 1nM E2 in the presence of either 50μM or 100μM fatty acids did not significantly alter $[^{3}H]$ PGF$_{2\alpha}$ uptake into BUECs compared to control group (Fig. 43). There was a stepwise decrease in $[^{3}H]$ PGF$_{2\alpha}$ uptake into BUECs in a pattern of EPA>DHA>AA for the 50μM fatty acid treatments (Fig. 423).

![Figure 43: Effect of a 24 hour co-incubation period with PUFA and 1nM E2 on the uptake of $[^{3}H]$ PGF$_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 3.](image)

Uptake of $[^{3}H]$ PGF$_{2\alpha}$ into BUECs was highest after a one day co- incubation with 1nM E2 and 50μM EPA, while uptake declined following treatment with 1nM E2 and 50μM DHA or 1nM E2 and 50μMAA (Fig. 43). Incubation with 1nM E2 and100μM PUFAs reduced $[^{3}H]$ PGF$_{2\alpha}$ uptake into BUECs compared to treatment with 50μM for all fatty acid treatments (Fig. 43). The most potent inhibitory effect of $[^{3}H]$ PGF$_{2\alpha}$ uptake into BUECs was when cells were incubated with 100μM AA in 1nM E2, possibly due to the increased synthesis of PGs in the presence of AA, which creates an intracellular concentration gradient that may prevent further uptake of PGs by BUECs.
3.4.6.5. $[^3]H$ PGF$_{2\alpha}$ uptake into BUECs after a 72 hour PUFA and 1nM E2 incubation

After a 72 hour PUFA incubation period in 1nM E2, uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs was highest following EPA treatment regardless of concentration of the PUFA added (Fig. 44). EPA increased uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs in a dose-dependent manner, from 0µM to 100µM (Fig. 44). DHA (50µM) treatment increased $[^3]H$ PGF$_{2\alpha}$ uptake but not as dramatically as the EPA treatment and reduced uptake after 100µM DHA incubation. AA decreased the uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs at 50µM and 100µM treatment in the presence of 1nM E2 (Fig. 44).

![Figure 44: Effect of a 72 hour co-incubation period with PUFA and 1nM E2 treatment on the uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 3.](image)
3.4.6.6. $[^3]H$ PGF$_{2\alpha}$ uptake into BUECs after a 168 hour PUFA and 1nM E2 incubation

PGF$_{2\alpha}$ uptake into BUECs after a 7 day treatment with PUFAs and 1nM E2 showed an inhibitory effect of 100μM EPA and 100μM AA (Fig. 45).

![Graph](image)

Figure 45: Effect of a 168 hour co-incubation period with PUFA and 1nM E2 on the uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 3.

Incubation for 7 days with PUFAs at a concentration of 50μM with 1nM E2 maintained a similar pattern of $[^3]H$ PGF$_{2\alpha}$ uptake as the control group (Fig. 45). EPA and AA at concentrations of 100μM reduced the uptake of $[^3]H$ PGF$_{2\alpha}$ into BUECs after 7 days, while 100μM DHA treatment in the presence of 1nM E2 maintained a similar pattern of $[^3]H$ PGF$_{2\alpha}$ uptake as the control (Fig. 45).
3.4.6.7. $[^3]$H PGF$_{2α}$ uptake into BUECs after a 24 hour PUFA and 10nM E2 incubation

Incubation with 10nM E2 and PUFAs maintained a basal level of $[^3]$H PGF$_{2α}$ uptake with discrete variations that were not significant after a one day incubation period (Fig. 46).

![Graph showing uptake of $[^3]$H PGF$_{2α}$](image)

Figure 46: Effect of a 24 hour co-incubation period with PUFA and 10nM E2 on the uptake of $[^3]$H PGF$_{2α}$ into BUECs. Values shown are mean ± sem, where n = 3.

BUECs co-incubated with EPA (50μM or 100μM) and 10nM E2 for 24hours showed a similar uptake of $[^3]$H PGF$_{2α}$ as the control (Fig. 46). E2 (10nM) treatment with 50μM or 100μM DHA moderately increased $[^3]$H PGF$_{2α}$ uptake compared to the control. AA treatment reduced uptake when BUECs were co-incubated with 50μM AA and 10nM E2 for 24hours, while uptake increased after incubation with 100μM AA and 10nM E2 (Fig. 46).
3.4.6.8. $[{}^{3}\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs after a 72 hour PUFA and 10nM E2 incubation

A 72 hour co-incubation with PUFAs and 10nM E2 showed a similar pattern of $[{}^{3}\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs for cells treated with the omega-3 PUFAs (EPA and DHA), compared to treatment with the omega-6 AA (Fig. 47).

![Uptake of $[{}^{3}\text{H}]$ PGF$_{2\alpha}$](image)

Figure 47: Effect of a 72 hour co-incubation period with PUFA and 10nM E2 on the uptake of $[{}^{3}\text{H}]$ PGF$_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 3.

Incubation with EPA and DHA increased the uptake of $[{}^{3}\text{H}]$ PGF$_{2\alpha}$ in a dose-dependent pattern (0µM to 100µM) when co-cultured with 10nM E2 (Fig. 47). Interestingly, BUECs treated with AA promoted uptake at 50µM while 100µM AA reduced uptake of $[{}^{3}\text{H}]$ PGF$_{2\alpha}$ relative to the control (Fig. 47).
3.4.6.9. \([^3]H\) PGF\(_{2\alpha}\) uptake into BUECs after a 168 hour PUFA and 10nM E2 incubation

Following a seven day co-incubation with PUFAs (50μM or 100μM) and 10nM E2, the uptake of \([^3]H\) PGF\(_{2\alpha}\) into BUECs was reduced compared to the control (Fig. 48). The pattern of uptake is similar for BUECs treated with EPA or DHA, where uptake was reduced at 50μM EPA or DHA. AA reduced the uptake of \([^3]H\) PGF\(_{2\alpha}\) into BUECs in a stepwise manner from 50μM AA to 100μM AA (Fig. 48).

Figure 48: Effect of a 168 hour co-incubation period with PUFA and 10nM E2 on the uptake of \([^3]H\) PGF\(_{2\alpha}\) into BUECs. Values shown are mean ± sem, where n = 3.
3.4.7. Effect of glucose incubation on the uptake of $[^3]$H PGs into BUECs

BUECs were incubated with different levels of glucose based on the concentration of glucose in cell culture media and uptake of $[^3]$H PGs was assessed. Lactate is the preferred exchange molecule for PG transport and is a by-product of cellular glucose metabolism. Studies were conducted to determine whether glucose has an effect on PG transport at different time points and co-incubated with estrogen or progesterone.

3.4.7.1. Effect of glucose concentration on the uptake of $[^3]$H PGE$_2$ into BUECs

There is a direct correlation between increasing the concentration of glucose in the incubations and increased $[^3]$H PGE$_2$ uptake into BUECs (Fig. 49).

Figure 49: Effect of glucose concentration on the uptake of $[^3]$H PGE$_2$ into BUECs. Values shown are mean ± sem, where n = 24.

Increasing the level of glucose concentration from 0mM to 43mM leads to a stepwise increase in $[^3]$H PGE$_2$ uptake into BUECs (Fig. 49), an effect that is statistically significant (p<0.05).
3.4.7.2. Effect of hormones in different glucose conditions on the uptake of $[^3]$H PGE$_2$ into BUECs after 2 minutes

Hormones promoted a rapid uptake of $[^3]$H PGE$_2$ into BUECS compared to cells that were not treated at all glucose levels (Fig. 50).

![Bar graph showing uptake of $[^3]$H PGE$_2$ in different glucose concentrations with hormones.](image)

Figure 50: Hormonal effect of estrogen or progesterone on the uptake of $[^3]$H PGE$_2$ into BUECs in media containing different glucose concentrations after 2 minutes. Values shown are mean ± sem, where n = 4.

Untreated control cells had significantly less uptake of $[^3]$H PGE$_2$ compared to BUECs treated with E2 or P4 after 2 minutes (Fig. 50). Uptake of $[^3]$H PGE$_2$ was highest when BUECs were treated with 10nM E2 in media containing 18mM glucose and treatment with 10nM P4 in 43mM glucose after 2 minutes (Fig. 50).
3.4.7.3. Effect of hormones in different glucose conditions on the uptake of \(^{3}\text{H}\) PGE\(_{2}\) into BUECs after 10 minutes

PGE\(_{2}\) uptake into BUECs at 10 minutes in different levels of glucose (Fig. 51) had an opposite trend compared to uptake at 2 minutes (Fig. 50). In effect, hormones promoted a rapid uptake of \(^{3}\text{H}\) PGE\(_{2}\) into BUECs at 2 minutes (Fig. 50), compared to less uptake observed in hormonally stimulated BUECs at 10 minutes (Fig. 51). In addition, unstimulated BUECs increased \(^{3}\text{H}\) PGE\(_{2}\) uptake at 10 minutes (Fig. 51) compared to 2 minutes (Fig. 50).

![Graph](image)

Figure 51: Hormonal effect of estradiol or progesterone on the uptake of \(^{3}\text{H}\) PGE\(_{2}\) into BUECs in media containing different glucose concentrations after 10 minutes. Values shown are mean ± sem, where n = 4.

Untreated BUECs showed a stepwise increase in \(^{3}\text{H}\) PGE\(_{2}\) uptake that correlated with an increased glucose concentration at 10 minutes (Fig. 51). BUECs treated with 10nM E2 or 10nM P4 in media containing either, 0mM, 18mM, 25mM or 43mM glucose maintained a constant level of \(^{3}\text{H}\) PGE\(_{2}\) uptake that was comparable to uptake in untreated cells at 10 minutes containing 0mM or 18mM glucose (Fig. 51).
3.4.7.4. Effect of glucose concentration on the uptake of $[^3 \text{H}] \text{PGF}_{2\alpha}$ into BUECs

Uptake of $[^3 \text{H}] \text{PGF}_{2\alpha}$ into BUECs was not altered significantly by glucose concentration but did show that incubation with 25mM glucose discretely increased the uptake of $[^3 \text{H}] \text{PGF}_{2\alpha}$ into BUECs (Fig. 52).

![Figure 52: Effect of glucose concentration on the uptake of $[^3 \text{H}] \text{PGF}_{2\alpha}$ into BUECs. Values shown are mean ± sem, where n = 24.](image)

Culturing BUECs with glucose did not show a stepwise increase in $[^3 \text{H}] \text{PGF}_{2\alpha}$ (Fig. 52) as observed in the uptake of $[^3 \text{H}] \text{PGE}_2$ (Fig. 49). Although, glucose at a concentration of 25mM enhanced $[^3 \text{H}] \text{PGF}_{2\alpha}$ uptake, the effect was not statistically significant. Incubation with 18mM glucose has a similar uptake of $[^3 \text{H}] \text{PGF}_{2\alpha}$ as the control (0mM glucose) while culture with 43mM glucose modestly reduced $[^3 \text{H}] \text{PGF}_{2\alpha}$ uptake compared to the control (Fig. 52).
3.4.7.5. Effect of hormones in different glucose conditions on the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs after 2 minutes

Uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs after 2 minutes was low in untreated cells, regardless of glucose concentration, while BUECs treated with hormones in different glucose concentrations enhance $[^3\text{H}]$ PGF$_{2\alpha}$ uptake (Fig. 53).

Figure 53: Hormonal effect of estradiol or progesterone on the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs in media containing different glucose concentrations after 2 minutes. Values shown are mean ± sem, where n = 4.

Uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into untreated BUECs showed a modest decrease in transport as glucose concentration increased (Fig. 53). Increasing the level of glucose increased the stimulatory effect that 10nM E2 had on the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ (Fig. 53). In contrast, increasing the glucose concentration decreased the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ in BUECs treated with 10nM P4 (Fig. 53). These results reveal opposing effects of E2 and P4 on the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs with increasing levels of glucose concentration.
3.4.7.6. Effect of hormones in different glucose conditions on the uptake of \( [^3\text{H}] \) PGF\(_{2\alpha}\) into BUECs after 10 minutes

Uptake of \( [^3\text{H}] \) PGF\(_{2\alpha}\) into BUECs after 10 minutes exposure increased transport in untreated cells (control) compared to BUECs hormonally challenged regardless of glucose level (Fig. 54).

Figure 54: Hormonal effect of estradiol or progesterone on the uptake of \( [^3\text{H}] \) PGF\(_{2\alpha}\) into BUECs in media containing different glucose concentrations after 10 minutes. Values shown are mean ± sem, where \( n = 4 \).

Untreated BUECs maintain elevated \( [^3\text{H}] \) PGF\(_{2\alpha}\) uptake at all glucose concentrations after 10 minutes (Fig. 54). Cells stimulated with either 10nM E2 or 10nM P4 reduced \( [^3\text{H}] \) PGF\(_{2\alpha}\) uptake compared to untreated BUECs at each glucose level after 10 minutes (Fig. 54).

This study is based on work carried out by Jones and Harper. (1983), on the accumulation of PGs into 6-day pregnant rabbit endometrium at different pH levels.

PG accumulation into the cytosol of BUECs was pH-dependent (Fig. 55) for both $[^3]$H PGs, where the assumption is that both PGs exist as anions at pH > 5.0 (Schuster, 1998).

Figure 55: Effect of pH on the uptake of $[^3]$H PGs into BUECs. Values shown are mean ± sem, n=8

Uptake of $[^3]$H PG into BUECs was pH-dependent (Fig. 55). Uptake is highest in an acidic solution (pH 3.0), where PGs are presented as the neutral lipid. As the pH level increased to pH 5.0, the uptake of $[^3]$H PGs was reduced significantly (p<0.01), showing that transport of the charged compound is less efficient than transport of the neutral compound.
3.4.8.1. Effect of an acidic solution (pH 3.0) on the cytosolic accumulation of [³H] PGE₂ across and into BUECs over time

Cytosolic accumulation of [³H] PGE₂ at pH 3.0 after 2 minutes is significantly higher than accumulation after 20 minutes (Fig. 56).

![Graph showing cytosolic accumulation of [³H] PGE₂ across treatments and time points](image)

Figure 56: Effect of pH 3.0 on the cytosolic accumulation of [³H] PGE₂ after 2 or 20 minutes incubation. Values shown are mean ± sem, where n = 4.

At pH 3.0, BUECs accumulated higher levels of [³H] PGE₂ at 2 minutes compared to 20 minutes regardless of treatment (Fig. 56), providing evidence for the rapid transport of the neutral compound. The lower levels of [³H] PGE₂ accumulated after 20 minutes may be attributed to efflux of [³H] PGE₂ from the BUECs over time. At 2 minutes, cytosolic accumulation was similar for all treatments, with the Indo&P4 treatment showing a modest increase in accumulation. The BUECs exposed to [³H] PGE₂ for 20 minutes had a similar pattern of accumulation except in the cells treated with indomethacin which revealed a discrete elevated accumulation of [³H] PGE₂ (Fig. 56).
3.4.8.2. Effect of physiological solution (pH 7.4) on the cytosolic accumulation of $[^{3}\text{H}]$ PGE$_2$ across and into BUECs after 2 or 20 minutes exposure.

Accumulation of $[^{3}\text{H}]$ PGE$_2$ at pH 7.4 (Fig. 57) showed a marked reduction in cytosolic accumulation compared to accumulation at pH 3.0 (Fig. 56) at either 2 or 20 minutes.

Figure 57: Effect of pH 7.4 on the cytosolic accumulation of $[^{3}\text{H}]$ PGE$_2$ after 2 or 20 minutes incubation. Values shown are mean ± sem, where $n = 4$.

Accumulation of $[^{3}\text{H}]$ PGE$_2$ at pH 7.4 into BUECs did not differ significantly between both time points (Fig. 57). Also, after 2 minutes, indomethacin promoted accumulation of $[^{3}\text{H}]$ PGE$_2$ compared to the control and other treatments. After 20 minutes, accumulation was highest in BUECs treated with Indo&P4 (Fig. 57).
3.4.8.3. Effect of an acidic solution (pH 3.0) on membrane incorporation of \[^3\text{H}\] \text{PGE}_2 across and into BUECs over time

After 2 minutes, treatment with Indo&P4 increased membrane incorporation of \[^3\text{H}\] \text{PGE}_2 compared to all other treatments at any time point at pH 3.0 (Fig. 58). After 20 minutes, \[^3\text{H}\] \text{PGE}_2 incorporation following treatment with indomethacin (E2 or P4) decreased \[^3\text{H}\] \text{PGE}_2 incorporation.

Figure 58: Effect of pH 3.0 on the membrane incorporation of \[^3\text{H}\] \text{PGE}_2 after 2 or 20 minutes incubation. Values shown are mean ± sem, where n = 4.
3.4.8.4. Effect of physiological solution (pH 7.4) on membrane incorporation of $[^{3}\text{H}]\text{PGE}_2$ across and into BUECs over time

At pH 7.4, incorporation of $[^{3}\text{H}]\text{PGE}_2$ into membranes is highly variable for both time points (Fig. 59).

![Graph showing membrane incorporation of $[^{3}\text{H}]\text{PGE}_2$ (dpm per mg protein) for different treatments at 2 and 20 minutes.](image)

Figure 59: Effect of pH 7.4 on the membranous incorporation of $[^{3}\text{H}]\text{PGE}_2$ after 2 or 20 minute incubations. Values shown are mean ± sem, where n = 4.

Membrane incorporation of $[^{3}\text{H}]\text{PGE}_2$ at 2 minutes and 20 minutes were similar for control BUECs, and BUECs treated with E2 and P4 (Fig. 59). Incorporation into BUECs treated at 2 minutes treated with Indo&E2 was significantly reduced compared to Indo&E2 treatment after 20 minutes. In addition, at 2 minutes, incorporation was higher following Indo&P4 treatment compared to incorporation at 20 minutes.
3.4.9. Efflux of \(^{3}\text{H}\) PGs from BUECs

\(^{3}\text{H}\) PGs was allowed to accumulate in BUECs for 20 minutes and then efflux into saline solutions was assessed.

3.4.9.1. Efflux of \(^{3}\text{H}\) PGE\(_{2}\) efflux from preloaded BUECs at pH 3.0 or pH 7.4

BUECs preloaded with \(^{3}\text{H}\) PGE\(_{2}\), effluxed significantly higher levels of \(^{3}\text{H}\) PGE\(_{2}\) at pH 3.0 than pH 7.4 (Fig. 60). Efflux at the acidic pH may be accounted for by increased \(^{3}\text{H}\) PGE\(_{2}\) uptake into the BUECs during the preloading period (Fig. 55).

![Figure 60: Effect of pH 3.0 and pH 7.4 on the efflux of \(^{3}\text{H}\) PGE\(_{2}\). Values shown are mean ± sem, where n = 240.](#)
3.4.9.2. Efflux of $[^3]H$ PGE$_2$ into saline or equimolar cold PGE$_2$ from preloaded BUECs at pH 3.0 or pH 7.4

$[^3]H$ PGE$_2$ effluxed at pH 3.0 was higher than efflux of $[^3]H$ PGE$_2$ from BUECs at pH 7.4 into either efflux solution (Fig. 61).

Figure 60: Efflux of $[^3]H$ PGE$_2$ into media containing saline or equimolar cold PG at different pH levels. Values shown are mean ± sem, where n = 120.

At pH 3.0, efflux into cold PG was higher than efflux into saline, and describes transport of $[^3]H$ PGE$_2$ against its concentration gradient (Fig. 60). At pH 7.4, efflux into the saline solution was higher than efflux into the solution containing 1nM cold PGE$_2$, and shows that at normal physiological pH, PGE$_2$ has a tendency to move from an environment of high concentration to a site of lower concentration to achieve equilibrium (Fig. 60).
3.4.9.3. Efflux of $[^3]$H PGE$_2$ into saline from preloaded BUECs at pH 3.0 and pH 7.4 over time

At pH 7.4, efflux of $[^3]$H PGE$_2$ is maintained at a constant level with a modest increase at 20 minutes (Fig. 62). Efflux of $[^3]$H PGE$_2$ from BUECs at pH 3.0 shares a similar profile as efflux at pH 7.4 but the peak observed at 20 minutes is significantly higher at pH 3.0 versus pH 7.4 (Fig. 62).

![Efflux of $[^3]$H PGE$_2$](image)

Figure 62: Time-dependent efflux of $[^3]$H PGE$_2$ from BUECs into saline at different pH levels. Values shown are mean ± sem, where n = 20.
3.4.9.4. Efflux of $[^3$H$]$ PGE$_2$ into cold PG from preloaded BUECs at pH 3.0 and pH 7.4 over time

For all time points, $[^3$H$]$ PGE$_2$ efflux into 1nM cold PGE$_2$ was higher at pH 3.0 compared to pH 7.4 (Fig. 63).

Figure 63: Time-dependent efflux of $[^3$H$]$ PGE$_2$ from BUECs into 1nM cold PG at different pH levels. Values shown are mean ± sem, where n = 20.

At pH 3.0, efflux of $[^3$H$]$ PGE$_2$ increased with respect to the duration of efflux experiment, in contrast to pH 7.4, where efflux remained at a constant lower level with a modest increase after 5mins (Fig. 63).
3.4.9.5. Localization of $[^3]$H PGE$_2$ in BUECs after a 20 minute efflux study into saline or cold PG at pH 3.0 and pH 7.4

$[^3]$H PGE$_2$ is localized more efficiently in the cytosolic compartment compared to incorporation into membranes after efflux into saline or 1nM cold PGE$_2$ (Fig. 64).

Figure 64: $[^3]$H PGE$_2$ remaining in BUECs after efflux into saline or cold PG. Values shown are mean ± sem, where n = 80.
3.4.9.6. Localization of $[^3]$H PGE$_2$ in BUECs after a 20 minute efflux study at pH 3.0 or pH 7.4

Localization of $[^3]$H PGE$_2$ after efflux at different pH levels, showed that $[^3]$H PGE$_2$ remaining in the BUECs was predominately confined to the cytosol (Fig. 65).

Figure 65: Compartmental localization of $[^3]$H PGE$_2$ at different pH levels following a 20 minute incubation. Values shown are mean ± sem, where n = 40.

$[^3]$H PGE$_2$ localization in the cytosolic domain at pH 3.0 was comparatively higher than $[^3]$H PGE$_2$ localization in the cytosol at pH 7.4 or $[^3]$H PGE$_2$ incorporated into membranes at either pH (Fig. 65). The high levels of $[^3]$H PGE$_2$ accumulation at pH 3.0 may be attributed to increased levels of $[3H^+]$ PGE$_2$ accumulated initially during the preloading period (Fig. 65).
3.4.10. Vectorial flux of $[^3$H] PGs between apical and basolateral compartments of a polarized BUEC monolayer

Transepithelial transport of $[^3$H] PGs between apical and basolateral compartments of a polarized monolayer were measured in order to gain insight to the permeability of BUECs to PGs.

3.4.10.1. Transepithelial transport of $[^3$H] PGE$_2$ and $[^3$H] PGF$_{2\alpha}$ from the apical to the basolateral surface

Transepithelial transport of $[^3$H] PGE$_2$ and $[^3$H] PGF$_{2\alpha}$ from the apical to the basolateral surface shared a similar profile, with increased PG transport after 1 minute that dissipated over time (Fig. 66). Vectorial transport at 1 minute was approx. 7-fold higher compared to transport at all the other time points for both PGs (Fig. 66).

![Figure 66: Transepithelial transport of $[^3$H] PGE$_2$ and $[^3$H] PGF$_{2\alpha}$ from the apical to the basolateral surface. Values shown are mean ± sem, where $n = 5.$](image-url)
3.4.10.2. Compartmental localization of $[^3\text{H}]$ PGE$_2$ and $[^3\text{H}]$ PGF$_{2\alpha}$ after a transepithelial flux from the apical to the basolateral surface

After a 20 minute transepithelial transport study, $[^3\text{H}]$ PGE$_2$ and $[^3\text{H}]$ PGF$_{2\alpha}$ are predominately localized in the cytosolic compartment compared to incorporation into membranes (Fig. 67).

![Compartmental Localization of $[^3\text{H}]$ PGs](image)

Figure 67: Level of $[^3\text{H}]$ PGE$_2$ and $[^3\text{H}]$ PGF$_{2\alpha}$ in cellular compartments after a 20 minute transepithelial flux from the apical to basolateral surface. Values shown are mean ± sem, where $n = 5$.

Accumulation of $[^3\text{H}]$ PGs in the cytosolic compartment was significantly greater ($p < 0.01$) than incorporation into BUEC membranes after a 20 minute apical to basolateral surface transport study (Fig. 67). Accumulation in the cytosol may be attributed to the increased cytosolic volume compared to the membrane or limited number of PG binding sites on the membranes.
3.4.10.3. Transepithelial transport of $[^3\text{H}]\text{PGE}_2$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ from the basolateral to the apical surface

The pattern of $[^3\text{H}]\text{PGE}_2$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ flux from the basolateral to the apical surface (Fig. 68) shares a similar profile as apical to basolateral flux (Fig. 65) but is approx. 10-fold higher.

![Graph showing transepithelial transport of $[^3\text{H}]\text{PGE}_2$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ from basolateral to apical surface.](image)

Figure 68: Transepithelial transport of $[^3\text{H}]\text{PGE}_2$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ from the basolateral to the apical surface. Values shown are mean ± sem, where n = 5.

$[^3\text{H}]\text{PGE}_2$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ transepithelial transport from the basolateral to the apical surface (Fig. 68) was comparable to apical to basolateral transport (Fig. 66), but is 10-fold higher suggesting that the basolateral surface is more permeable to $[^3\text{H}]$ PGs than the apical membrane.
3.4.10.4. Compartmental localization of $[^3]H$ PGE$_2$ and $[^3]H$ PGF$_{2a}$ after a transepithelial flux from the basolateral to the apical surface

Cytosolic accumulation of either $[^3]H$ PG was significantly higher than incorporation into membranes after a 20 minute basolateral to apical transport study (Fig. 69).

![Graph showing compartmental localization of [3H+] PGs](image)

Figure 69: Level of $[^3]H$ PGE$_2$ and $[^3]H$ PGF$_{2a}$ in cellular compartments after a 20 minute transepithelial flux from the basolateral to the apical surface. Values shown are mean ± sem, where n = 5.

$[^3]H$ PGE$_2$ and $[^3]H$ PGF$_{2a}$ was localized in the cytosolic region compared to membrane incorporation following basolateral to apical flux (Fig. 69). Incorporation of $[^3]H$ PGs into the membrane was significantly less than accumulation in the cytosol (p<0.05), possibly due to the increased volume of the cytosol.
3.5. PGT mRNA Expression in BUECs

3.5.1. Hormonal effects on PGT mRNA expression in cultured BUECs after a 24 hour treatment period in phenol red free media supplemented with 0.1% BSA

BUECs were cultured in T25 flasks (75cm\(^3\)) and treated with hormones to elucidate whether PGT mRNA was constitutively expressed or corresponds to differential expression. Bands of expected size are shown on the gel that correlate with the DNA marker (1), negative control (2), positive control (3) and PGT (Lanes 4,5,6,7,8 and 9). All treatments were prepared in media containing 0.1% BSA (Fig. 70).

Figure 70: RT-PCR analysis of total RNA isolated from hormonally stimulated BUECs for 24 hours in 0.1% BSA medium, using a gene specific primer for bPGT.

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<td>Mif &amp; P4 0.1% BSA</td>
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<td>9</td>
<td>Indomethacin in 0.1% BSA</td>
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Table 14: Lane constituents in 0.1% BSA media.
bPGT mRNA expression in BUECs cultured with 0.1% BSA is downregulated compared to BUECs treated with hormones (Fig. 70). Furthermore, bPGT mRNA was expressed uniformly following a 24 hour hormone treatment in 0.1% BSA (Fig. 70).

3.5.2. Hormonal effects on PGT mRNA expression in cultured BUECs after a 24 hour treatment period in phenol red free media supplemented with 10% FCS

In order to discover if there was an effect of serum, BUECS were treated with hormones in the presence of 10% FCS for 24 hours (Fig. 71). Bands of expected size are shown on the gel that correlates with the DNA marker (1), negative control (2), positive control (3) and PGT (Lanes 4, 5, 6, 7, 8 and 9).

![Figure 71: RT-PCR analysis of total RNA isolated from hormonally stimulated BUECs for 24 hours in 10% FCS medium, using a gene specific primer for bPGT.](image)

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Table 15: Lane constituents in 10% FCS media
Control BUECs cultured in phenol red free DMEM supplemented with 10% FCS did not express bPGT mRNA (Fig. 7). BUECs displayed a differential pattern of PGT mRNA expression following hormonal treatment, when cultured in phenol red free DMEM supplemented with 10% FCS. E2 reduced bPGT expression to a level similar to the control, an effect that was reversed by Fulv&E2 treatment (Fig. 7). BUECs cultured with indomethacin in media containing 10% FCS elevated bPGT mRNA expression compared to all other treatments (Fig. 7).
3.5.3. Effects of PKC activators and indomethacin on PGT mRNA expression in cultured BUECs after a 24 hour treatment period in phenol red free media supplemented with 0.1% BSA

BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA. TPA, an activator of PKC, upregulated bPGT mRNA, while indomethacin and oxytocin maintained a low level of expression (Fig. 72). Bands of expected size are shown on the gel that correspond with the DNA marker (1), negative control (2), positive control (3) and PGT (Lanes 4,5,6 and 7). All treatments were prepared in media containing 0.1% BSA (Fig. 72).

![Figure 72: RT-PCR analysis of total RNA isolated from hormonally stimulated BUECs for 24 hours in 0.1% BSA medium, using a gene specific primer for bPGT.](image)

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Table 16: Lane constituents in 0.1% BSA media
3.5.4. Effects of PKC activators and indomethacin on PGT mRNA expression in cultured BUECs after a 24 hour treatment period in phenol red free media supplemented with 10% FCS

bPGT mRNA expression in hormonally stimulated BUECs in 10% FCS showed a differential pattern of expression (Fig. 73) and differed from BUECs cultured with the same hormones but with 0.1% BSA conditions (Fig. 72). Firstly, control BUECs, cultured in phenol red free DMEM supplemented with 10% FCS, reduced bPGT expression. Oxytocin increased bPGT mRNA, indomethacin maintained a basal pattern of expression, while expression was dramatically reduced after TPA treatment (Fig. 73). Bands of expected size are shown on the gel that correspond with the DNA marker (1), negative control (2), positive control (3) and PGT (Lanes 4, 5, 6, 7 and 8), and all treatment were carried out in media containing 10% FCS (Fig. 73).

Figure 73: RT-PCR analysis of total RNA isolated from hormonally stimulated BUECs for 24 hours in 10% FCS medium, using a gene specific primer for bPGT

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Table 17: Lane constituents in 10% FCS media
Discussion
4.1. Discussion

4.1.1. Identification of PGT in uterine tissue

PGT protein expression was examined in bovine uterine tissue from the luteal phase (Fig. 20) and follicular phases of the oestrous cycle (Fig. 21) and in line with a study by Banu et al., 2003, PGT protein was confined to the luminal epithelium (LE), which is the major site of PG synthesis (Asselin et al., 1996). Bovine uterine tissue from the luteal phase expressed PGT protein (Fig. 20), with negligible expression observed during the follicular phase (Fig. 21). The stage specific expression of PGT protein highlights an efficient transport mechanism that indicates the transport of newly synthesized PGs during the luteal phase, possibly to induce luteolysis and subsequent resumption of the oestrous cycle. The results obtained here are in agreement with a previous study conducted by Banu et al., (2003). Progesterone is the dominant hormone during the luteal phase of the bovine oestrous cycle and ensures uterine quiescence (Inskeep., 2004) and maintains a functional CL (Section 1.3.3). In addition, progesterone has been shown to increase PG synthesis in cultured bovine endometrial cells (Asselin et al., 1996; Skarzynski et al., 1999), as well as upregulating the expression of oatps in various systems (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006).

Also, Bao et al., (2002) confirmed the expression of PGT in tissues that synthesize and secrete prostanoids. In cows, endometrial responsiveness to oxytocin increases during the luteal phase, with an initial spike occurring at days 13-16 postestrous and maximum expression at the onset of luteolysis (Mirando et al., 1993). Maximum expression of oxytocin receptor (OTRs) at luteolysis coincides with elevated oxytocin secretion from the CL (Schams et al., 1985) and increased PGF$_{2 \alpha}$ secretion (Mirando et al., 1993). Immunohistochemical studies show increased PGT expression in bovine uterine tissue treated with oxytocin on the ipsilateral side of the CL (Fig. 20B), implying a role for oxytocin induced luteal regression (Section 1.13.1.1.). Increasing the density of PGT protein in uterine tissue during the luteal phase may aid in the compartmental transport of PGs from the luminal epithelia towards the CL to induce luteal regression and resumption of the bovine oestrous cycle.
Taken together, these previous studies and the results obtained from this study provide an insight into the fate of PGs in bovine uterine tissue. Elevated progesterone observed during the luteal phase can promote PG synthesis in uterine luminal epithelial cells and increase the expression of PGT. By increasing the components of PG transport, there is potentially increased transport of PGs which in vivo can carry PGs back to the CL to induce luteolysis at the end of the luteal phase (PGF$_{2\alpha}$) or a luteotropic effect during pregnancy (PGE$_2$).

A progesterone priming period is necessary for estradiol to promote PGF$_{2\alpha}$ (LaFrance and Goff, 1988) and results from immunohistochemical studies during the assumed follicular phase (Fig. 21), showed the absence of PGT protein regardless of proximity to the large follicle or whether tissue was subject to an oxytocin challenge. Bovine uterine tissue from the follicular phase used in these studies may only have been exposed to progesterone for a short period of time and therefore did not upregulate the PGT protein.

Rat uterine tissue displayed a different pattern of PGT expression (Fig. 22 and 23) compared to expression in bovine uterine tissue (Fig. 20 and 21), indicating species differences in the expression of PGT. Interestingly, and similar to bovine uterine, PGT protein was localized to the uterine luminal epithelium, which demonstrates a role for PGT in the rapid transfer of PGs, synthesized in these cells. The pattern of PGT protein expression was examined from each stage of the rat oestrous cycle (Fig. 22), where PGT protein was upregulated in luminal cells at proestrous (Fig. 22C) compared to all other stages indicating rapid PG transport at this stage, when progesterone levels begin to drop and estradiol levels start to increase. PGT was also expressed in rat uterine tissue from the metestrous stage (Fig. 22B) and at estrous (Fig. 22D) but to a lesser extent. In ovariectomized rats, it has been previously shown that progesterone priming for 2 days followed by an estradiol injection stimulated an early peak in PG concentration at 1 hour that declined gradually over-time reaching basal levels by 12 hours (Castrance and Jordan, 1975). In line with that study (Castrance and Jordan, 1975), in this study, rat uterine tissue obtained from the proestrous stage (Fig. 22C), had been primed with progesterone during metestrous and diestrous and exposed to low levels of ovarian estradiol at proestrous, which could potentially increase PG synthesis. Newly synthesized PGs can therefore utilize the PGT expressed at proestrous to exert their effect.
Stimulation of rat uterine tissue from different stages of the oestrous cycle with oxytocin also increased PGT protein expression in an estradiol rich-environment (Fig. 23). PGT protein was highest during the estrous stage (Fig. 23D) compared to the proestrous stage (Fig. 23C) with negligible expression during metestrous (Fig. 23B) and diestrous (23A). In effect, oxytocin administration during the rat oestrous cycle delayed the expression of PGT protein by downregulating protein expression at proestrous and elevating its expression at estrous (Fig. 23) compared to rat uterine tissue not exposed to oxytocin (Fig. 22).

The species-specific pattern of PGT protein expression may be due to the different lengths of uterine exposure to progesterone, where bovine uterine tissue is in a progesterone-rich environment for approx. 13-15 days (Section 1.3), while rat uterine tissue is exposed to progesterone for only 2 days (Suckrow et al., 2006). In addition, the differential expression between animals could be attributed to the ovulatory process of each animal. Cows are monovulatory animals that release one oocyte per cycle, while rats are polyovulatory and release several oocytes per cycle. The subtle difference between the species with respect to their reproductive cycles may affect expression of PGT at different stages of the cycle.

4.1.2. Effects of Culture Conditions on PGT expression and $[^3H]^+$ PG uptake into BUECs

In order to identify the control mechanisms of PG transport in BUECs, a simple in vitro model was developed. BUECs were enzymatically and mechanically isolated and cultured to form a monolayer (Fig. 24). These cells stained positively for the epithelial cell marker, cytokeratin with minimal vimentin staining (Fig. 25), thus confirming the epithelial morphology of the BUECs.

BUECs were cultured in phenol red free or phenol red containing DMEM in the presence or absence of sera to investigate whether different culture conditions affected the transport of $[^3H]$ PGs. Uptake of $[^3H]$ PGF$_{2\alpha}$ into BUECs cultured in DMEM containing phenol red was significantly (p<0.01) higher compared to BUECs cultured in phenol red free DMEM (Fig. 30). The effect of phenol red DMEM on the uptake of $[^3H]$ PGF$_{2\alpha}$ was two-fold. Phenol red which is used in some cell culture models as a pH indicator, is also a weak estrogen (Berthois et al., 1986) that may illicit some hormonal responses in BUECs. In contrast, BUECs cultured in phenol red free media may lie in a hormonally non-responsive state and uptake of
[\textsuperscript{3}H] PGF\textsubscript{2α} may be limited. Secondly, phenol red DMEM used for these studies was glucose rich (25mM) compared to 18mM glucose available in phenol red free DMEM. Overnight incubation with increasing concentrations of glucose caused a similar pattern of [\textsuperscript{3}H] PGF\textsubscript{2α} uptake into BUECs (Fig. 30) as observed in a previous similar study by Chan et al., (2002), where higher concentrations of overnight glucose incubation increased PG uptake into cells. This effect of phenol red DMEM could be potentially due to elevated lactate turnover in cells cultured in high glucose conditions.

Further experiments demonstrate increased [\textsuperscript{3}H] PGF\textsubscript{2α} uptake into BUECs that were cultured in phenol red DMEM supplemented with different sera compared to [\textsuperscript{3}H] PGF\textsubscript{2α} uptake in BUECs grown in phenol red free conditions (Fig. 31). Serum contains different concentrations of fatty acids and hormones that can vary between batches. The PUFA concentration of FCS was estimated in 1984 (Stoll and Spector., 1984) and a PUFA content of 27.8\% was calculated, composed of 10.4\% arachidonic acid (AA) and 6.2\% linoleic acid (LA). The high AA and LA content can have many and varied effects and in terms of PGs, AA and LA can help regulate PG synthesis in cultured cells via their incorporation into the cell membrane, thus providing immediate precursors for PG production (Section 1.6.). LA itself has also been found to have estrogenic properties and can stimulate ERβ mRNA expression and upregulate the progesterone receptor (PR) (Liu et al., 2004). The role of LA in providing precursors for PG synthesis and acting in an estrogen-like manner in culture may alter PG synthesis and transport properties across BUECs membranes. Also, FCS contains glucose which is negligible in BSA which may generate lactate to act as an exchange anion for PG uptake. The contaminant effects of lipophillic material, including steroids, can be removed from serum by charcoal stripping the serum, without affecting amino acid, glucose or salt content. Thus, CS-FCS can be used with confidence to analyse PGF\textsubscript{2α} in the absence of steroid hormones.
Uptake was consistently higher in BUECs cultured with phenol red DMEM, where BUECs supplemented with 0.1% BSA in phenol red media had the highest uptake, followed by charcoal-stripped FCS then FCS alone (Fig. 30). The stimulatory effect of phenol red DMEM cultured BUECs supplemented with 0.1% BSA (Fig. 30) may be attributed to low PG precursors in BSA and elevated glucose obtained in phenol red DMEM. The combined effects, generate an inward PG gradient, owing to reduced fatty acid precursors in BSA, that use lactate (by-product of glucose metabolism) as an exchange molecule, thus providing optimal conditions for [³H] PGF₂α uptake into BUECs (Fig. 30).

The inhibitory role of FCS supplementation on [³H] PGF₂α uptake may be attributed to the high AA and LA content of FCS (Stoll and Spector., 1984) acting as precursors for PG synthesis, thus generating outward gradient that impedes additional uptake of exogenous [³H] PGF₂α (Fig. 30).

It was also demonstrated that [³H] PGF₂α uptake into BUECs was higher for all time points when phenol red DMEM was used as culture medium as opposed to phenol red free DMEM (Fig. 31). The pattern of [³H] PGF₂α uptake into BUECs was similar for both culture media, and revealed a discrete peak after 10mins that dissipated over-time. This was consistent with a study carried out by Chan et al., (1998), that showed highest levels of [³H] PG uptake into PGT transfected HeLa cells after 10mins, which plateaued after 20mins and decreased gradually over time. The time-course of [³H] PGF₂α is consistent with an exchange model (Hagenbuch and Meier., 2004), whereby the uptake of PG is coupled to the counter-exchange of another substrate down its concentration gradient that dissipates over time (Schuster., 1998; Schuster., 2002). Again, the higher level of [³H] PGF₂α uptake in BUECs cultured in phenol red DMEM, may be attributed to an estrogenic component or possibly the glucose content of phenol red DMEM compared to phenol free DMEM.

The effect of sera in different phenol-containing media was examined to determine an interaction over time (Fig. 33). Uptake of [³H] PGF₂α into BUECs cultured in phenol free DMEM with different sera (Fig. 33a) was consistently lower than uptake into BUECs cultured in phenol red DMEM with different sera (Fig. 33b). FCS reduced the uptake of [³H] PGF₂α into BUECs compared to other sera supplements in either phenol free or phenol red DMEM (Fig. 33), suggesting an inhibitory factor, possible fatty acids, present in FCS that can ultimately generate an electrochemical gradient the prevents further uptake of PGs.
Highest uptake was observed after 10 minutes in phenol red free cultured BUECs supplemented with 10% CS-FCS (Fig. 33a), while uptake of [\(^3\)H] PGF\(_{2\alpha}\) into BUECs cultured in phenol red free DMEM, supplemented with 0.1% BSA peaked at 10 mins (Fig. 33a), both of which showed a similar pattern of [\(^3\)H] PG uptake as reported by Chan et al., (1998). [\(^3\)H] PGF\(_{2\alpha}\) uptake into BUECs in phenol red DMEM was higher for all different sera compared to phenol free DMEM but did not reveal a sera effect (Fig. 33b). Supplementation of phenol red cultured BUECs with 0.1% BSA showed highest uptake after 5 minutes that declined with respect to time; this uptake pattern may be attributed to a low level of PG precursors present in BSA compared to FCS and the generation of an inward PG gradient.

BUECs cultured in phenol red DMEM supplemented with 10% CS-FCS or 10% FCS showed a similar profile for [\(^3\)H] PGF\(_{2\alpha}\) uptake (Fig. 33b) as their respective effects in phenol free cultured BUECs (Fig. 33a) and illustrated low uptake at 5 minutes with a modest peak after 10 minutes that declined gradually towards 20 minutes, consistent with antiport transport.

4.1.3. Effect of hormones on PGT protein expression and uptake of [\(^3\)H] PG in BUECs

BUECs were cultured with steroid hormones to mimic the uterine environment at different stages of the bovine oestrous cycle. Progesterone is the dominant hormone during the luteal phase of the oestrous cycle and pregnancy and is secreted from a functional CL (Section 1.3.). In contrast, estradiol is the hormone produced at estrous from developing follicles and sporadically coinciding with the onset of a follicular wave during the cycle (Section 1.3.). Treating BUECs with progesterone distinguishes the uterine environment as the secretory phase, which corresponds to the ovarian luteal phase, compared to when BUECs are treated with estradiol, to mimic the proliferative uterine phase (ovarian follicular phase).

Progesterone maintained a similar pattern of PGT protein expression in isolated cultured BUECs as the control after a 24 hour treatment (Fig. 26), 48hour (Fig. 27) and 72 hour (Fig. 28) treatment periods with slight differences. In addition, following P4 treatment, PGT protein expression was localized near the nuclei, suggesting protein expression on the nuclear envelope as opposed to insertion into cellular membranes. Comparatively, P4 treatment after 24 hours, showed less PGT expression than BUECs cultured with E2 for 24hours (Fig. 26), while co-incubation with the progesterone receptor antagonist, mifepristone and P4 (Mif&P4) increased expression of PGT protein compared to the control (Fig. 29). Mifepristone is an
antiprogestin that inhibits progesterone action at the receptor level and is used commercially as an abortifacient (Hazra and Pore., 2001). Although, P4 has been shown to increase PGT protein expression in bovine uterine tissue (Fig. 20) and in other systems (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006), this stimulatory effect was not observed in cultured BUECs treated with P4. Treatment with P4 maintained a similar pattern of protein expression as the control for these studies, thus introducing a role for other contributory factors in the up- and down-regulation of PGT protein expression in BUECs aside from progesterone.

The inhibitory effect of P4 on PG transport in isolated cultured BUECs was also observed in transport studies that examined the effect of P4 treatment on [³H] PG uptake into BUECs, when cells were treated for 24hours (Figs. 34 and 37) and 72hours (Figs. 35 and 39).

BUECs cultured with 10nM P4 in phenol free DMEM supplemented with 10% FCS for 24hours reduced the uptake of [³H] PGF₂α into the cells (Fig. 34) but the effect was not statistically significant. Co-incubation of BUECS with mifepristone and progesterone (Mif & P4) in 10% FCS supplemented phenol free DMEM for 24 hours increased [³H⁺] PGF₂α uptake into BUECs (Fig. 34) indicating a regulatory role for progesterone in the transport of PGs in uterine cells.

Steroid hormone treatment for 72 hours reduced [³H] PGF₂α uptake into BUECs for all treatments (Fig. 35) compared to the 24hour treatment period (Fig. 34). P4 treatment in phenol free DMEM supplemented with 10% FCS significantly reduced uptake after 72hours, while co-incubation with Mif & P4 in the same culture conditions reversed and surpassed the uptake potential of BUECs for [³H] PGF₂α by these cells compared to P4 treatment alone (Fig. 35). Antiprogestins (mifepristone) reduce PGF₂α secretion in bovine endometrial epithelial cells (Skarzynski et al., 1999), and in this study the mifepristone has the potential to inhibit PG synthesis, thereby generating an increased electrochemical gradient that drives intracellular PGF₂α uptake into BUECs. Mifepristone inhibits PG synthesis, probably via its anti-proliferative properties (Cameron et al., 1996; Narvekar et al., 2004; Goyeneche et al., 2007) thereby, preventing the progression of the cell cycle at the G1-S transition (Goyeneche et al., 2007). Suppressing the cell cycle inhibits COX-2 expression and subsequent PG synthesis (Gilroy et al., 2001).
These obvious P4 effects are not observed in BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA compared to supplementation with 10% FCS (Fig. 34 and 35) and reveals a P4 and sera interaction. The effects of P4 treatment in different sera are twofold. It has previously been demonstrated that P4 can promote PG synthesis in endometrial cells (Asselin et al., 1996; Skarzynski et al., 1999), while contaminants of FCS, including AA, LA and other steroids (Stoll and Spector., 1984) may act as precursors in the synthesis of PGs in BUECs. The combined effect of increasing PG synthesis may be attributed to the ability of P4 to mobilize and utilize lipid droplets from FCS and supply sufficient esterified AA for PG synthesis (Brinsfield and Hawk, 1973). The proposed elevation of PG production in BUECs generates an outward electrochemical gradient that prevents further accumulation of PGs applied extracellularly.

An antagonistic relationship between P4 treatments (1nM; 10nM or 100nM) and phenol-red containing DMEM was revealed after a 24 hour treatment period (Fig. 37). BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA and treated with P4 for 24 hours, significantly increased (p<0.05) the uptake of [³H] PGF₂α into BUECs from 0nM to 100nM P4 (Fig. 36). This dose-dependent increase in the level of [³H] PGF₂α uptake into BUECs in phenol red free conditions that were treated with P4 for 24 hours may be due to inhibition of PG production due to reduced bioavailability of precursors for PG synthesis. In contrast, BUECs treated with P4 (1nM, 10nM or 100nM) for 24 hours in phenol red DMEM supplemented with 0.1% BSA exhibited a stepwise decrease in the level of [³H⁺] PGF₂α uptake into BUECs (Fig. 37). Phenol red, acting as a weak estrogen, has the potential to upregulate PRs in these BUECS, making them more responsive to administration of P4. P4 induces rapid phospholipid turnover via a non-genomic mechanism (Brinsfield and Hawk, 1973; Baldi et al., 1995) eliciting a more profound stimulatory effect to promote PG synthesis thus generating a direct outward PG gradient that repels further PG uptake.

In line with immunohistochemical studies where FCS and P4 treatment maintained a basal level of PGT expression (Section 3.3), a co-incubation system of BUECs with P4 in FCS supplemented media more than likely functions synergistically to promote PG synthesis and generate a net outward PG flux to prevent further uptake of PGs (Figs. 34 and 35).
BUECs primed with P4 for 71.5 hours and then subjected to an oxytocin challenge for 0.5 hours or primed only with P4 for 72 hours reduced $[^3]$H PGF$_{2a}$ uptake into BUECs (Fig. 39) in comparison to E2 primed BUECs (Fig. 38). An oxytocin effect was not observed in BUECs primed with P4 before exposure to oxytocin for 0.5 hours, where uptake into BUECs not subject to an oxytocin challenge was similar for both experimental groups (Fig. 39). Again, estrogen is necessary to induce oxytocin receptors (OTRs) in uterine cells (Bathgate et al., 1995), and these non-responsive BUECs cannot mount an effect to alter $[^3]$H PGF$_{2a}$ uptake into BUECs. A previous study demonstrated that P4 inhibited oxytocin-stimulated production of PGF$_{2a}$ in bovine endometrial cells (Kowalik et al., 2009), which could upregulate the uptake of $[^3]$H PGF$_{2a}$ by generating an inward PG gradient. However, the studies are not comparable since subtle differences between both studies may have contributed to the results obtained.

Extrapolation of the inhibitory effects of P4 on PG transport in vitro back to the in vivo model provides an insight into the fate of PGs during the luteal phase of the bovine oestrous cycle and pregnancy. Reduced PG uptake during these stages could potentially inhibit the transfer of PGF$_{2a}$ towards the CL, thus preventing luteolysis and maintaining pregnancy. This is especially important during the maternal recognition of pregnancy, when PGF$_{2a}$ can initiate luteolysis subsequent to early embryonic loss (Kastelic et al., 1991). Also, an oxytocin challenge in a P4-rich environment (Fig. 39) failed to alter PG uptake, indicating the potent inhibitory effect of P4 on PG transport. Interestingly, the fatty acid content in uterine cells appears to be another factor of PG transport leading to the generation of PGs thereby preventing further accumulation of extracellular PGs.

Estradiol (E2) maintained or modestly increased PGT protein expression in isolated, cultured BUECs as observed through immunohistochemical techniques. Treatment with 1nM or 100nM E2 for 24 hours, shared a similar pattern of PGT protein expression as BUECs cultured with 0.1% BSA, while 10nM E2 for 24 hours increased the density of PGT protein in BUECs (Fig. 26). Furthermore, while 10nM E2 promoted PGT protein expression, co-culture with the E2-receptor antagonist, fulvestrant and E2 (Fulv&E2) for 24 hours down regulated expression of PGT in BUECs (Fig. 29), thus introducing a role for E2 in the regulation of PGT expression in vitro after a 24 hour incubation period.
Fulvestrant is an ER antagonist with a binding affinity to ERs of 89% (Wakeling et al., 1991). Fulvestrant displays no agonistic activity and completely blocks ER-mediated transcriptional activity (Osborne et al., 2004).

After a 48 hour treatment period there was a dramatic decrease in the expression of PGT protein for all steroid hormone treatments (Fig. 27). Expression was lost after 1nm E2 treatment, while a discrete increase in PGT expression following treatment with 10nM and 100nM E2 compared to the control was observed (Fig. 27). PGT protein expression was upregulated in BUECs, after a 72 hour priming period with 1nM and 10nM E2, while expression is absent when treated with 100nM E2 for 72 hours (Fig. 28). In these isolated BUECs, E2 treatment had a stimulatory effect on the regulation of PGT protein after 24 hour and 72 hour treatments. Previous studies indicate an inhibitory effect (Asselin et al., 1996) or no effect of estradiol (Skarzynski et al., 1999) on PG synthesis by bovine endometrial epithelial cells. The results from the immunohistochemical staining in this study suggested that these cells maintained a basal level of PG synthesis following estradiol treatment and require PGT for the transport of these PGs (Skarzynski et al. 1999). Furthermore, in agreement with a study by Bao et al., (2001), demonstrating that PGT is expressed in all cell types that synthesize PGs, these BUECs expressed elevated PGT in preparation for rapid PG transport. Similar to rat uterine tissue (Fig. 22 and 23), BUECs in an estradiol rich environment expressed PGT compared to a progesterone rich environment.

The effect of E2 on the uptake of $^3$H PGF$_{2\alpha}$ into BUECs was examined and showed only minor alterations in transport. BUECs cultured to 70-90% confluence and treated with 10nM estradiol (E2) or co-incubated with Fulv&E2 for 24hours in phenol red free DMEM supplemented with different sera (Fig. 34), did not reveal an estrogenic effect on the uptake of $^3$H PGF$_{2\alpha}$. Uptake of $^3$H PGF$_{2\alpha}$ into BUECs was similar for both estrogen treatments and the control after 24hours (Fig. 34). BUECs primed with E2 or Fulv&E2 for 72 hours in phenol free DMEM supplemented with 0.1% BSA or 10% FCS maintained the same pattern of $^3$H PGF$_{2\alpha}$ uptake into the cells as the control and did not reveal a significant effect of treatment (Figs. 35).
Interestingly, BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA and treated with E2 (1nM, 10nM or 100nM) for 24 hours showed a non-significant stepwise decrease in the uptake of [³H] PGF₂α into BUECs as E2 concentration increased (Fig. 36), indicating a more potent inhibitory effect of E2 on PG synthesis at higher doses. The effect of E2 treatment in phenol red DMEM was indeterminate (Fig. 36) and may be attributed to exposure of the BUECs to supra-concentrations of E2 that may be toxic to the cells. Comparatively, the effects of different concentrations of P4 (Fig. 37) and E2 (Fig. 36) in phenol red free DMEM supplemented with 0.1% BSA demonstrated opposite uptake profiles. Incubation with P4 exhibited a dose-dependent increase in [³H] PGF₂α uptake into BUECs from 0nM to 100nM P4, while E2 treatment revealed a dose-dependent decrease in [³H] PGF₂α uptake from 1nM to 100nM E2.

BUECs primed with E2 for 72 hours in phenol red free DMEM supplemented with 0.1% BSA showed highest uptake of [³H] PGF₂α into BUECs, when treated with 10nM E2 (Fig. 38), but all E2 concentrations increased [³H] PGF₂α uptake with respect to the control.

Priming BUECs for 71.5 hours with E2, prior to an oxytocin challenge suppressed the stimulatory effects of 1nM E2 (Fig. 38). Previous studies demonstrate that E2 does not alter (Skarzynski et al., 1999) or modestly reduces PG synthesis in endometrial cells (Asselin et al., 1996). Therefore, BUECs treated with E2 only caused a discrete change in the synthesis of PG compared to the control, which ensured a similar pattern of [³H] PGF₂α uptake into BUECs as the control. The uptake studies obtained here are in contrast to the results obtained in the immunohistochemical studies such that bovine tissue may confer a role for other contributory factors in the regulation of components of the PG transport mechanism. E2 increased PG uptake into cells at higher doses after a 72 hour culture period (Fig. 38), possibly due to inhibition of PG synthesis by BUECs under these conditions.
In relation to the bovine oestrous cycle, E2 does not inhibit PG transport and when E2 concentrations are highest during estrous, efficient permeability of uterine cells to PGs is necessary to allow the transport of endometrial PGE$_2$ towards the dominant follicle to induce ovulation (Espey., 1980). Also, after BUEC priming with low concentrations of E2 (1nM) followed by an oxytocin challenge (Fig. 38), PG uptake into BUECs was suppressed. This phenomenon *in vivo* could be attributed to elevated OTRs at low E2 concentrations (Bathgate *et al.*, 1995) that promote intracellular PG synthesis after an oxytocin challenge mediated via iPLA$_2$ (Tithof *et al.*, 2008) and prevent further accumulation of PGs.

### 4.1.4. PUFAs and [$^3$H] PG uptake into BUECs

The hydrophobic nature of the plasma membrane is almost entirely due to the lipid content, which accounts for $\approx$50% of mammalian membrane mass and contains approximately $1 \times 10^9$ lipid molecules per membrane (Alberts *et al.*, 2008). Phosphoglycerides are the predominant phospholipids of the cell membrane and are composed of a glycerol esterified to 2 fatty acids and a phosphorylated alcohol (Murray *et al.*, 2000). The unsaturated fatty acid is usually AA but can be displaced by members of the n-3 PUFAs. Metabolic cleavage of membrane bound omega-6 fatty acid AA yields 2-series PGs, while omega-3 fatty acids, especially EPA is the precursor of 3-series PGs (Wada *et al.*, 2007). Inclusion of a tailored feeding regime that ensures proficient uterine synthesis of PGs by dairy cows has been proposed as a novel and insightful method of reversing the failing trend of herd fertility. However, this has had limited success because the basic biology at all levels of the cascade is unknown.
In the BUEC model employed for these studies, uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs, following a 1day (Fig. 40) or 3day (Fig. 41) incubation period revealed similar profile of uptake for both incubation periods, despite a considerable reduction in uptake after 3days PUFA incubation (Fig. 41). BUECs cultured with EPA and DHA show a progressive decline in $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake into cells as PUFA concentration increases from 50μM to 100μM after 1day (Fig. 40) and 3days (Fig. 41). Furthermore, 50μM AA reduced the uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ into BUECs after one (Fig. 40) or 3day (Fig. 41) incubation and 100μM AA incubation restored $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake to control levels for both one day (Fig. 40) and three day culture periods (Fig. 41).

Treatment of BUECs with either EPA or DHA for seven days at both concentrations (50μM or 100μM) increased the uptake of $[^3\text{H}]\text{PGF}_{2\alpha}$ with respect to 0μM PUFA and treatment with either concentration of AA (Fig. 42).Similar to one day (Fig. 40) and three day (Fig. 41) PUFA incubations, 50μM AA inhibited uptake and 100μM AA promoted $[^3\text{H}]\text{PGF}_{2\alpha}$ uptake into BUECs (Fig. 42). The extended incubation period assumed elevated PUFA incorporation into cellular lipid membranes. Uptake in the control group revealed that $[^3\text{H}]\text{PGF}_{2\alpha}$ transport was significantly less compared to the DHA and EPA treatments at 50μM (p<0.05) and significantly reduced in the control group compared to the DHA treatment group at 100μM (p<0.05). The most likely reason for a 24 hour treatment showing a non-significant effect of PUFA incubation on PG transport (Fig. 40) was due to the reduced level of PUFA incorporation into plasma membranes, although strict protocols to ensure maximum PUFA delivery were followed (Mattos et al., 2003).

After a 3day incubation (Fig. 41), PUFA treatment at a concentration of 50μM revealed a significant effect (p<0.05) and a Tukey post-hoc test shows that PGF$_{2\alpha}$ uptake was reduced in BUECs treated with 50μM AA compared to treatment with 50μM EPA (p<0.05) and 50μM DHA (p<0.05). The inhibition of PGF$_{2\alpha}$ uptake observed with 50μM AA after a 3day incubation with PUFAs (Fig. 41) was more than likely due to elevated synthesis of 2-series PGs produced from the AA substrate (Section 1.7.1). The intracellular synthesis of 2-series PGs, eliminated an electrochemical gradient that prevented further accumulation of extracellular PGs. EPA and DHA generate 3-series PGs, and may introduce an electrochemical gradient for uptake of 2-series PGs.
BUEC incubation with PUFAs for 7 days (Fig. 42) revealed a significant effect at 50μM (p<0.05) and 100μM concentrations (p<0.05). The extended incubation period elevated PUFA incorporation into cellular lipid membranes, where n-3 PUFAs direct the synthesis of 3-series PGs, thus creating an inward 2-series PG gradient (Fig. 42). Inhibition of [³H] PGF₂α uptake into BUECs was also observed when cells were treated with 50μM AA, which suggests increased synthesis of 2-series intracellularly to prevent further uptake of the PGs.

From previous transport studies, estradiol was shown to act as a facilitator (Figs. 34; 354; 36; 38) for [³H⁺] PGF₂α uptake into BUECs.

Following on from these studies it was decided whether PUFAs can alter [³H⁺] PGF₂α uptake in an estradiol rich environment at different co-incubation intervals.

PUFA treatment (Fig. 40) or PUFA co-culture with 1nM E2 (Fig. 43) or co-culture with 10nM E2 (Fig. 46) for one day showed high variability between treatments. However, an inhibitory effect of 100μM AA was observed at 1nM E2 (Fig. 43). This inhibitory effect on [³H] PGF₂α uptake into BUECs may be attributed to increased AA incorporation into cell membrane and its catalytic cleavage from the membrane via increased activity of cPLA₂ following E2 stimulation (Dey et al., 1982). After a three day PUFA co-culture period with 1nM E2 (Fig. 44) or co-culture with 10nM E2 (Fig. 47), there was a stepwise increase in [³H] PGF₂α uptake into BUECs from 0μM to 100μM, possibly due to n-3 directed synthesis of 3-series PGs and possible generation of a gradient to promote [³H] PGF₂α uptake. PUFA co-culture with AA and 1nM E2 were inhibitory after 3 days (Fig. 44) but only inhibitory at a high dose when BUECs were co-cultured with PUFAs and 10nM E2 (Fig. 45). The inconclusive results here require further investigation in order to gain a better understanding of the effects of PUFA co-culture with E2 for 3 days had on the PG cascade in uterine tissue. A day PUFA treatment (Fig. 40) or PUFA co-culture with 1nME2 (Fig. 43) or culture with 10nM E2 (Fig. 46) for seven days demonstrated an inhibitory effect of 50μM AA on [³H] PGF₂α uptake into BUECs and inhibition of [³H] PGF₂α uptake at either AA concentration on the presence of 1nM E2 (Fig. 43) of 10nM E2 (Fig. 46). This inhibitory effect of AA after a seven day incubation or co-incubation with E2 demonstrates was more than likely due to increased incorporation of AA into plasma membranes over time and the high level of subsequent 2-series PG synthesis that inhibited further accumulation of [³H] PGF₂α into BUECs.
4.1.5. Effects of Glucose and [\(^3\)H] PG uptake into BUECs

Incubation of BUECs with glucose provides two mechanisms that might promote increased PG transport. Firstly, hyperosmolar concentrations of glucose have been shown to increase intracellular PG synthesis (Williams and Schrier., 1993; Sitter et al., 1998; Kim and Han., 2008) and cells that produce PGs express PGT (Bao et al., 2002). Secondly, lactate is a by-product of glucose metabolism and has been identified as an exchange molecule for PG transport via PGT (Chan et al., 2002). [\(^3\)H] PGE\(_2\) uptake into BUECs was significantly altered (p<0.05) by glucose concentration, illustrating a progressive increase in [\(^3\)H] PGE\(_2\) uptake as glucose concentration increased from 0mM to 43mM (Fig. 49). The stepwise increase in [\(^3\)H] PGE\(_2\) uptake into BUECs was most probably due to generation of higher concentrations of lactate as glucose concentration increased and coincides with a similar study conducted by Chan et al., (2002). Also, hyperosmolar glucose concentrations promote PG synthesis, which can promote PGT expression. PGT protein has been positively observed in BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA for 24hours (Fig. 26), therefore a limiting factor of PG transport, such as an exchange molecule may be influencing [\(^3\)H] PG transport in BUECs. Generation of lactate by metabolizing BUECs, may increase intracellular concentrations of lactate that exchange with extracellular PGs to promote uptake of [\(^3\)H] PGE\(_2\) by BUECs. Incubation of BUECs with increasing concentrations of glucose moderately affected [\(^3\)H] PGF\(_{2\alpha}\) uptake, where uptake increased from 0mM to 25mM glucose and fell when incubated with 43mM glucose (Fig. 52). Although, it has been reported that PGT transports eicosanoids in the following affinity order PGE\(_2\) = PGF\(_{2\alpha}\) = PGD\(_2\) >>Arachidonic acid (Banu et al., 2003), the results here implied that PGT carries [\(^3\)H] PGE\(_2\) (Fig. 49) more efficiently than [\(^3\)H] PGF\(_{2\alpha}\) (Fig. 52), in glucose rich environments. Uptake of [\(^3\)H] PGE\(_2\) after 2 minutes (Fig. 50) or 10 minutes (Fig. 51) exposure in untreated BUECs with different glucose concentrations demonstrated elevated [\(^3\)H] PGE\(_2\) uptake at 10mins in contrast to 2mins. Uptake of PGs after 10 minutes is in agreement with a study carried out by Chan et al., (1998) and from an uptake study as part of this research project (Fig. 32). Interestingly, increased [\(^3\)H] PGE\(_2\) uptake into BUECs was higher at 2 minutes following either E2 or P4 stimulation at all levels of glucose compared to untreated cells (Fig. 50). This may be accounted for by a non-responsive state the BUECs are in owing to sera and hormone withdrawal. Comparatively, at 10 minutes, PG uptake into BUECs in untreated BUECs, showed a stepwise increase in [\(^3\)H] PGE\(_2\) transport as glucose
concentration increases from 0mM to 43mM (Fig. 51), which may be accounted for by elevated lactate turnover from higher concentrations of glucose, which acts as an exchange molecule for the PGT to increase $[^3\text{H}]$ PGE$_2$ uptake into BUECs. Hormone treatment (E2 or P4) reduced $[^3\text{H}]$ PGE$_2$ uptake into BUECs after 10mins compared to control (Fig. 51) and uptake after 2mins (Fig. 50). This effect of hormones indicates rapid transport of $[^3\text{H}]$ PGE$_2$ in hormonally challenged BUECs, that dissipates by 10minutes.

Glucose concentration did not significantly alter the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ into BUECs (Fig. 52). After a 2 minute $[^3\text{H}]$ PGF$_{2\alpha}$ exposure, uptake is higher in hormonally treated BUECs compared to untreated BUECs in different concentrations of glucose (Fig. 53). Untreated BUECs did not increase $[^3\text{H}]$ PGF$_{2\alpha}$ uptake at any glucose concentration. In contrast, E2 showed a dose-dependent increase in $[^3\text{H}]$ PGF$_{2\alpha}$ uptake, while P4 demonstrated a dose-dependent decrease in $[^3\text{H}]$ PGF$_{2\alpha}$ uptake, from 0mM to 43mM glucose (Fig. 53). Previous results from this research project, demonstrated increased PGT protein expression in BUECs following E2 treatment after a 24hour treatment period (Fig. 26) and in line with the results of that immunostaining study, it can be deduced that E2 increased PGT expression in these cells and that uptake of $[^3\text{H}]$ PGF$_{2\alpha}$ increased as glucose concentration increased due to elevated lactate turnover. The combined effects of elevated PGT protein expression (Fig. 26) and increased lactate turnover, may have promoted $[^3\text{H}]$ PGF$_{2\alpha}$ uptake. P4 reduced the uptake of $[^3\text{H}]$ PGF$_{2\alpha}$, after 2 minutes, as glucose concentration increase from 0mM to 43mM (Fig. 53). Hyperosmolar concentrations of glucose (Williams and Schrier., 1993; Sitter et al., 1998; Kim and Han., 2008) working in conjunction with P4 (Asselin et al., 1996; Skarzynski et al., 1999) possibly increase intracellular PG concentration, thereby generating a net outward electrochemical gradient that prevents further uptake of PGs.

In the absence of hormones, $[^3\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs, remained consistently lower at 2mins (Fig. 53) compared to 10mins (Fig. 53), thus providing evidence for slow uptake of PGs in unchallenged BUECs. In contrast, E2 and P4 maintained equal $[^3\text{H}]$ PGF$_{2\alpha}$ uptake into BUECs for 2 minutes (Fig. 53) and 10mins (Fig. 54), indicating a similar level of $[^3\text{H}]$ PGF$_{2\alpha}$ uptake at both time points. In essence, steroid hormones in the presence of high glucose concentrations promoted early uptake of $[^3\text{H}]$ PGs, when compared to untreated BUECs and a longer transport study time.
In non-pregnant cows, the rate of glucose metabolism to lactate in endometrial cells does not change with respect to oestrous cycle stage (Chasse et al., 1992). Interestingly a study on rabbits demonstrated reduced production of lactate by pregnant and pseudopregnant animals compared to non-pregnant animals (Murdoch and O’Shea., 1970). Glucose metabolism of the bovine embryo is low during the first cleavage stages and only increases after reaching the uterus (Le Quarre et al., 1997). The low levels of lactate produced by the endometrium (Murdoch and O’Shea., 1970) and the embryo (Le Quarre et al., 1997) may confer a role for reduced intracellular lactate to abolish the uptake of PGs into the endometrium back to the CL or into the foetus to prevent detrimental accumulation. Furthermore, studies in sheep demonstrate that at the onset of parturition there is an increase in maternal and foetal plasma glucose that precedes elevated lactate production (Comline and Silver., 1972), while during early postpartum there is elevated PGF$_{2\alpha}$ production by bovine endometrium (Guilbault et al., 1984). It is well established that PGF$_{2\alpha}$ is necessary for prepartal luteolysis (Kindahl et al., 2002), while elevated glucose metabolism and lactate generation may be involved in the transport of PGF$_{2\alpha}$ to induce its luteolytic effects. In addition, a study in rats demonstrated that a diet rich in glucose was essential for normal parturition (Koski and Hill., 1986). Taken together, glucose metabolism and lactate generation are important mediators of PG transport and may influence bovine reproductive efficiency, therefore, close attention to the addition of glucose to bovine diets needs to be investigated.
4.1.6. Effects of pH and [3H⁺] PG uptake into BUECs

Having a pKa of 5.0, at normal physiological pH, PGs predominate as the charged organic anion bound to albumin (Schuster., 1998). At lower pH levels, PGs are present as the neutral lipid that that accept free H⁺, but as the pH increases they dissociate yielding the anionic molecule through liberation of the H⁺. The pH of the solution in which [³H] PGs are prepared significantly altered uptake into BUECs (Fig. 54). At pH 3.0, where the PG is presented as the neutral lipid, [³H] PG uptake into BUECs exceeded that at all other pH solutions (p<0.01), where the PG is in the anionic state. Therefore, BUECs are poised to transport neutral PGs more effectively than charged PGs. The results obtained here are in line with a previous study by Jones and Harper (1983), that highlighted the efficient uptake of [³H] PGs at acidic pH and poor [³H] PG uptake as the pH increased in endometrial epithelial cells from six day pregnant rats.

Cytosolic accumulation and membrane incorporation of [³H] PGE₂ were examined when PGs were prepared in an acidic pH (3.0) or at physiological pH (7.4). Accumulation of [³H] PGE₂ was significantly higher (p<0.05) for all treatments at 2 minutes compared to 20 minutes (Fig. 56), thereby indicating rapid transport of the neutral lipid. In addition, there was a slight increase in cytosolic accumulation of [³H] PGE₂ at 2 minutes, when BUECs were co-incubated with indomethacin and progesterone (Indo&P4). Indomethacin is a COX inhibitor that reduces PG synthesis, while P4 has the ability to upregulate oatps (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006). The combined effects of indomethacin and progesterone induced an inward PG gradient that may promote [³H] PGE₂ cytosolic accumulation via a transporter. Also, after 20mins, cytosolic accumulation of [³H] PGE₂ is marginally higher in BUECs cultured with indomethacin (Fig. 56), possibly due to reduced intracellular PG synthesis and the generation of a gradient for [³H] PGE₂ accumulation.

Cytosolic accumulation of [³H] PGE₂ at physiological pH (57) was considerably less for both time points compared to accumulation at pH 3.0 (Fig. 56) and did not show a treatment effect (Fig. 57). Membrane incorporation of [³H] PGE₂ at pH 3.0 at 2 or 20 minutes (Fig. 58) was less than cytosolic accumulation at pH 3.0 (Fig. 56). Two possibilities may explain increased accumulation (Fig. 56) versus incorporation (Fig. 58) at pH 3.0. Firstly, the cytosol volume was considerably higher compared to the membranous compartments and secondly there may only be a limited number of PGTs available in BUEC membranes for [³H] PGE₂ to occupy.
Furthermore, at 2 minutes in an acidic environment, hormone co-incubation with indomethacin (Indo&E2 or Indo&P4), elevated \[^3\text{H}\] PGE\(_2\) membrane incorporation (Fig. 58), an effect that was more than likely due to an increased inward gradient owing to the ability of indomethacin to reduce PG synthesis. At pH 7.4, membrane incorporation of \[^3\text{H}\] PGE\(_2\) (Fig. 59) was similar to incorporation at pH 3.0 (Fig. 58). BUECs co-incubated with Indo&E2 delayed \[^3\text{H}\] PGE\(_2\) incorporation at 2mins, while indo&P4 promoted rapid \[^3\text{H}\] PGE\(_2\) incorporation (Fig. 59).

Efflux of \[^3\text{H}\] PGE\(_2\) from BUECs preloaded with the tritiated PG for 20 minutes was higher when \[^3\text{H}\] PGE\(_2\) was prepared in an acidic (pH 3.0) compared to a physiological (pH 7.4) saline solution (Fig. 60). This phenomenon may be attributed to elevated \[^3\text{H}\] PGE\(_2\) uptake during the pre-loading period as previously investigated (Fig. 55). Efflux of \[^3\text{H}\] PGE\(_2\) from BUECs into saline or equimolar cold PG was higher at pH 3.0 compared to efflux at pH 7.4 (Fig. 61). Interestingly, efflux of \[^3\text{H}\] PGE\(_2\) into cold PG at pH 3.0 was uncharacteristically higher than efflux into saline (Fig. 61). This effect may be attributed to rapid influx and efflux of the neutral \[^3\text{H}\] PGE\(_2\) across the plasma membrane. At pH 7.4, efflux was greater into the saline solution compared to the cold PG solution (Fig. 61), demonstrating transport of the anionic PG down its concentration gradient into saline (Hagenbuch and Meier., 2004).

\[^3\text{H}\] PGE\(_2\) efflux from BUECs was time-dependent and showed highest efflux at both pH levels after 20 minutes (Fig. 62). \[^3\text{H}\] PGE\(_2\) was exported from BUECs into saline at a constant basal level from 2 to 15 minutes and peaked at 20 minutes for both pH levels but \[^3\text{H}\] PGE\(_2\) efflux into saline at 20mins in pH 3.0 was significantly higher than all other time-points at either pH (Fig. 62). Furthermore, \[^3\text{H}\] PGE\(_2\) efflux into a solution of cold PG was significantly higher for all time points in a pH 3.0 environment (Fig. 63), and efflux increased slowly over-time at pH 3.0 (Fig. 63). Efflux of \[^3\text{H}\] PGE\(_2\) into cold PG at pH 7.4 was maintained at a basal level overtime that peaked discretely at 5 minutes (Fig. 63). Taken together, it can be deduced that BUECs export the neutral PG more efficiently than the anionic PG, indicating increased permeability of the BUEC membrane to neutral compounds.

After the 20 minute efflux studies, there was increased \[^3\text{H}\] PGE\(_2\) localized in the cytosol compared to the membrane compartment, regardless of efflux solution (Fig. 64) or pH level (Fig. 65).
Transport of PGs via PGT involves the binding of carboxyl moieties of the anionic PG (Schuster et al., 2002). However, the neutral lipid does not contain carboxyl moieties and transport is therefore more than likely due to diffusion into the BUECs. This indicates that these BUECs are more permeable to neutral PGs (pH 3.0) and that the concentration of PGT protein in cellular membranes may be low.

4.1.7. Transepithelial transport of [3H+] PG in BUECs

PGs are synthesized in endometrial tissue (Asselin et al., 1996) or can be produced by foetal trophoblastic cells after the establishment of pregnancy (Knickerbocker et al., 1986). Due to their potent effects on CL function, PG transport from uterine compartments towards the CL needs to be fully elucidated in order to ascertain the mechanisms involved in the bovine estrous cycle and maintenance of pregnancy. BUECs cultured on SnapWell inserts form a polarized monolayer, giving rise to 2 functional domains, apical and basolateral membranes. Apical surfaces are directed towards the lumen of the tube, while basolateral surfaces face the interstitium. The confirmation of a tight epithelial BUEC monolayer provides a model for measuring the vectorial transport of PGs between the apical and basolateral compartments and vice versa. BUECs were cultured until the transepithelial electrical resistance (TER) measurement reached at least 2.5 kΩ prior to transport study. Both PGs ([3H] PGE$_2$ and [3H] PGF$_{2\alpha}$) shared a similar pattern of transepithelial transport across the polarized monolayer. The study measuring basolateral to apical vectorial PG flux (Fig. 68) is 10-fold higher than apical to basolateral vectorial PG flux (Fig. 66). Both systems showed high transport of both [3H] PGE$_2$ and [3H] PGF$_{2\alpha}$ at one minute that declined over-time. The obvious difference is the elevated PG flux from basolateral to apical surface (Fig. 68) in comparison to PG flux from apical to basolateral surfaces (Fig. 66), indicating that the basolateral membrane is more efficiently poised for [3H] PGs uptake compared with the apical membrane. Banu et al., (2003), identified expression of PGT protein at the basolateral surface of endometrial luminal epithelial cells and in line with that study, the increased basolateral to apical flux implies expression of PGT at the basolateral surface (Fig. 68).
At the end of the transepithelial transport studies, $[^3\text{H}]$ the PG content in BUECs was measured and revealed highest $[^3\text{H}]$ PGE$_2$ and $[^3\text{H}]$ PGF$_{2\alpha}$ concentration in the cytosol compared to the membrane compartments, regardless of the direction of flux of both (Fig. 67 and 69). $[^3\text{H}]$ PGE$_2$ was moderately higher than $[^3\text{H}]$ PGF$_{2\alpha}$ in the cytosolic compartment following the apical to basolateral PG flux study (Fig. 67), suggesting increased permeability of the apical membrane to $[^3\text{H}]$ PGE$_2$. Conversely, $[^3\text{H}]$ PGF$_{2\alpha}$ concentration in the cytosol was somewhat higher compared to concentration of $[^3\text{H}]$ PGE$_2$ in the cytosol (Fig. 69), which indicated increased permeability of the basolateral membrane to $[^3\text{H}]$ PGF$_{2\alpha}$. The elevated $[^3\text{H}]$ PGs localized in the cytosolic compartments may be accounted by the greater volume of the cytosol in comparison to the membranes or the presence of only a limited number of PG binding sites on cellular membranes.

**4.1.8. Analysis of PGT mRNA expression BUECs**

PGT mRNA expression displayed a uniform level using RT-PCR techniques, when BUECs were stimulated for 24 hours with steroid hormones and their receptor antagonists in phenol red free DMEM supplemented with 0.1% BSA (Fig. 70). In contrast, control BUECs, cultured in phenol red free DMEM supplemented with 0.1% BSA, downregulated bPGT (Fig. 70). In order to investigate bPGT mRNA expression further, BUECs were stimulated with indomethacin (PG synthesis inhibitor), oxytocin (cPLA$_2$ activator) and TPA (PKC activator) for 24 hours. In phenol red free media supplemented with 0.1% BSA (Fig. 72), bPGT is again uniformly expressed after incubation with these series of drugs, with expression highest in the BUECs cultured in phenol red free DMEM supplemented with 0.1% BSA and stimulated with TPA for 24 hours (Fig. 72). Upregulation following TPA stimulation substantiates a role for PKC activation in the expression of bPGT mRNA.
Discussion

FCS (10%) downregulated the expression of bPGT mRNA (Fig. 71), indicating the presence of an inhibitory factor in FCS that prevents bPGT mRNA expression that is overcome in the presence of steroid hormones (Fig. 71). Treatment of BUECs with hormones in phenol red free DMEM containing 10% FCS showed highest bPGT mRNA expression in a P4 rich environment consistent with previous studies that show increased oatp expression following P4 treatment (Kullak-Ublick et al., 2001; St. Pierre et al., 2002; Grube et al., 2006). In vivo, elevated bPGT mRNA expression may be important at the end of the luteal phase to enable the transport of endometrial PGs towards the CL to induce luteolysis and subsequent resumption of the oestrous cycle. Indomethacin treatment increased bPGT mRNA expression in BUECs (Figs. 71 and 72). Interestingly, two bands of bPGT mRNA were identified when BUECs were challenged with indomethacin, TPA and oxytocin in phenol red free media supplemented with 10% FCS (Fig. 73). A number of conclusions can be drawn from the paradoxical expression of bPGT mRNA and protein. Firstly, bPGT mRNA analyses were carried out on isolated cultured uterine epithelial cells that differ to an in vivo system. Multiple extra-uterine factors can affect the endometrial environment from an in vivo study that cannot be applied to a cell culture system. For example, mechanical stretch upregulates COX-2, that ultimately drives PG synthesis (Akai et al., 1994; Sooranna et al., 2004). Spermatozoan also stimulate COX-2, PGES and PGFS expression in a dose and time-dependent manner in the bovine oviduct that may ultimately regulate PG synthesis and transport in uterine tissue (Kodithuwakku et al., 2007). Alterations in endometrial epithelial cell fatty acid composition can affect the synthesis of PGs by affecting the type of fatty acid in the cellular membranes (Cheng et al., 2001).
Conclusion
5.1. Conclusion

The steady decline in bovine reproductive performance, in the region of 0.45-1% annually has led to extensive research to reverse this trend and allow modern dairy farmers to select reproductively viable cows with high milk yield ensuring respectable profit margins without compromising fertility. The bovine embryonic and foetal mortality rate is substantial at approximately 40%, with an estimated 70-80% of loss occurring between day 8 and 16 post-fertilization (Diskin and Sreenan., 1980). The high incidence of embryonic loss occurring within the first 3 weeks of pregnancy coincides with the maternal recognition of pregnancy.

Maternal recognition of pregnancy is a critical period for the maintenance of a conceptus and requires the presence of a healthy embryo. At the establishment of pregnancy, synthesis of endometrial PGs is under trophoblastic control. Embryonic IFN-τ, directs PGE₂ synthesis at the expense of PGF₂α. However, unhealthy embryos or those with retarded growth may not secrete sufficient IFN-τ to direct the synthesis of luteotropic PGE₂. Endometrial synthesis of PGF₂α at the establishment of pregnancy can be relayed back towards the CL to induce luteolysis and subsequent early embryonic loss.

There are numerous factors that may affect embryonic mortality at the early stages of pregnancy. One area receiving a lot of attention is the adoption of bovine feeding regimes that alter aspects of the PG synthesis and transport cascade in order to reduce the incidence of embryonic loss. Feeding plans that guarantee the maintenance of a successful pregnancy until term, followed by prompt uterine involution and return to estrous are desirable to ensure herd reproductive performance and producer profitability (Mattos et al., 2000). Supplemental PUFAs were initially added to bovine feedstuffs to increase energy density and abolish postpartum NEB. However, it has also been shown that supplemental PUFAs obtained in the diet may offer a mechanism of improving reproductive performance by altering aspects of the PG synthesis and transport system in foetal and uterine tissue.
Conclusions

PG synthesis and subsequent transport in uterine tissue is a complex event because a multitude of factors have the potential to affect the sensitive cascade. These factors include hormonal stimuli, anion exchangers, electrochemical gradients, electrical charge of PG, compartmental transport of PGs between epithelial surfaces, fatty acid bioavailability and viability of BUECs in an in vitro culture model, all of which are examined carefully in this thesis in a series of experiments. Understanding PG synthesis and transport mechanism at a cellular level may prove an important factor for the design of feeding regimes that reverse poor bovine fertility trends.

This study investigated PGT protein expression in bovine and rat uterine tissue at different stages of their respective oestrous cycles and identified the localization of PGT in uterine luminal epithelial cells that was stage and species specific. PGT is expressed in bovine uterine tissue during the luteal phase (progesterone dominance), while expression is highest in rat uterine tissue during the estradiol dominance. Identification of PGT protein in uterine luminal cells from the luteal phase highlights the requirement of a carrier for the transport of PGs, that is synthesized under the influence of progesterone. In addition, the absence of a transporter during the follicular phase of the bovine oestrous cycle indicates that PGT is not necessary when PGs are not synthesized. For rat uterine tissue, PGT expression is highest during proestrous and estrous, indicating PG transfer during estradiol dominance. The difference in PGT expression may be attributed to the different durations of the oestrous cycles, in which uterine tissue are exposed to steroid hormones for distinct periods of time or the number of oocytes ovulated for either animal per cycle. In addition, bovine uterine tissue for PGT protein expression during the follicular phase was selected on the basis of the presence of a large follicle, however, there was a possibility that follicle may not have been dominant, which could affect the expression of PGT, although strict protocols were adhered to.

PGT protein expression in BUECs and the transport of $[^{3}H]$ PGs in BUECs was measured in relation to culture conditions, hormonal stimuli, PUFA supplementation, glucose treatment, pH and eventual transepithelial flux of $[^{3}H]$ PGs.
Conclusions

Culture of BUECs in phenol red DMEM supplemented with 0.1% BSA (Fig. 31), increased $[^3$H] PGF$_{2\alpha}$ in comparison to all other phenol red and sera treatments. The reasons for this phenomenon are several fold and imply that reduced fatty acid content and increased glucose or hormonally responsive BUECs are more permeable to extracellular PGs.

BUECs treated with P4 for different time periods maintain a basal level of PGT protein and its expression is only upregulated when BUECs are treated with 10nMP4 for 48hours or primed with 10nM P4 for 48hours and then stimulated with estradiol, revealing a complex method of PGT protein regulation in BUECs (Appendix B). Progesterone inhibits the uptake of [3H$^+$] PGF$_{2\alpha}$, an effect that is exacerbated after a 3day treatment period and reversed and exceeded when BUECs were co-cultured with mif&P4 for 3days in phenol red free media supplemented with 10% FCS (Fig. 35). Furthermore, an antagonistic relationship develops when BUECs are treated with different levels of P4 in phenol-free or phenol red media. Progesterone treatment in phenol red free media shows a dose-dependent increase in [$^3$H] PGF$_{2\alpha}$ uptake, while uptake shows a dose-dependent decrease in [$^3$H] PGF$_{2\alpha}$ uptake in phenol red media (Fig. 37). The opposing effects, may be due to upregulation of PRs induced by phenol red. BUECs treated with progesterone maintain a similar expression of PGT mRNA as the control in phenol red free media supplemented with either 0.1% BSA or 10% FCS, revealing that BUECs are able to synthesize PGT at each stage of the cycle and by possible post-translational modifications, alter its protein expression.

Estradiol does not significantly alter [$^3$H] PGF$_{2\alpha}$ uptake into BUECs and maintains a relatively constant uptake pattern as the control (Figs. 34, 35, 36). However, BUECs cultured in media containing phenol red show an indeterminate profile for [3H$^+$] PGF$_{2\alpha}$ uptake compared to uptake into BUECs cultured in phenol red free media. Most likely, this effect is due to exposure of the BUECs to supra-concentrations of E2 that may be toxic to the cells.
Oxytocin increases the expression of PGT protein from bovine uterine tissue ipsilateral to the CL (Section 3.1). Progesterone in conjunction with oxytocin has stimulatory effects on PG synthesis, and the upregulation of the PGT suggests elevated PGT protein when PG production is raised. In rat uterine tissue, oxytocin delays the expression of PGT protein, an effect that is species specific (Fig. 23). Similarly, in BUECs, oxytocin reduces $[^3]H$ PGF$_{2\alpha}$ uptake when BUECs were primed with low levels of estradiol and then subject to an oxytocin challenge (Fig. 38), again indicating an inhibitory effect of oxytocin in environments of low estradiol concentration.

BUECs treated with PUFAs show a variable profile for $[^3]H$ PGF$_{2\alpha}$ uptake (Section 3.4.6). Omega-3 PUFAs (EPA and DHA) decrease uptake after a one day treatment (Fig. 40) and uptake is reduced again only after a three day PUFA incubation at high concentrations (100µM), while low doses (50µM) of n-3 PUFAs after three days treatment elevated uptake of $[^3]H$ PGF$_{2\alpha}$ (Fig. 41). Furthermore, after a seven day PUFA incubation period, both concentrations increased $[^3]H$ PGF$_{2\alpha}$ uptake (Fig. 42). The altered uptake of $[^3]H$ PGF$_{2\alpha}$ with different levels n-3 is most likely due to the bioavailability of PUFAs for PG synthesis. Arachidonic acid (n-6) inhibits $[^3]H$ PGF$_{2\alpha}$ uptake at low doses after a one, three and seven day treatment but increases uptake at higher doses. Incubation of BUECs with PUFAs plus estradiol does not offer any conclusive results of $[^3]H$ PGF$_{2\alpha}$ uptake. In effect, there appears to be no effect of this combination of treatment (E2 and PUFA), with inconclusive and highly variable results.

Glucose addition has a potent effect on the uptake of $[^3]H$ PGE$_2$ and $[^3]H$ PGF$_{2\alpha}$ into BUECs, with increasing levels of glucose, initiating a dose-dependent increase in $[^3]H$ PGE$_2$ uptake. This is a significant result, since lactate is a by-product of glucose metabolism and has been reported to be the preferred exchange molecule for PG transport via PGT. In addition, uptake of $[^3]H$ PGE$_2$ and $[^3]H$ PGF$_{2\alpha}$ into BUECs, treated with progesterone or estradiol is increased at 2mins compared to 10mins, while the reverse is true for unstimulated cells.
The pH of the solution in which \([3\text{H}^+]\) PGE\(_2\) and \([3\text{H}^+]\) PGF\(_{2\alpha}\) are prepared impacts greatly on transport of the tritiated PGs. Neutral PGs (pH 3.0) are transported more efficiently than anionic PGs (pH 7.4). In addition, efflux of \([3\text{H}^+]\) PGE\(_2\) and \([3\text{H}^+]\) PGF\(_{2\alpha}\) is time-dependent that increases linearly overtime. \([3\text{H}^+]\) PGE\(_2\) and \([3\text{H}^+]\) PGF\(_{2\alpha}\) compartmentalization is higher in the cytosol compared to membranes, possibly due to the greater volume or limited number of PG binding sites available on membranes.

Vectorial flux is higher from the basolateral to the apical surfaces compared to apical to basolateral flux, suggesting greater permeability of the basolateral compartment to PGs. This result is significant for in vivo model and the transport of PGs towards the CL to induce their effects.

Expression of PGT mRNA and protein was also assessed to gain a more accurate understanding of the uterine control of PG transport and enable the development of a more concise summary of events. PGT mRNA expression in BUECs is maintained at a basal level after all treatments, indicating that these cells are capable of producing PGT protein at any time and that other contributory factors not explored in this study may impact on PGT mRNA synthesis.

The complexity of the PG transport mechanisms system in bovine uterine tissue has been observed in these studies and indicates that tissue and cell models offer rather different results showing that other factors may be involved in the mechanism. Hormone and glucose treatment offer conclusive results in BUEC transport studies investigating PG transport, while vectorial transport studies demonstrate greater permeability of the basolateral surface to PGs than permeability at the apical surface. Further studies involving PUFA treatment and gene expression studies will need to be carried out to generate an improved picture of the PG transport mechanism. Interestingly, glucose supplementation, fatty acid content of phospholipid membrane, simple PG transport gradients, expression of PGT are vital for the cellular uptake of PGs and a feeding regime that offers glucose and fatty acids may prove to be one method of altering the fate of newly synthesized endometrial PGs.
5.2. Limitations and Future Work

Critical analysis highlights certain limitations to the work and that future studies could be carried out to provide a more concise understanding. In hindsight, confocal microscopy could have been used to detect the PGT protein in tissue sections to increase resolution and contrast thereby isolating PGT protein in uterine cell membranes. The principle of confocal microscopy would in effect give sharp localization of PGT in precise regions of the cell membrane. Fluorescent microscopy that was used in this study does detect PGT but some images appeared blurred and detect rogue staining, confocal microscopy eliminates the limitations of fluorescent microscopy and provides more conclusive results. Transport could be carried out over a shorter duration that measures initial rate of PG transport in seconds as opposed to minutes since a considerably level of $[^3]$H PG is transported early by bovine uterine epithelial cells. Also, it is noteworthy to realise that transport studies involving changes in pH may induce cell lysis and to combat this, an MTT assay of cells after the transport study could determine cell viability and eliminate this limitation. Polarized monolayers provide a more accurate account of the activity of BUECs in vivo and perhaps additional transepithelial studies could be carried out that include the effects of serum and hormones have on $[^3]$H PG transepithelial transport between apical and basolateral domains could be performed to gain more insight to the fate of PG transport in vivo. Future studies in the area could use and develop the results obtained from this study and by overcoming the limitations expand the knowledge of PG transport in bovine uterine tissue thereby offering new methods to reduce bovine infertility rates by controlling PG transport.


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