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INHIBITION OF DIRECT PROSTAGLANDIN $F_{2\alpha}$ EFFECTS ON PRE-ATTACHMENT EMBRYOS IMPROVES REPRODUCTIVE EFFICIENCY IN CATTLE

A Dissertation

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Degree

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Fernando Scenna

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DEDICATION

This dissertation is dedicated to my beautiful and loving wife Magdalena, my precious daughter Julia and all my family in Argentina. Magda, only you know exactly how tough it was for me to be able to reach this point, I want to thank you for all your patience and unconditional support. Without you, this accomplishment would have never been possible. Julia, since you were born my life has another meaning. You were my daily injection of inspiration to finish my degree. Coming home after working at school was my favorite part of the day, thanks! Mama, Papa y Sil, thanks for your unwavering support no matter what I endeavors I embark on. I can't wait to see you again!

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ABSTRACT

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to have detrimental effects on embryonic development, quality and hatching ability of embryos and pregnancy rates in cows. However, information about $PGF_{2\alpha}$ receptor (FPr) mRNA and protein in the pre-attachment bovine embryo is absent in the literature. The first experiment was design to identify the period of time during *in vitro* embryo development that is most susceptible to $PGF_{2\alpha}$ and to determine FPr mRNA and protein in bovine embryos. Prostaglandin $F_{2\alpha}$ decreased development of embryos to compact morula, but had no effect on development to blastocyst. In addition, FPr mRNA and protein was confirmed by real time PCR and Western Blot analysis, respectively, suggesting that $PGF_{2\alpha}$ is having a direct negative effect on *in vitro* development of bovine embryos by activating its receptor.

Discovery of FPr in bovine embryos allowed for development of new therapeutic strategies aimed at improving reproduction in bovines. Therefore, in a second experiment, the effects of a selective FPr antagonist, AL-8810, on *in vitro* development of bovine embryos was evaluated. The antagonist did not have toxic effects on development of embryos. Subsequently, efficacy of AL-8810 to prevent $PGF_{2\alpha}$ effects on pre-compacted embryos was investigated. Results showed that addition of AL-8810 to the culture medium inhibited $PGF_{2\alpha}$ effects on development to morula stage.

In a third experiment, embryonic development and gene expression of Na^+/K^+ ATPase α1 and zonula occludens-1 (ZO-1), two important genes participating in blastocyst formation and hatching, was examined after culture of *in vivo*-derived frozen/thawed bovine embryos with AL-8810, PGF, AL-8810+PGF, or Control. Thereafter, pregnancy rates of embryos recovered with medium containing AL-8810 was

evaluated. Results indicated that AL-8810 inhibited negative effects of $PGF_{2\alpha}$ on development of embryos; however, no differences in gene expression were observed. Furthermore, recovery of embryos with medium containing AL-8810 improved pregnancy rates following transfer to recipient cows. In conclusion, inhibition of $PGF_{2\alpha}$ binding to its receptors on *in vitro*- and *in vivo*-derived bovine embryos increases embryonic development and pregnancy rates after transfer to recipient animals; respectively. These findings will likely increase efficiency of *in vitro* production of embryos and embryo transfer programs in cattle.

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CHAPTER 1

INTRODUCTION

Reproductive losses associated with the beef industry in the United States are estimated to cost $500 million dollars annually (Bellows et al., 2002). Early embryonic death accounts for 75 to 80% of all reproductive losses (Sreenan and Diskin, 1983). The majority of these losses occur when the embryo enters the uterus (5-8 days after mating or insemination) and the morula stage embryo is developing into blastocyst (Ayalon, 1978; Maurer and Chenault, 1983; Sreenan and Diskin, 1983; Wiebold, 1988; Dunne et al., 2000).

Prostaglandins are potent chemical mediators derived from arachidonic acid metabolism that participate in several physiological processes. Studies have linked prostaglandins with several pathological conditions such as cancer, inflammation, and neurodegenerative diseases (Ishihara et al., 2004; Hoozemans and O'Banion, 2005). In cattle, elevated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentrations in the uterine lumen has also been shown to lower reproductive efficiency by decreasing embryonic survival and development (Harper and Skarnes, 1972; Maurer and Beier, 1976; Breuel et al., 1993; Schrick et al., 1993; Buford et al., 1996; Hockett et al., 2004; Scenna et al., 2004), decreasing the ability of bovine embryos to escape from the zona pellucida (hatching; (Scenna et al., 2004)), and by decreasing pregnancy rates (Lemaster et al., 1999; Sales et al., 2004; Scenna et al., 2005). Further implications for $PGF_{2\alpha}$ as an embryotoxic agent was observed when administration of several prostaglandin synthesis inhibitors increased pregnancy rates after embryo transfer in cows (Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005).

To date, the mechanisms through which $PGF_{2\alpha}$ reduces embryonic survival and development in the cow are still unclear. In addition, the presence of prostaglandin $PGF_{2\alpha}$ receptors (FPr) in pre-attachment bovine embryos has not been documented in the literature. Therefore, the objectives of this dissertation were 1) to identify the period of time during *in vitro* embryo development where the embryo is most susceptible to detrimental effects of $PGF_{2\alpha}$, 2) to determine the presence of FPr mRNA and protein in bovine embryos; 3) to inhibit detrimental effects of $PGF_{2\alpha}$ on embryo development by addition of a selective FPr antagonist, AL-8810, to the culture medium of embryos treated with $PGF_{2\alpha}$; and lastly 4) to improve pregnancy rates after bovine embryo transfer by transferring embryos treated with AL-8810.

In conclusion, $PGF_{2\alpha}$ is a primary cause of embryonic loss in cattle. Therefore, understanding the mechanism/s by which $PGF_{2\alpha}$ causes its effects on the embryo will allow the development of new therapeutic strategies to improve embryo quality, development and survival. These improvements will result in better production efficiency (due to an increase in pregnancy rates and subsequent calving rates) and higher monetary earnings to the cattle industry (due to an increase in cattle sales).

CHAPTER 2

LITERATURE REVIEW

1. Early embryonic development

Establishment of a viable pregnancy in cattle depends on many complex processes. Functional gametes must be produced by both sexes, the female must show estrus, mating must occurr within the functional lifespan of the gametes, and proper uterine environment and corpus luteum functionality must ensure embryonic and fetal development.

In the cow, a population of primordial follicles containing an oocyte arrested at prophase of the first meiotic division (immature oocyte) is established during fetal life (van Wezel and Rodgers, 1996). During each estrous cycle, two or three follicular waves occur in which a group of follicles are recruited to initiate growth (Savio et al., 1988; Ginther et al., 1989; Fortune, 1993). During each follicular wave, only one of these follicles becomes dominant and continues to grow, while subordinate follicles regress (Knopf et al., 1989). Upon luteolysis (destruction of the corpus luteum by $PGF_{2\alpha}$), the dominant follicle increases in size and produces estradiol 17β to "trigger" a preovulatory peak of luteinizing hormone (LH) and estrous behavior. Luteinizing hormone causes the oocyte in the dominant follicle to resume meiosis (progresses to metaphase of the second meiotic division) and ovulation to take place (Espey, 1994).

After expulsion of the matured oocyte from the ovulatory follicle, the oocyte is collected by the oviduct. The oviduct is comprised of four distinct regions: infundibulum, ampulla, ampullary-isthmic junction, and isthmus (Ellington, 1991). The mature oocyte is

captured by the infundibulum and transported to the ampullary-isthmus junction where capacitated sperm bind to the zona pellucida surrounding the oocyte, undergoes acrosome reaction and penetrates the oocyte's plasma membrane (Saling, 1991). After fertilization and organization of the male and female pronuclei, the zygote becomes an embryo that undergoes a series of synchronized cell divisions (also called cleavage divisions; Figure 1).

The first cleavage division generates a two-cell embryo, in which cells are called blastomeres. As the early embryo is transported through the isthmus region of the oviduct, each blastomere undergoes subsequent divisions yielding 4-, 8-, 16- and 32-cell embryos (Figure 1). The embryo enters the uterine horn on day 5-6 after estrus; at which time, blastomeres flatten against each other forming a "solid ball" of cells called a morula (Figure 1). The morula stage embryo begins to form a fluid filled cavity (the blastocoele) and the embryo is classified as a blastocyst (Watson et al., 1992). Throughout all these developmental stages, blastomeres become smaller with no net increase in size of the embryo. Further accumulation of fluid within the blastocoele allows the embryo to expand (expanded blastocyst) and finally hatch (escape) from the zona pellucida (hatched blastocyst) on approximately day 9 after estrus (Van Soom et al., 1997).

In cattle, hatching is a critical step for a successful pregnancy because it allows filamentous development of the embryo and posterior attachment of the embryo to the uterine endometrium. Similarly, low implantation rates of *in vitro*-fertilized human embryos are largely due to impaired development and hatching of blastocyst (Magli et al., 1998).

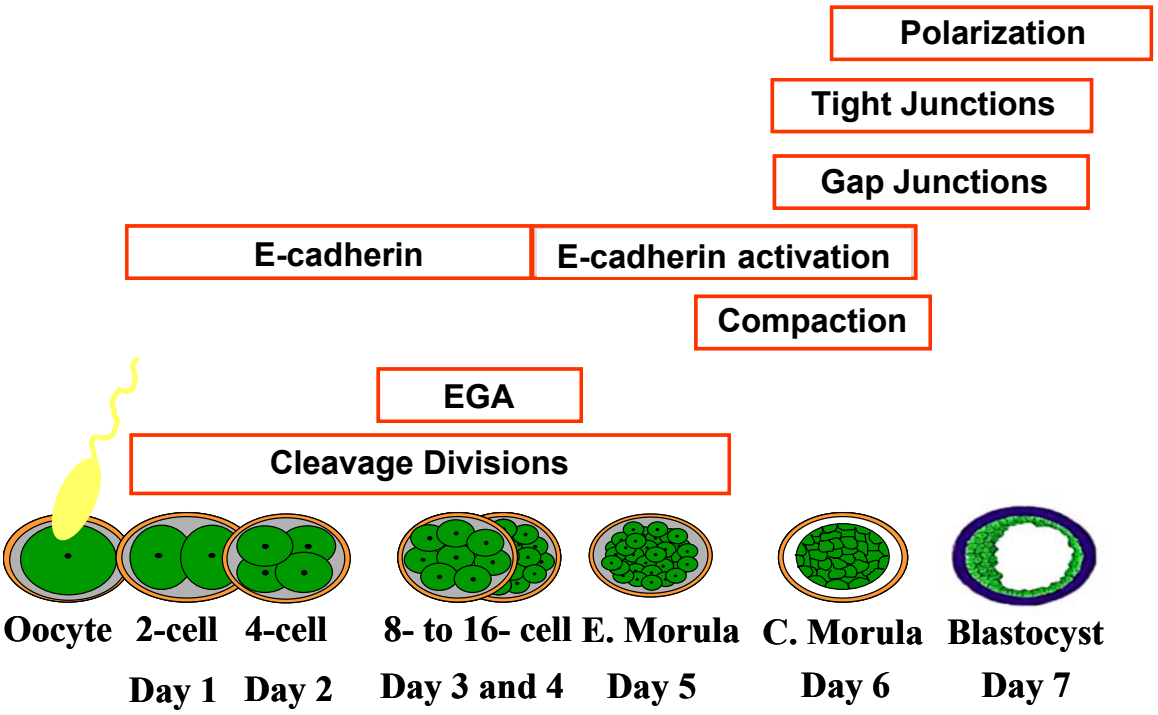


Figure 1. Important events during development of bovine embryos. After fertilization, the zygote suffers a series of well orchestrated cell divisions (cleavage divisions) leading to the formation of first a compact morula (C. Morula) and later a blastocyst stage embryo. Presence of maternally inherited and embryonic E-cadherin allows compaction to occur. Following compaction, outer cells of the compact morula develop tight junctions, whereas inner cells develop gap junctions. Outer cells polarize and differentiate in trophectoderm cells, whereas inner cells remain apolar and differentiate into the inner cell mass. EGA= embryonic genome activation. Approximate days after insemination are depicted for every stage of development.

2. Embryonic genome activation and gene expression in bovine embryos

After fertilization of the oocyte, several important events occur during development of an early bovine embryo. These include the first cleavage division, the activation of the embryonic genome, compaction, formation of the blastocoele and cell differentiation. Embryonic genome activation is considered the most important of these events due to the fact that it leads the way to further development. Without genome activation, an early bovine embryo stops development at the 8 to 16-cell stage embryo, a phenomenon known as "developmental block" (Meirelles et al., 2004). Maternal mRNAs and proteins, accumulated in the oocyte's cytoplasm during its growing phase within the follicle, are able to support early embryonic divisions (Briggs et al., 1951; Fair et al., 1997). However, further development is achieved only by activation of the embryonic genome, which allows the embryo to synthesize its own mRNA and proteins. Initiation of this process differs between species: 1-cell stage in the mouse (Aoki et al., 1997), 4-cell stage in humans (Braude et al., 1988), and 8- to 16-cell stage in cattle (Camous et al., 1986), sheep (Crosby et al., 1988), and rabbits (Manes, 1973).

In the mouse, the majority of maternal proteins and mRNA are degraded during transition from maternal to embryonic genome activation (Latham et al., 1991). In fact, adenylation and degradation of maternal mRNA are initiated during meiotic maturation and results in a net loss of 30% of polyadenylated mRNA pools between fully grown oocytes and ovulated oocytes (Bachvarova et al., 1985). Active turnover of mRNA continues and the majority of poly-A tail mRNA is degraded by the 2-cell stage embryo (Piko and Clegg, 1982). At the same time, a major activation of the embryonic genome occurs (Aoki et al., 1997). About 50% of these newly synthesized transcripts are absent

from the maternal population of mRNA (Taylor and Piko, 1987). During maternal to embryonic genome activation in the mouse, ribosomal RNA and ribosome numbers decrease only 25% (Piko and Clegg, 1982). Moreover, small nuclear RNAs (snRNAs) from maternal origin, responsible for processing new transcripts into functional mRNA, are not degraded during the transition from maternal to embryonic genome activation (Dean et al., 1989). This suggests that in the mouse, mRNA synthesized during embryonic genome activation appears to be processed by snRNAs of maternal origin.

In the cow, a gradual decrease in protein concentration is observed from the oocyte through the 8-cell stage (Frei et al., 1989). Following embryonic genome activation at the 8-cell stage embryo, a marked increase in total mRNA and proteins occurs (Camous et al., 1986; Frei et al., 1989). Memili and coworkers (1998) suggested a minor genome activation between the 1- and the late 4-cell stages and a major genome activation at the 8-cell stage in bovine embryos. There is a positive correlation between genome activation and the duration of the cell cycle in bovine embryos (Barnes and Eyestone, 1990). The total length for the first cell cycle is 28 h, 24 h for the second cell cycle (without G_1 or G_2), 14 h for the third cell cycle (without G_1), and 24-28 h for the fourth cell cycle (with both G_1 and G_2, coinciding with the activation of the embryonic genome). During embryonic genome activation, several important genes for cell proliferation, compaction and blastocyst formation are activated. Some of those genes include expression of growth factors and their receptors (Rappolee et al., 1990), cell adhesion molecules (Kidder, 1987) and Na^+/K^+ ATPase enzyme (Watson and Kidder, 1988).

3. Protein content of bovine oocytes and embryos

A major feature of mammalian embryogenesis is a marked change in the pattern of protein synthesis as the embryo proceeds through early cleavage divisions. During this period of time, populations of both maternal RNA and proteins that had been stored in the oocyte during oogenesis are sufficient to support early embryonic development to the 2 cell stage in mouse (Golbus et al., 1973), 4-8 cell stage in humans (Braude et al., 1988), 8-cell stage rabbits (Manes and Daniel, 1969; Schultz et al., 1973) and 8 to16-cell stage in sheep and bovine embryos (Crosby et al., 1988; Frei et al., 1989). Due to degradation of these stored molecules, subsequent development is dependent upon transcriptional activation of the embryonic genome.

Protein synthesis of bovine oocytes and early stage embryos (up to 16-cell stage) was first analyzed by Frei et al. (1989) after culturing *in vivo*-derived embryos in medium containing radiolabelled methionine. In this study, marked changes in the pattern of synthesis were observed at the 8-16-cell stage of development. Quantitatively, a gradual decrease in rate of protein synthesis occurred between the zygote and the 8-cell stage and then increased progressively to the blastocyst stage. Grealy and coworkers (1996) were the first to actually report the amount of protein in bovine oocytes and *in vivo*-derived embryos. Authors concluded that protein content of oocytes and 2-cell stage embryos was very similar (0.126 and 0.132 ug per oocyte and embryo, respectively), higher in morula and blastocyst stage embryos (0.183 and 0.185 μg per embryo, respectively), and even higher for the expanded blastocyst stage embryo (0.367 μg). Also, a 160- and more than 2000-fold increase in protein content of hatched blastocysts recovered on day 13 and 16, respectively, was observed when compared to protein content of expanded blastocysts on

day 8. These findings are in agreement with those reported by Frei et al. (Frei et al., 1989) and suggest that the increase in protein content from the 2-cell stage embryo onwards is related to the activation of the embryonic genome at the stage of 8- to 16-cell (Camous et al., 1986; Memili et al., 1998), an event followed by a sharp increase in protein synthesis in the embryo. The protein content of bovine oocytes and *in vitro*-derived embryos was recently reported by Thompson and collaborators (1998). Despite using different techniques to determine protein content in the embryo (BCA vs. Lowry methods), the amount of protein in ova and pre-compacted stage embryos reported by these two studies were similar. On the other hand, protein content between *in vivo*-derived (Grealy et al., 1996) and *in vitro*-derived (Thompson et al., 1998) expanded blastocysts differed between these two studies (367 ± 28 vs. 152 ± 10 ng per embryo, respectively). However, it is worth noting that Thompson and coworkers utilized *in vitro*-derived zona pellucida-free embryos to determine protein content, and when using zona pellucida-enclosed expanded blastocyst, the total amount of protein was estimated to be 337 ± 58 ng per embryo. Therefore, the authors estimated that the protein content of the zona pellucida from *in vitro*-derived embryos is approximately 200 ng.

In summary, data demonstrate that protein content decreases prior to compaction and increases after compaction and during the formation of the blastocyst (Frei et al., 1989; Thompson et al., 1998). This pattern suggests that during pre-compaction development, levels of protein degradation are higher than protein synthesis and that net growth is observed only after the activation of embryonic genome.

4. Compaction and blastocyst formation

Compaction represents a key critical event for continued embryonic development. It allows polarization and differentiation of blastomeres into two different cell populations (by induction of cell adhesion molecules between blastomeres that enhance cell communication and adherence) and facilitates fluid accumulation within the embryo that directly contributes to blastocyst formation and subsequent hatching from the zona pellucida (Watson, 1992). Compaction appears to occur in all mammalian embryos but timing differences exist between species: mouse at 8-cell stage (Ducibella and Anderson, 1975), humans at 16-cell stage (Edwards et al., 1981), and bovine (Van Soom et al., 1997) and rabbit (Koyama et al., 1994) at 32-cell stage.

During compaction, blastomeres adhere to and flatten against each other and boundaries between cells cannot be recognized (Van Soom et al., 1997). Development of the Ca^{++}-dependent cell adhesion molecule E-cadherin allows the establishment of gap and tight junctions leading to cellular and morphogenetic differentiation of blastomeres (Braga, 2002). Outer blastomeres polarize and form the trophectoderm (TE), while inner blastomeres remain apolar and generate the inner cell mass (ICM;(Koyama et al., 1994). The inner cell mass forms the embryo proper and some extra-embryonic membranes; whereas, trophectoderm cells will combine with ICM-derived extra-embryonic membranes to form the fetal placenta (Schlafer et al., 2000). The increase in interblastomeric contacts during compaction is mediated mainly by the action of E-cadherin, a Ca^{++}-dependent cell adhesion molecule (Pratt et al., 1982; Riethmacher et al., 1995), but also by action of microfilaments and microtubules (Pratt et al., 1981), high intracellular levels of Ca^{++} (Pey et al., 1998), and activation and phosphorylation of

proteins by protein kinase C (Winkel et al., 1990). In fact, compaction can be inhibited or disrupted by addition of cytochalasin D (antimycotic drug that depolymerizes microfilaments), colcemid (a derivate of colchicines, a plant toxin that inhibits microtubule polymerization) or by culturing embryos in Ca^{++}-free medium (Pratt et al., 1981; Pratt et al., 1982; Reima, 1990). In addition, activation of PKC with phorbol ester or treatment with diacylglycerides caused 4-cell mouse embryos to compact ahead of normal timing, while PKC inhibitors prevented compaction (Winkel et al., 1990).

During compaction, the free or apical membrane surfaces of the outer cells develop a microvillus cap with several Na^+/dependent transport systems (Miller and Schultz, 1985; Fleming and Johnson, 1988). These transport systems play a central role in establishment of an ion concentration gradient across the epithelium that facilitates osmotic accumulation of water into the blastocoelic space to form the nascent blastocoele (Biggers et al., 1988). Basolateral surfaces of TE remain free of microvilli, but become distinguished from apical surfaces by localization of tight junctions (Fleming et al., 1989), gap junctions (Kidder, 1987), adherent junctions (Vestweber et al., 1987), and acquisition of an active Na^+/K^+ ATPase pump (Watson et al., 1999).

In summary, during compaction, increase of interblastomeric contacts mediated by several adhesion molecules (mainly E-cadherin) induces cell polarization and differentiation of blastomeres into two cell populations, the TE epithelium and the ICM. Moreover, the trophectoderm develops the capacity to initiate and regulate blastocoele formation by the action of Na^+/K^+ ATPase enzyme (especially in the basolateral surface TE epithelium) and the presence of tight junction at the apical part of the TE epithelium

that prevents uncontrolled leakage of this fluid from the blastocoele cavity (Watson et al., 1992).

5. Role of E-cadherin during embryonic development

E-cadherin has three major domains or regions: an extracellular domain responsible for specific recognition of the same cadherin present in neighboring cells, a transmembrane domain that spans the cell membrane, and a cytoplasmic domain that extends into the cell and associates with cytoplasmic proteins called catenins, which in turn bind cadherins to actin filaments in the cytoskeleton (Ivanov et al., 2001). In the mouse, E-cadherin is expressed in the oocyte and during the early period of embryonic genome activation (at 2-cell stage; (Vestweber et al., 1987). E-cadherin-mediated adhesion between blastomeres initiates compaction and plays a critical role in differentiation of the trophectoderm and morphogenesis of the mouse blastocyst (Fleming and Hay, 1991). E-cadherin is uniformly distributed on the surface of all mouse blastomeres during early cleavage but redistributes to cell-cell contact sites upon activation at the 8-cell stage (when compaction begins) suggesting post-translational regulations. In fact, E-cadherin becomes phosphorylated for the first time at compaction (Sefton et al., 1992). In addition, homozygous null mutant mouse embryos for the E-cadherin gene initially compact (an event attributed to the presence of maternal or oogenetic E-cadherin proteins) but then proceed to decompact, with cells becoming apolar due to interference in the formation of apical junctional complexes (Laure et al., 1994; Riethmacher et al., 1995). Moreover, these embryos fail to develop a normal blastocoele cavity and do not hatch from the zona pellucida (Riethmacher et al., 1995).

Fibroblast cells lost cell-cell contacts (Erez et al., 2004) and mouse embryos decompacted (Reima, 1990) when exposed to antibodies directed against Ca^{++}-dependent molecules, further suggesting involvement of E-cadherin during compaction.

In cattle, E-cadherin is evenly distributed around cell margins of 1-cell zygotes and cleavage stage embryos and becomes restricted to basolateral membranes of outer cells at morula stage, while maintaining apolar distributions in the ICM (Barcroft et al., 1998). This suggests an involvement of E-cadherin in the formation of junctional apical complexes during the bovine compaction process. In a recent study, targeted disruption of the E-cadherin gene in bovine preimplantation embryos by RNA interference technology resulted in less embryos reaching compact morula or blastocyst stage (Nganvongpanit et al., 2006). These findings indicate a critical role of E-cadherin not only during compaction, but furthering embryonic development.

6. Role of tight junctions during embryonic development

Tight junctions are a "belt-like", multiprotein complex around each cell and represent a site of close intercellular adhesion including partial membrane fusion. Tight junctions form a barrier against paracellular diffusion, generate a trans-epithelial resistance, and allow accumulation of fluid during blastocoele formation (Biggers et al., 1988). Tight junctions comprise several proteins (both transmembrane and cytoplasmic) with the most important being zonula occludens 1 (ZO-1α+ and ZO-1α-; (Willott et al., 1992)) and cingulin (Citi, 1993). Although tight junction formation does not begin until compaction of the 8-cell stage mouse embryo (a process dependent upon E-cadherin activation), immunoblotting of unfertilized oocytes and preimplantation mouse embryos

showed high levels of cingulin in oocytes and early cleavage stages up to the 16-cell (Javed et al., 1993), indicating a maternal inheritance of these transcripts and protein.

During compaction in the mouse, outer cells gradually envelope inner cells causing ZO-1 in the inner cells to disappear. Thus, symmetrical cell contact appears to initiate ZO-1 down regulation in the ICM lineage (Fleming and Hay, 1991). In cattle, ZO-1 is undetectable until the morula stage when it first appears as punctuate points between outer cells. In blastocysts, ZO-1 is localized as a continuous ring at apical points of trophectoderm or outer cell contacts, whereas it remains undetectable in the ICM (Barcroft et al., 1998). Miller and coworkers (2003) reported that the amount of tight junction mRNA in *in vitro*-derived bovine embryos is dependent on the ability to undergo a well-orchestrated compaction process. In this study, when blastocyst developed after a short compaction period, expression of total tight junction mRNA was lower than in blastocysts derived from a longer compaction process. Moreover, a dramatic increase in ZO-1 was observed during transition from morula to blastocyst stage both in *in vivo* and *in vitro*-derived embryos, suggesting a stage-dependent rather than a time-dependent up-regulation of embryonic transcription just prior to blastocyst formation.

7. Role of the Na^+/K^+ ATPase pump during embryonic development

The Na^+/K^+ ATPase is composed of two obligatory subunits, a catalytic, nonglycosylated α-subunit and a glycosylated β-subunit (Jorgensen, 1982). The α-subunit is responsible for the physiological role of the enzyme (Jorgensen, 1986), while the β-subunit may facilitate processing and insertion of the α-subunit into the plasma

membrane (Geering, 1991). These subunits are encoded by multi-gene families as four α-subunits (α1, α2, α3, α4) and two β-subunit (β1, β2) have been cloned (Kent et al., 1987).

In the mouse, this enzyme is first detected at the late morula stage within the cytoplasm of each blastomere; but when cavitation begins, its distribution changes dramatically to a ring encircling the blastocoele, and restricted to the basolateral cell margins (Watson and Kidder, 1988). Moreover, northern hybridization experiments have detected transcripts for only the α1-subunits in all stages of mouse preimplantation development (Watson et al., 1990). In contrast, *in vitro*-produced bovine embryos expressed multiple α- and β-subunits of this enzyme throughout early development (Betts et al., 1998). Furthermore, the bovine Na^+/K^+ ATPase enzyme is more sensitive to effects of ouabain (an inhibitor of Na^+/K^+ ATPase) than the one present in murine embryos (Betts et al., 1998). In addition, Na^+/K^+ ATPase transcripts were detected in the ICM cells and trophectoderm cells of bovine blastocysts (Wrenzycki et al., 2003). Wrenzycki et al. (2003) investigated gene expression directing bovine blastocyst formation in TE and ICM cells and reported that Na^+/K^+ ATPase, E-cadherin, ZO-1 and glucose transporter-3 transcripts were higher in TE cells when compared to ICM cells. In contrast, desmocollin II and glucose transporter-1 and -4 were more abundant within ICM cells. These results demonstrate a specific gene expression pattern between these two cell lines in the early embryo regulating preimplantation development.

8. Role of gap junctions during embryonic development

Gap junctions are channels present in opposing plasma membranes of neighboring cells (Kidder and Wintergaher, 2001). The cell membranes come close together, but are

separated by 2-4 nanometers. These junctions are composed of at least 13 different proteins called connexins (Bruzzone and Goodenough, 1996; De Sousa et al., 1997); however, several studies identified connexin 43 as the most important gap junctional protein expressed in mouse (De Sousa et al., 1993; Valdimarsson and Kidder, 1995), bovine (Wrenzycki et al., 1996; Wrenzycki et al., 1999), and rat (Reuss et al., 2002) preimplantation embryos.

Intercellular communication through gap junctions is believed to be essential for cellular growth, differentiation and development (Warner, 1987; Zhang and Thorgeirsson, 1994). At the plasma membrane, six copies of the same connexin form a hexagonal ring-shaped channel called connexon. Connexons in adjacent cell membranes align together to form a hydrophilic channel that allows direct passage of molecules up to 1 kDa such as ions, cyclic AMP, Ca^{++}, and inositol trisphosphate (IP_3) between cells, and thereby coordinate metabolic and electrical activities.

In mouse, assembly of functional gap junctions is first observed during compaction in 8-cell stage embryos (Lo and Gilula, 1979; McLachlin et al., 1983) and represents a critical step for maintenance of compaction and subsequent blastocyst formation (Lee et al., 1987; Bevilacqua et al., 1989). Injection of gap junction antisense RNA to 2- and 4-cell mouse embryos reduced the percentage of compacted embryos to less than 20%, while 90% of uninjected embryos compacted (Bevilacqua et al., 1989). Also, when the same antisense was injected to 8-cell mouse embryos, only 5% of the embryos developed to blastocyst stage, while blastulation occurred in 90% of control embryos. Similarly, injection of gap junction antibodies inhibited dye transfer and dramatically reduced electrical coupling of 8-cell mouse embryos (Lee et al., 1987).

Moreover, preimplantation mouse embryos deficient in connexin 43 retained the capacity for cell-to-cell transfer of fluorescent dye (dye coupling), but at a severely reduced dye coupling (De Sousa et al., 1997).

Literature on intercellular communication through gap junctions in early bovine embryos is limited. Utilizing polymerase chain reaction (PCR), Wrenzycki and colleagues (1996) reported that transcripts for connexin 43 were present in immature oocytes and in all developmental stages of *in vitro*-produced embryos up to the morula stage. In contrast, only *in vivo*-derived morula and blastocyst stage embryos expressed connexin 43. These findings agree with reports from Boni et al. (1999) that demonstrated diffusion of dye between ICM cells of *in vivo*-derived morula stage embryos and ICM cells of *in vitro*-produced blastocyst stage embryos. Moreover, junctional conductance was higher in *in vivo* embryos than in *in vitro* embryos, with isolated ICM cells from both groups of embryos showing greater conductance compared to intact blastocyst. Higher junctional conductance was also correlated with higher density of gap junctions in *in vivo*-derived embryos than their *in vitro* counterparts. Prather and First (1993) also reported that *in vivo*-derived morula and blastocyst stage embryos showed dye transfer indicating the existence of gap junctions. However, *in vitro*-produced morula and blastocyst stage embryos did not show spreading of dye. Differences in dye transfer between Boni et al. (1999) and Prather and First (1993) studies may be explained by the fact that in the later, injection of dye to *in vitro* embryos was only performed on TE cells, and as Boni et al. (1999) demonstrated, no transfer of dye was observed in TE cells derived from *in vitro* embryos.

9. The zona pellucida, structure and functions

The zona pellucida (ZP) is an extracellular matrix surrounding growing oocytes, ovulated oocytes, and early stage embryos (Baker, 1972). The ZP exerts several important functions such as binding to spermatozoa in a specie-specific manner, induction of sperm acrosome reaction, blocking polyspermy, preventing the dispersion of blastomeres during preimplantation development, facilitating the passage of the embryo through the oviduct, and protecting the embryo during early stages of development (Wassarman, 1990, 1990; Epifano and Dean, 1994).

The majority of data generated on the structure and function of the ZP were obtained from studies performed in mouse and has been used as a model for other species. The mouse ZP is approximately 7 μm thick, and consists of three glycoproteins called mouse (m) ZP1, mZP2, and mZP3, with molecular weight of approximately 200, 120, and 83 kD, respectively (Bleil and Wassarman, 1980). All these ZP glycoproteins are exclusively secreted by the growing oocyte until time of ovulation (Bleil and Wassarman, 1980). These glycoproteins are organized in a specific manner within the extracellular matrix. Dimers composed of mZP2 and mZP3 polymerize into long filaments that constitute the ZP. The mZP1, a dimer of identical polypeptides, serves as the cross-linker between filaments. Ovaries from homozygous null female mice for mZP3 ($mZP3^{-/-}$) contained growing oocytes without ZP, while oocytes from heterozygous null females ($mZP3^{+/-}$) had a ZP half the thickness of those from wild-type female mice (Liu et al., 1996; Rankin et al., 1996). Homozygote female mice for mZP3 exhibited normal reproductive behavior, but failed to become pregnant after mating with fertile wild type male mice.

In addition, mZP3$^{-/-}$ female mice carrying a human ZP3 (hZP3) transgene possessed a thick ZP that consisted of mZP1, mZP2 and hZP3 (Rankin et al., 1998). In addition to the structural role of mZP3 during ZP formation, the mZP3 serves also as a receptor for sperm (Bleil and Wassarman, 1980). Only purified mZP3 binds specifically to heads of acrosome-intact sperm and prevents sperm from binding to ovulated oocytes *in vitro* (Bleil and Wassarman, 1980). On the other hand, mZP3 from fertilized oocytes or early embryos did not prevent binding of sperm to the ZP of ovulated oocytes *in vitro* (Bleil and Wassarman, 1980), which suggests that protein changes in ZP3 after fertilization inhibit further binding of sperm to the ZP. Acrosome intact sperm recognize and bind to specific O-linked oligosaccharides located on serine residues (serine 332 and 334) near the carboxy terminus of mZP3 polypeptide (Kinloch et al., 1995). Moreover, certain oligosaccharides inhibited binding of mouse sperm to oocytes *in vitro* (Litscher et al., 1995) indicating that binding of sperm to oocytes is a carbohydrate-mediated event. The mZP3 also triggers the acrosome reaction in sperm (Bleil and Wassarman, 1983), which allows sperm to penetrate the zona pellucida and fuse with the oocyte's plasma membrane. During acrosome reaction, the sperm remains bound to the ZP by binding to mZP2 (Bleil et al., 1988).

Zona pellucida glycoproteins are found in ZP of oocytes from virtually all mammalian species, from mice to humans (Wassarman et al., 1999; Wassarman, 1999) with a remarkable similarity in amino acids homology between species. In bovine, the ZP is composed of only a few highly modified glycoproteins; bovine (b) ZP1, bZP2, bZP3α, bZP3β, and bZP4 (Topper et al., 1997). The sperm receptor activity is attributed to ZP3α, while ZP2 serves as a secondary receptor for acrosome-reacted sperm. After sperm-egg

fusion, enzymes released by the cortical granules during the cortical reaction convert ZP2 and ZP3 to $ZP2_f$ and $ZP3_f$, respectively. These modifications inhibit further fusion of sperm with the oocyte's plasma membrane, a phenomenon called block of polyspermy (Kopf, 1990). In addition to ZP glycoproteins, passage of an early embryo through the oviduct allows the incorporation of several other proteins secreted by the oviductal epithelium (Buhi et al., 2000; Buhi, 2002).

Contrary to the mouse, where the growing oocyte is the only source of zona glycoproteins (Flechon et al., 1984), investigations in other species such as human (Grootenhuis et al., 1996), monkey (Grootenhuis et al., 1996), rabbit (Grootenhuis et al., 1996), dog (Tresoriero, 1981), pig (Sinowatz et al., 1995) and cow (Kolle et al., 1998) have shown that both the oocyte and the follicle cells (corona radiate cells) contribute to the synthesis of ZP glycoproteins. These finding have been supported by *in vitro* cell culture of granulosa cells that were able to secrete ZP glycoproteins (Skinner et al., 1984). During follicular development in the bovine ovary, the ZP3β protein and its mRNA are primarily localized in the oocytes of primordial and primary follicles (Sinowatz et al., 1995; Kolle et al., 1998). In secondary follicles, both the oocyte and granulosa cells express ZP3β protein and transcripts (Sinowatz et al., 1995; Kolle et al., 1998). In tertiary and pre-ovulatory follicles, both protein and transcripts are found in the cytoplasm of granulosa cells (Sinowatz et al., 1995; Kolle et al., 1998). In addition, investigations in fetal bovine ovaries have shown that ZP proteins are synthesized by the oocyte and granulosa cells during prenatal development (Totzauer et al., 1998).

When visualized by scanning electron microscopy (SEM), the mammalian ZP is found to be composed of a network dispersed with numerous pores (Dudkiewicz and

Williams, 1977) and with morphologically dissimilar internal and external surfaces. The external surface displays a fenestrated lattice-like appearance and the internal surface shows a regular rough appearance (Phillips and Shalgi, 1980). When pores are compared among species, the largest pores are observed in the ZP of the rabbit and the cat, and the smallest in the cow. The pores are largest at the outer surface of the ZP but decrease in size centripetally (Dudkiewicz and Williams, 1977). Vanroose and coworkers (2000) studied the outer ZP surface of bovine oocytes, zygotes, 8-cell stage and morula stage embryos. In this study, the outer ZP surface of bovine oocytes and morulae showed a rough and spongy appearance with numerous pores. On the other hand, the outer ZP surface of zygotes was found to have a smooth, melted appearance with only a few pores and 8-cell stage embryos presented both surface patterns. The mean number of pores was 1511 in oocytes, 1187 in zygotes, 1658 in 8-cell stage embryos, and 3259 in morulae, with mean diameters of 182, 223, 203, and 155 nm, respectively. In the same study, the ability of fluorescent microspheres of 40-50 nm and 180-200 nm to traverse the ZP was evaluated. Authors concluded that for all stages evaluated, the smallest beads were detected half way through the thickness of the ZP, whereas the biggest beads remained only at the outer surface of the ZP.

10. Hatching process

In cattle, accumulation of fluid (between days 8 and 10 after fertilization) within the blastocoele cavity in the blastocyst distends the zona pellucida (ZP), which becomes gradually thinner until it ruptures allowing escape of the blastocyst by protrusion through the opening in the ZP (Flechon and Renard, 1978; Massip and Mulnard, 1980).

Accumulation of fluid within the blastocoele cavity is achieved by a sodium concentration gradient created in the blastocoele cavity by the presence and function of the Na^+/K^+-ATPase pump (Biggers et al., 1988) at the basolateral surface of the trophectoderm epithelium. Additionally, the presence of a junctional apical complex (formed by tight junction at the apical part of the TE epithelium) prevents uncontrolled leakage of this fluid from the blastocoele cavity (Watson et al., 1992; Bavister, 1995).

In hamster and mouse embryos, hatching results from degradation of the ZP by enzymes originated from the embryo and/or the uterus (Gordon and Dapunt, 1993; Gonzales and Bavister, 1995). In fact, hatching of *in vitro*-produced mouse blastocyst has been related to a trypsin-like proteinase associated with cells of the trophectoderm (Perona and Wassarman, 1986). In the cow, there is no evidence of an enzymatic mechanism during hatching since zona pellucida-intact embryos, as well as empty ZP, can be found from uterine flushings up to day 16 after ovulation (Betteridge et al., 1980). These findings suggest that in the cow, hatching of the fully expanded bovine blastocyst occurs through an increase in hydrostatic pressure in the blastocoele cavity which is followed by rupture of the ZP.

Several studies during *in vitro* blastocyst expansion and hatching have observed one or more partial or total collapse(s) of the blastocoele cavity followed by re-expansion of the blastocoele. This pulsatile activity has been observed in various species including rabbit (Lewis and Gregory, 1929), mouse (Cole, 1967), and cow (Massip and Mulnard, 1980). The origin and possible roles of these collapses are unknown; however, excessive pulsatility might be related with poor embryo viability due to the fact that blastocyst exhibiting frequent collapses failed to hatch (Massip and Mulnard, 1980). In a recent

study (van Heule et al., 2001), the effect of serum supplementation on pulsatile activity and hatching of *in vitro* produced bovine blastocyst was investigate by time-lapse cinematography. Addition of serum increased pulsatile activity before ZP rupture, reduced the time of hatching and increased hatching rates when compared to embryos cultured in the same medium but without serum supplementation. Moreover, pulsatile activity was not involved in the hatching process due to the fact that blastocyst showing no collapses hatched. Finally, hatching occurred at various times post insemination (average of 153h 35 min ± 1h 59 min) and at various embryo diameters (ranging from 179 to 235 μm).

11. Developmental differences between in vivo and in vitro embryos

Before making inferences about similarities and differences between *in vitro* and *in vivo*-derived embryos, it worth noting that literature cited to prepare this discussion can be considered "old", with the majority of these publications published in the 90'. Since then, several changes on *in vitro* development of bovine embryos have occurred to more closely resemble the events occurring during *in vivo* development of bovine embryos in an effort to increase the quality of *in vitro*-derived embryos.

The percentage of putative zygotes reaching the blastocyst stage after *in vitro* production (IVP) of embryos is only 20 to 30% (Thompson and Duganzich, 1996). In addition to this poor developmental capacity, *in vitro*-derived embryos present lower quality than those derived from *in vivo*. In an attempt to determine such differences, Van Soom and Kruif (1992) cultured *in vivo*-derived zygotes and 2-cell stage embryos and *in vitro*-derived putative zygotes under identical conditions. In this study, *in vivo*-derived

zygotes and early embryos cleaved and developed better than *in vitro*-derived putative zygotes (84.3 vs. 62% cleavage and 69 vs. 35% development, respectively). Moreover, *in vivo*-derived embryos compacted better and presented better blastomere morphology (rounded vs. square and swollen) than *in vitro* counterparts. In addition to this, Iwasaki and Nakahara (1990) reported that allocation of cells to the inner cell mass is lower at the blastocyst stage for *in vitro*-derived embryos. In fact, Van Soom et al. (1997) demonstrated that rapid transition between morula to blastocyst stage of *in vitro*-derived embryos is responsible for lowering the numbers of cells at the inner cell mass of the embryo. To the contrary, the longer transition from morula to blastocyst stage of *in vivo*-derived embryos allows sufficient time for allocation of inner cells to the inner cell mass of the embryo.

Other differences between *in vivo* and *in vitro* embryos include chromosomal abnormalities, morphology under electron microscopy, embryo metabolism, gene expression and survival after handling and freeze/thawing techniques. Conditions utilized during *in vitro* production of embryos affected the incidence of chromosomal abnormalities, from 7-10% in *in vivo*- to 15% in *in vitro*-derived embryos (Kawarsky et al., 1996). Moreover, under electron microscopy, *in vitro*-produced embryos were characterized by a lack of desmosomal junctions, a reduction of surface microvilli, an increase in the number of lipid droplets, mitochondrial degeneration, and cytoplasmic vacuolation (Shamsuddin and Rodriguez-Martinez, 1994; Rizos et al., 2002). Compared with their *in vivo* counterparts, *in vitro*-produced embryos tend to have darker cytoplasm and a lower buoyant density as a consequence of their higher lipid content (Pollard and Leibo, 1994) and a more fragile zona pellucida (Duby et al., 1997). With regard to

embryo metabolism, ATP production, oxygen uptake and glucose uptake between *in vivo* and *in vitro*-derived embryos appear to be the same (Thompson et al., 1996). On the other hand, glucose oxidation (Thompson et al., 1991) and amino acid uptake (Partridge and Leese, 1996) for *in vitro*-derived embryos appeared to be lower than *in vivo*-derived embryos. The quality and cryotolerance of *in vivo*-derived embryos are higher than embryos produced *in vitro*. These differences may be explained by differences in gene expression and lipid content between these two groups of embryos.

Recent studies have shown that development of *in vitro*-derived embryos depends upon developmental competence of the oocyte (Rizos et al., 2002); however, the post-fertilization culture environment is known to be the most important factor determining quality of resulting embryos (Rizos et al., 2002; Rizos et al., 2003). Several studies have reported expression patterns of developmentally competent genes in preimplantation bovine embryos (Wrenzycki et al., 1996; Wrenzycki et al., 1998, 1999; Lazzari et al., 2002; Rizos et al., 2002; Rizos et al., 2002; Wrenzycki et al., 2003; Mohan et al., 2004; Tesfaye et al., 2004; El-Halawany et al., 2005); however, the end point of these studies has generally been measured in blastocyst stage embryos. Lonergan et al. (2003), in an attempt to determine temporal divergence in mRNA expression of *in vitro* and *in vivo*-cultured bovine embryos, reported that changes in gene expression are strongly influenced by the culture environment and that changes in the amount of mRNA in blastocyst stage embryos are a consequence of lowered transcription earlier in development. In a similar study, Tesfaye et al. (2004) also reported temporal divergences in mRNA between *in vivo* and *in vitro*-derived bovine embryos. These findings are in agreement with those from Rizos et al. (2002; 2003), where culture medium of *in vitro*

produced embryos determined quality of resulting embryos measured in terms of their cryotolerance and relative abundance of transcripts. Moreover, Lazzari et al. (2002) demonstrated that *in vitro* culture of bovine embryos using animal proteins (bovine serum albumin, BSA) alters kinetics of embryo development (greater amount of cells and overall size), the expression of developmentally important genes, and when transferred to suitable recipients, resulted in higher birth weight of calves compared to embryos produced *in vivo*. Furthermore, when embryos were cultured in medium without BSA (replaced by polyvinyl alcohol, PVA), gene expression patterns resembled that of *in vivo*-derived embryos. Therefore, alterations in gene expression of *in vitro*-produced embryos, caused by the culture environment, are probably responsible for lowering the quality of these embryos when compared to the quality of *in vivo*-derived embryos.

12. Synthesis of prostaglandins

Prostaglandins are involved in several physiological processes such as inflammation, production of pain and fever (Seibert et al., 1994; Blatteis and Sehic, 1997), control of blood vessel tone and arterial blood pressure (Nett et al., 1976), luteolysis (Goding, 1974), muscle growth and differentiation (Horsley and Pavlath, 2003), and blood clotting (Moncada and Vane, 1981). With exception of red blood cells, prostaglandins are synthesized and released by all mammalian cells and tissues. However, unlike other hormones, prostaglandins are not stored in cells but are produced and released immediately after synthesis (Bito, 1975).

Phospholipase A_2 (PLA_2) enzymes catalyze hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid (AA), a precursor of

prostaglandins, tromboxane A_2, and leukotrienes. So far, at least 19 enzymes that possess PLA_2 activity have been identified in mammals (reviewed by (Murakami and Kudo, 2004). Since arachidonic acid contains 20 carbon atoms, prostaglandins are referred as eicosanoids.

The formation of $PGF_{2\alpha}$ begins with release of the precursor of all prostaglandins, arachidonic acid (AA), from the sn-2 position of membrane phospholipids principally by the action of the cytosolic, high molecular weight, calcium-dependent phospholipase A_2 ($cPLA_2$; (Flint et al., 1986), but also by the action of phospholipase C (PLC), phospholipase D (PLD), and mono- and diacy-glycerol lipases (Flower and Blackwell, 1976; Irvine, 1982; Kozawa et al., 1997; Kozawa et al., 1997). Cleavage of AA from membrane phospholipids is the rate-limiting step in prostaglandin synthesis. Several phospholipases A_2 (PLA_2s) can release AA from membrane phospholipids (as reviewed by (Khan et al., 1995; Capper and Marshall, 2001). These PLA_2s are basically divided in two groups: 1) Low molecular weight PLA_2s (the largest group): are secreted to the extracellular space and their activity depends on high levels of calcium. Their functions are not very well known with the exception of the IIA and IB PLA_2s. Phospholipase IIA has direct bactericidal effect on gram-negative bacteria, whereas PLA_2IB (also called pancreatic PLA_2) participates in lipid digestion. The other group 2) High molecular weight PLA_2s are constituted by intracellular PLA_{2s}, that are also divided in calcium-dependent ($cPLA_{2\alpha}$ or IVA $cPLA_2$, $cPLA_{2\beta}$ or IVB $cPLA_2$) and calcium-independent PLA_2 (VIA $iPLA_2$ and IVC $cPLA_2$)

Arachidonic acid released by the action of PLA_2s is converted to prostaglandin H_2 (PGH_2) by the action of cyclooxygenase enzymes-1 or -2 (COX-1 and COX-2) at the

membrane of the endoplasmic reticulum (Smith and Dewitt, 1996) or at the nuclear envelope (Parfenova et al., 2001). Cyclooxygenase-1 is a constitutive enzyme found in almost all cells (O'Neill et al., 1994), whereas COX-2 is an inducible form in response to inflammatory reactions, hormonal and environmental stimulus (Smith and Dewitt, 1996). Conversion of AA to PGG_2 is performed in two sequential enzymatic steps: oxygenation, which converts free arachidonic acid to the unstable intermediate PGG_2, followed by a peroxidation that converts PGG_2 to PGH_2 (Smith and DeWitt, 1996). These two enzymatic reactions (oxygenation and peroxidation) are catalyzed by the enzymes cyclooxygenase-1 and –2 (COX-1 and COX-2). Finally, PGH_2 is transformed into $PGF_{2\alpha}$ by tissue-specific prostaglandin $F_{2\alpha}$ synthase (Watanabe et al., 1985). Following its synthesis, $PGF_{2\alpha}$ is transported out of the cell by a prostaglandin transporter (Chan et al., 1998) where it exerts an autocrine or paracrine function through its G protein coupled receptor. Catabolism of $PGF_{2\alpha}$ is accomplished by the enzyme prostaglandin 15-dyhydrogenase (PGDH), which converts $PGF_{2\alpha}$ into the inactive $PGF_{2\alpha}$ metabolite, PGFM (Keirse et al., 1978).

Different specific enzymes, whose presence varies depending on the cell type, mediate formation of the D, E, I, and F series from PGH_2 in different tissues. Synthesis of prostaglandins can be blocked by administration of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, flunixin meglumine, indomethacin and phenylbutazone and by steroidal anti-inflammatory drugs like hydrocortisone, prednisone and prednisolone. Nonsteroidal anti-inflammatory drugs act through inhibition of COX-1 and COX-2 (Vane and Botting, 1996), while steroidal anti-inflammatory drugs interfere with mobilization of

arachidonic acid from the plasma membrane by inhibition of phospholipase A_2 (Rothhut and Russo-Marie, 1984).

13. Mechanism of action of prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ interacts with its G protein coupled receptor at the cell membrane and activates Gq Protein/phospholipase C (PLC-β)/Calcium release/Protein Kinase C (PKC) signaling pathway (Wiltbank et al., 1989; Wiltbank et al., 1990; Abramovitz et al., 1994; Sugimoto et al., 1994; Sales et al., 2004). G protein receptors contain 3 subunits called α, β, and γ (Findlay et al., 1993). The β and γ subunits remain together and are usually referred as the Gβγ subunit, whereas the α subunit is referred to the Gα subunit as reviewed by Findlay et al. (1993). Following $PGF_{2\alpha}$ binding to its Gq protein-coupled receptor (Lee et al., 1987), the Gα subunit suffers a conformational change that allows exchange of GDP for GTP and dissociation of the Gα from the Gβγ subunit. Next, the Gα subunit binds to PLC-β causing its activation. After activation of PLC-β, the intrinsic GTP-ase activity of the Gα subunit hydrolyzes bound GTP to GDP and dissociates from PLC-β allowing re-association of the Gα subunit with the Gβγ subunits (inactive Gq form). Next, PLC-β cleaves phosphatidyl inositol 4,5 bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG), that remains associated to the plasma membrane and inositol 1,4,5 trisphosphate (IP_3) that diffuses to the cytoplasm. Inositol trisphosphate releases calcium from the endoplasmic reticulum to the cytosol (Berridge, 1993).

The increase of intracellular calcium levels recruits PKC to the cell plasma membrane where DAG causes its activation. Protein Kinase C phosphorylates several

other proteins involved in cellular growth and metabolism. Exposure of bovine luteal cells and osteoblasts to $PGF_{2\alpha}$ activates Raf/MEK/MAPK signaling cascade (Chen et al., 1998). Mitogen activated protein kinases (MAPK) form homodimers that translocate to the nucleus where they regulate activity (through phosphorylation) of many nuclear transcription factors such as TCF or SRF (reviewed by (Hunter, 2000). Phosphorylated TCF and SRF form a transcription factor complex that binds to SRE in DNA and stimulate gene transcription of c-fos and c-jun genes (Chen et al., 2001). In addition, Gq protein may not be the only G-protein involved in induced MAPK activation, since studies involving pertussis toxin (a selective inhibitor of G-protein G_i) demonstrated inhibition of $PGF_{2\alpha}$ -dependent MAPK stimulation by this toxin (Melien et al., 1998). In agreement with this, G_i $\beta\gamma$ subunits mediated activation of MAPK in $PGF_{2\alpha}$ -stimulated uterine myometrial cells (Melien et al., 1998). Mitogen activated protein kinases were directly activated through Ras or indirectly activated by the formation of the Shc, Grb2 and SOS complex leading to activation of Ras followed by activation of the Raf/MEK/MAPK signaling cascade. Furthermore, $PGF_{2\alpha}$-induced proliferation of neoplastic human endometrial cells by activating MAPK signaling via PLC-β and trans-activation of the epidermal growth factor receptor (EGFR; (Sales et al., 2004). Lastly, the increase in cytosolic calcium by IP_3 and activation of PKC can lead to activation of other protein kinases such as PI_3K, p38 and Junk, which can also mediate cellular responses.

The bovine CL represents an excellent model for understanding the mechanisms of action of $PGF_{2\alpha}$. Protein Kinase C is believed to mediate many of the antisteroidogenic actions of $PGF_{2\alpha}$ in large luteal cells (McGuire et al., 1994). In addition to this, $PGF_{2\alpha}$ may interfere with ability of LH to activate PKA, an enzyme needed for phosphorylation

and stimulation of other key enzymes in the luteal steroidogenic pathway, such as cholesterol esterase (hydrolyzes the stored cholesterol) and steroidogenic acute regulatory protein (StAR), which transports cholesterol to the mitochondria (Garverick et al., 1985). In contrast to early signaling events, downstream signals in response to $PGF_{2\alpha}$ are poorly understood. Chen et al. (1998) reported that $PGF_{2\alpha}$ activates the Raf/Mek1/mitogen-activated protein kinase (MAPK) signaling cascade in bovine luteal cells. Therefore, signals on the cell surface may be responsible for transcription of luteal genes. Prostaglandin $F_{2\alpha}$ promotes apoptosis in luteal cells by activation of endonucleases after influx of Ca^{++} from intracellular stores (Juengel et al., 1993). Also during luteolysis in cattle, mRNA encoding Bax (a pro-apoptotic gene) is elevated resulting in an increased ratio of Bax to Bcl-2 (anti-apoptotic gene), an event consistent with Bax-mediated apoptosis (Rueda et al., 1997). Prostaglandin $F_{2\alpha}$ induces apoptosis in luteal cells by generation of oxygen radicals such as superoxide anion, hydroxyl radical and hydrogen peroxide (Riley and Behrman, 1991; Carlson et al., 1993). Exposure of rat luteal cells to these oxidants resulted in oxidation and loss of fluidity in cellular membranes (Sawada and Carlson, 1991). Decreased membrane fluidity affects cellular function including receptor binding and membrane-bound enzyme activity as demonstrated by Wu et al. (1992), who suggested that superoxide radical production inhibited LH stimulation of cyclic AMP and decreased activity of protein kinase A. In pigs, $PGF_{2\alpha}$ depleted the CL of vitamin C, a natural antioxidant that prevents oxygen radical formation (Petroff et al., 1999).

Prostaglandin $F_{2\alpha}$ is a potent, bioactive compound. As such, its production and metabolism must be tightly regulated. The lungs appear to play an important role in

inactivating prostaglandins. In the ewe, $PGF_{2\alpha}$ in the blood stream has a half-life of less than 1 min, since 99% of it is metabolized to 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) in a single passage through the lung (Hansel and Dowd, 1986). In the cow, only 16% of $PGF_{2\alpha}$ remained in the blood after three passages through the lung (Davis et al., 1984).

14. Prostaglandin $F_{2\alpha}$ receptor

Prostaglandin $F_{2\alpha}$ receptors (FPr) have been cloned from cattle (Sakamoto et al., 1994), sheep (Graves et al., 1995), mouse (Sugimoto et al., 1994), rat (Kitanaka et al., 1994) and human (Abramovitz et al., 1994) cDNA libraries. In the cow and ewe, the clones encode for 362 amino acids residues in the open reading frame (Sakamoto et al., 1994; Graves et al., 1995); while in mouse and rat, FPr have 366 amino acids (Kitanaka et al., 1994; Sugimoto et al., 1994) and human FPr consist of 359 amino acids (Abramovitz et al., 1994). The molecular weight of the FPr is similar between species, with estimated molecular weights ranging from 40 (humans) to approximately 41 KDa (bovine). The hydropathy profile indicates that the deduced amino acid sequence consists of seven hydrophobic segments, representing seven transmembrane helices, a structure common to known prostanoids receptors and other G protein-linked receptors. The amino acid sequences of FP receptors are very similar between different species. For example, the bovine FPr (Sakamoto et al., 1994) shares 98% homology with the ovine FPr (Graves et al., 1995), 86% with the human (Abramovitz et al., 1994), 80% with the mouse FPr (Sugimoto et al., 1994), and 78% with the rat FPr (Kitanaka et al., 1994). Sequence homology between species is highly conserved in the transmembrane domains of the FPr protein. In contrast, the NH_2-terminal and COOH-terminal regions show considerable

sequence diversity. The NH_2-terminal region contains two potential N-glycosylation sites as observed with other members of the G protein coupled receptor family (Hubbard and Ivatt, 1981). Several serine and threonine residues, potential phosphorylation sites for protein kinase C, are commonly conserved in the COOH-terminal region or in the second intracellular loop (Sakamoto et al., 1994). Sequence comparisons of the FPr and other members of the prostanoids receptor family reveal significant homologies between them. For example, the bovine FPr shares 34, 33, and 36% amino acid sequence homology with the human, mouse, and bovine TXA2 receptors, respectively (Sakamoto et al., 1994). Similarly, the bovine FPr shares 31 and 32% homology with the bovine and mouse EP_3 receptors (Sakamoto et al., 1994). It is worth noting that these homologies are mostly localized to transmembrane domains, particularly domain VII.

In order to elucidate the mechanisms of receptor-ligand interactions at the molecular level, Sakamoto and coworkers (1995) constructed a model for the bovine FPr based on its amino acid sequence, the known helical arrangement of rhodopsin (a G-protein coupled receptor; (Findlay et al., 1993), and a model of the human TXA_2 receptor (Yamamoto et al., 1993). The authors concluded that the ligand binding pocket of the FPr includes argenine-291 and a large hydrophobic pocket. The carboxylic acid group of $PGF_{2\alpha}$ may interact with arginine-291. The C-9 and C-11 hydroxyl groups of the $PGF_{2\alpha}$ may interact with the serine and threonine residues in the transmembrane domains III and VII, respectively. Another amino acid, histidine-81, in the transmembrane domain II, may also be important for $PGF_{2\alpha}$ binding as shown in transfection studies with mutant rat FPr expressed in COS-7 cells (Rehwald et al., 1999). This may be explained in part by the close proximity of histidine-81 to arginine-291 and also by the ability of histidine as an

hydrogen bond donor (Rehwald et al., 1999; Anderson et al., 2001). Cysteine residues (Cys-109 and Cys-186) in the first and second extracellular loops of the FPr are likely to form a disulfide bond that stabilizes the protein structure (Probst et al., 1992; Abramovitz et al., 1994; Lake et al., 1994). These two cysteines are highly conserved among all prostanoids receptors and many other G-protein coupled receptors (Probst et al., 1992).

15. Gene structure and regulation of the FPr

The gene size of the FPr is approximately 10, 11 and 40 kilobases in humans, mouse and cow, respectively (reviewed by Anderson et al., 2001). The FPr gene is located in chromosome 1 in humans (Duncan et al., 1995) and near the gene for prostaglandin E receptor-subtype 3 in chromosome 3 in mice (Ishikawa et al., 1996). The exon/intron organization of the FPr gene is conserved among humans (Betz et al., 1999) and cattle (Ezashi et al., 1997).

All known members of the prostaglandin receptor family have similar organization of exons and introns (Ogawa et al., 1995) and support the idea of a common origin from a single ancestor gene. In humans and cattle, the FPr gene contains three exons and 2 introns with translated regions localized at exons 2 and 3 (Ezashi et al., 1997; Betz et al., 1999). The first exon is the smallest (164 and 194 base pairs in humans and cattle, respectively) and includes most of the 5' untranslated region. The second exon (approximately 870 base pairs for both species) contains a small fraction of untranslated region and the majority of translated region for the receptor. The third and largest exon (1459 and 3977 base pairs in humans and cattle, respectively) includes the remainder of the translated region and a large untranslated region.

Multiple transcription initiation sites have been identified in mouse and bovine FPr gene. In the mouse, the major transcription site is located at the 5' end of exon 1, 232 bp upstream of the ATG that marks the translation start site. In the bovine, multiple transcription initiation sites were identified in exon 1. In addition, other minor transcription initiation sites were identified in intron 1 (reviewed by (Anderson et al., 2001).

Two potential promoter regions were identified in the bovine FPr gene (Ezashi et al., 1997). A 1.6-kb region upstream of exon 1 contained sequences for the transcription factors: TRE/AP-1 (TPA-responsive element/activator protein-1), NF-IL6 (nuclear factor-interleukin 6), Sp 1, and GCF (GC binding factor). The second potential promoter in intron 1 contained sequences for Sp 1, TRE/AP-1, CRE (cyclic AMP-responsive element), NF-IL6, and AP-2 (activator protein-2) as well as CAAT-like and TATA-like boxes (Ezashi et al., 1997). The activities of these two promoters were investigated by Ezashi et al. (1997) after transfecting bovine luteal cells with these promoters linked to a reporting luciferase gene. Treatment of transfected cells with TPA (a phorbol ester known to activate protein kinase C), but not with forskolin (an activator of protein kinase A), resulted in transcriptional activation of the luciferase gene. It is well known that TPA directly activates protein kinase C (Comb et al., 1986) and that PKC can modulate the activity of nuclear transcription factors such as AP-1, which can increase gene expression by binding to TRE. In contrast to the findings of Ezashi and coworkers (1997), Tsai and colleagues (1998) reported that phorbol ester and forskolin decreased and increased FPr mRNA concentration in ovine luteal cells, respectively. These results also agreed with several other studies (Sakamoto et al., 1995; Juengel et al., 1996; Mamluk et al., 1998;

Hoyer et al., 1999) and suggest that additional regions of the genome are needed to regulate transcription of the FPr gene.

16. Messenger RNA for FPr

Several studies have identified expression of FPr mRNA using Northern Blot hybridization and polymerase chain reaction techniques. Although a great variety of tissues express FPr mRNA in ovine, the steady-state concentration of FPr was highest in the corpus luteum with quantities of FPr mRNA at least 100-fold greater than any other tissue (Tsai et al., 1998). In the corpus luteum, mRNA coding for the FPr is localized mostly on large luteal cells of bovine and ovine mid cycle corpora lutea (Fitz et al., 1982; Juengel et al., 1996). In fact, Tsai and coworkers (1998) revealed that the concentration of FPr mRNA was greater in large luteal cells compared with small luteal cells. Large luteal cells contained 7,314 ± 829 copies of FPr mRNA per cell, whereas small luteal cells contained 226 ± 54 copies per cell.

Expression of FPr mRNA in the corpus luteum was shown to progressively increase during the estrous cycle, but was markedly reduced upon luteal regression (Sakamoto et al., 1995). In addition, constant amounts of FPr mRNA were observed in early and middle pregnant corpus luteum, with a significant reduction by late pregnancy (Sakamoto et al., 1995). The expression of FPr mRNA was decreased by treatment of ewes with $PGF_{2\alpha}$ or by treatment of large luteal cells with $PGF_{2\alpha}$ *in vitro* (Tsai et al., 1998). Therefore, mRNA encoding FP receptor appears to be downregulated by its homologous ligand. In fact, the number of FPr in corpus luteum from non-pregnant sheep has been shown to decrease at the time of luteolysis (Wiepz et al., 1992). Several other

studies have shown a decrease of FPr after $PGF_{2\alpha}$ administration or natural luteolysis in cattle (Sakamoto et al., 1995), ovine (Hoyer et al., 1999), and pigs (Estill et al., 1995). In contrast, the FPr mRNA was increased after treatment of rats with a $PGF_{2\alpha}$ analogue (Olofsson et al., 1996), which will indicate specific species differences in the regulation of the FPr mRNA.

17. Prostaglandin $F_{2\alpha}$ receptor isoforms

Prostaglandins, are ubiquitously produced and act locally in an autocrine or paracrine manner by binding to G-protein coupled receptors (Findlay et al., 1993; Hata and Breyer, 2004). All eight prostanoid receptors identified to date (TP: tromboxane receptor; DP; prostaglandin D receptor; EP: prostaglandin E receptor [EP1, EP2, EP3, EP4]; IP: prostaglandin I receptor, and FP: prostaglandin F receptor) are integral membrane proteins that possess seven transmembrane domains (α helices). These receptors are coupled to specific G proteins mediating the formation of second messengers such as cAMP, DAG and inositol trisphosphate (IP_3; as reviewed by Tsuboi and coworkers, (2002). The extracellular parts of the receptor can be glycosylated or form disulfide bonds between highly conserved cysteine residues to stabilize the receptor. The intracellular loops between α helices 5 and 6 and the C-terminal segment are important for interactions with G proteins. The prostanoids receptors are classified according to the prostanoid ligand that each binds with greatest affinity. Recently, a ninth prostaglandin receptor, CRTH2, was identified on Th2 cells (Hirai et al., 2001).

Two differentially spliced variants of the sheep FP receptor have been reported, the original FPr re-named FP_A and a truncated form called FP_B (Pierce et al., 1997).

These variants exhibit similar ligand binding profiles and G protein coupling preferences (Pierce et al., 1997). However, the C terminal tail of the FP_A, but not the truncated FP_B, can be phosphorylated by PKC, leading to agonist-induced desensitization and internalization (Fujino et al., 2000). In contrast, FP_B does not undergo agonist-induced internalization but instead experiences constitutive internalization (Srinivasan et al., 2002). In addition, both isoforms are able to stimulate IP_3 accumulation to the same maximum, but the basal level of hydrolysis is 130% higher for the FP_B than the original FP_A isoform (Pierce et al., 1997).

Another type of FP receptor isoform, FP_a, has recently been cloned in bovine (Ishii and Sakamoto, 2001). FP_a is generated by alternative splicing at the middle of the sixth transmembrane domain, resulting in the lack of a seven transmembrane domain and an intercellular carboxyl tail. Transfected cells with FP_a failed to stimulate PKC after treatment with $PGF_{2\alpha}$; whereas, cotransfection of an excess amount of FP_a markedly reduces the original FP-mediated PKC response, suggesting that FP_a could play a role as a negative regulator to attenuate normal FPr function (Ishii and Sakamoto, 2001).

Recently, Sakamoto et al. (2002) identified the cDNA clones for five novel FPr isoforms from a bovine corpus luteum cDNA library. The sequence analysis revealed that these clones encoded the amino acid sequences of the original FPr (Sakamoto et al., 1994) and the following clone-specific regions. Because only one copy of the FPr gene has been identified in the bovine (Ezashi et al., 1997), these clones named FP-α, FP-β, FP-γ, FP-δ, and FP-ε were identified as novel FPr isoforms generated by alternative mRNA splicing. These proteins are constituted of 325, 326, 339, 293, and 298 amino acids, respectively. These isoforms can be classified based in the location of the splicing

site into two groups. FP-α, FP-β, FP-γ can be potentially spliced within the fourth intracellular C-tail. These isoforms can be classified into the same group (Type I), which is composed of the common overall sequences from the N-terminus through transmembrane segment VII and the isoform-specific tail. The other isoforms, FP-δ and FP-ε, are classified into the second group (Type II), which contains the variation from the fourth extracellular domain through the C-tail. Hydrophobicity analysis revealed that Type I isoforms possess intact seven transmembrane domains, while the Type II isoforms carry truncated structures lacking transmembrane domain VII and the intracellular C-tail (Sakamoto et al., 2002). In fact, the C-terminal region of the Type II isoforms may be buried in the plasma membrane or protrude into the extracellular region and therefore their ability to interact with G proteins and stimulate a cell signaling cascade would be totally inhibited. As previously demonstrated by Ishii et al. (2001), these Type II isoforms may function as suppressors for the normal activation of PKC after $PGF_{2\alpha}$ binding to the original FPr. The mRNA of these novel FPr isoforms and the original FPr isoforms were shown to be expressed in the bovine corpus luteum at the same time during the estrous cycle and pregnancy (Sakamoto et al., 2002). This suggests a cooperative role of these isoforms with the original FP during the estrous cycle and pregnancy.

18. Prostaglandin $F_{2\alpha}$ receptor agonists and antagonists

Development of potent and selective FP receptor agonists has resulted in the ability to investigate specific cellular responses coupled to the activation of these receptors (for example, activation of phospholipase C, increases in intracellular levels of calcium and activation of PKC). Some of the FPr agonists include bimatoprost,

latanoprost and travoprost (Sharif et al., 2002). These substances possess similar chemical structures and shapes as the endogenous ligand and therefore are able to bind to the receptor and trigger a cellular response by "mimicking" the interaction of the endogenous ligand to the receptor (Jasper and Insel, 1992). Agonists can be classified as full or partial agonists (Jasper and Insel, 1992). A partial agonist binds to the specific receptor, but does not cause as much of a physiological change as does a full agonist (Franciosa, 1989). Moreover, a partial agonist can bind to a large number of receptors and competitively inhibit action of a full agonist for the same receptor (Franciosa, 1989).

Antagonists for the FPr are being used to positively identify FPr mediating functional responses *in vitro* or *in vivo*. Some FPr antagonists include AL-3138, AL-8810, $PGF_{2\alpha}$ dimethylamine, dimethylamide, glibenclamide and phloretin (Griffin et al., 1999; Sharif et al., 2000). Antagonist substances bind to receptors and do not produce any physiological responses. Antagonists are classified as competitive antagonists or non-competitive antagonists. Competitive antagonists compete with the endogenous ligand/s for the active site of the receptor. Once the antagonist is bound to the receptor, it does not produce any physiological response in the cell and does not allow the endogenous ligand to bind to the same receptor. Competitive antagonists produce a concentration dependent shift in the endogenous ligand concentration-response curve, without decreasing the agonist-induced maximal response. Another effect of a competitive antagonism is the increase in the K_d of an agonist when increasing concentrations of the competitive antagonist. An example of this is the effect of AL-8810 concentration on the concentration-response curves of fluprostenol, a full agonist of the FP receptor (Sharif et al., 2002). Griffin et al. (1999) characterized the pharmacological activities of AL8810 at

the FP receptor. Authors concluded that previous exposure of mouse fibroblast to AL-8810 abolished the production of IP_3 associated to FPr stimulation by FPr agonist (fluprostenol) or its endogenous ligand ($PGF_{2\alpha}$). In addition, AL-8810 did not have any antagonistic activity at several other prostanoid receptors such as TP, DP, EP2 and EP4. In agreement with these findings, several other studies indicated that AL-8810 inhibited production of IP_3 after activation of FPr (Sharif et al., 2002; Kelly et al., 2003; Sharif et al., 2003; Sharif et al., 2003). During non-competitive antagonism, an antagonist binds to its receptor on a different site from the endogenous ligand. In this case, binding of the antagonist does not block the endogenous ligand binding (or vice versa), but the receptors can not get activated when the antagonist is bound to it. The antagonist also lowers the concentration of free ligand and has no effect on the K_d for the agonist.

19. Prostaglandin $F_{2\alpha}$ association with early embryonic loss

The first indications that $PGF_{2\alpha}$ were involved in embryonic loss occurred in a series of experiments in the "short cycle" cow at West Virginia University. In the postpartum cow, the first ovulation is followed by a short luteal phase (short cycle) due to premature release of $PGF_{2\alpha}$ (Cooper et al., 1991). Cows with short luteal phase failed to maintain pregnancy even when exogenous progestogen was provided (Breuel et al., 1993). Moreover, when good quality frozen-thawed embryos were transferred to progestogen-supplemented animals on day 7 after estrus, pregnancy was maintained in 28% of short cycle cows compared to 58% of cows with normal luteal phase (Butcher et al., 1992). However, pregnancy rates did not differ when day-6 embryos from short cycle or normal cycling cows were transferred to normally cycling recipients (Schrick et al.,

1993), suggesting that an improper uterine environment appears to play a role in lower survival of embryos in cows with short cycle as compared to cows with normal luteal phases. Schrick et al. (1993) observed that concentrations of $PGF_{2\alpha}$ in flushing media from cows with short luteal phases were more than double those from cows with normal luteal phases (636 ± 82 and 288 ± 90 pg/ml, respectively). Furthermore, embryo quality tended to be negatively correlated with concentrations of $PGF_{2\alpha}$ in the flushing media (r = -0.42; $P=0.07$). Thus, a direct negative effect of $PGF_{2\alpha}$ may be implicated in lowering embryonic survival as demonstrated *in vitro* in the mouse (Harper and Skarnes, 1972), rabbit (Maurer and Beier, 1976), and rat (Breuel et al., 1993).

Effects of $PGF_{2\alpha}$ on embryo survival have been examined in several studies using progestogen-supplemented cows to replace the regressing corpus luteum. Buford et al. (1996) demonstrated that $PGF_{2\alpha}$ was detrimental to embryonic survival when 15 mg of $PGF_{2\alpha}$ every 8 h was injected to non-lactating cycling beef cows during days 4 through 7 or 5 through 8 after insemination, but pregnancy rates remained high when lutectomy (removal of the corpus luteum) was performed before $PGF_{2\alpha}$ treatment begun. Similarly, $PGF_{2\alpha}$ administration to progestogen-supplemented beef cows on days 5 to 8 postbreeding reduced pregnancy rates (23%) compared to control (72%), whereas lutectomy prior to $PGF_{2\alpha}$ administration tended to improve pregnancy rates (55%; Seals et al., 1998). Removal of the corpus luteum eliminates oxytocin release from the corpus luteum and blocks the positive feedback loop between oxytocin and $PGF_{2\alpha}$. As a result, $PGF_{2\alpha}$ concentration in the uterine lumen would remain low, allowing embryonic development to occur. Furthermore, Seals et al. (1998) reported that detrimental action(s) of $PGF_{2\alpha}$ occurred during early embryonic development (days 5 to 8) since

administration of $PGF_{2\alpha}$ during filamentous embryo development (beyond day 10) had no effect on maintenance of pregnancy.

Buford et al. (1996) demonstrated that postpartum cows bred at their first estrus after weaning had higher pregnancy rates when lutectomy was used in conjunction with flunixin meglumine (an inhibitor of COX-1 and COX-2), but lutectomy alone failed to maintain pregnancy rates. The authors suggested that both premature secretion of $PGF_{2\alpha}$ and a factor released by the regressing corpus luteum may contribute to embryonic death in the postpartum cow with a short luteal phase.

Oxytocin, which is released by large cells of the regressing corpus luteum in response to $PGF_{2\alpha}$, can increase secretion of $PGF_{2\alpha}$ from the uterus (Milvae and Hansel, 1980). Lemaster et al. (1999) treated progestogen-supplemented cows with oxytocin rather than $PGF_{2\alpha}$ in the same type of experiment performed by Seals et al. (1998). Administration of oxytocin on days 5 through 8 every 8 h reduced pregnancy rates to 33% compared to the control group (80%). In the same study, administration of flunixin meglumine blocked the negative effects of oxytocin, while lutectomy did not; thus, the role of oxytocin on embryonic death may involve further release of $PGF_{2\alpha}$ rather than having a direct negative effect on the embryo.

Numerous factors such as heat stress (Putney et al., 1989; Malayer et al., 1990), nutrition (Butler, 1998), mastitis (Hockett et al., 2000), manipulation of the reproductive tract during embryo transfer and artificial insemination (Odensvik et al., 1993; Scenna et al., 2005), and plant toxins (Woclawek-Potocka et al., 2005) may contribute to early embryonic losses (before day 8) through premature release of uterine luminal concentrations of $PGF_{2\alpha}$ in cattle. Administration of flunixin meglumine or ibuprofen

lysinate (inhibitors of COX-1 and COX-2) at time of embryo transfer improved pregnancy rates in cows (Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005). Furthermore, low-dose aspirin treatment improved implantation and pregnancy rates in human patients undergoing *in vitro* fertilization and transfer (Rubinstein et al., 1999). Thus, *in vivo* and *in vitro* studies have shown that $PGF_{2\alpha}$ decreased embryonic development and survival in several species.

20. Embryo transfer

The objective of a multiple ovulation and embryo transfer (MOET) program in livestock production is the rapid acquisition of superior genetics and to increase the productive life of superior donor cows by increasing the number of its valuable progeny through this technique (i.e. obtaining 20 transferable embryo per year resulting in 10 pregnancies instead of only having one calf per year when not using ET). Currently, the ET technique is widely used around the world, with more than 500,000 embryos being transfer annually (Thibier, 2000). Briefly, during any MOET program, the estrous cycle of a donor cow is synchronized, the growth of follicles in each ovary is stimulated by injecting follicle stimulating hormone (FSH), and the donor cow is observed for estrus and artificially inseminated with frozen semen 12 and 24 h after heat detection (Seidel, 1981). Embryo recovery is performed seven days after estrus by placing a catheter through the cervix and up to the upper third of each uterine horn or in the uterine body (Drost et al., 1976). The uterus is flushed several times with phosphate buffered solution (PBS) containing antibiotics and bovine serum albumin or a synthetic surfactant such as polyvinyl alcohol (PVA) and the embryos are recovered in a filter. Next, embryos are

searched under a microscope, loaded in holding medium into 0.25 mL straws and transfer fresh to recipient cows or frozen in glycerol or ethylene glycol for future use.

The first successful ET in mammals was recorded in 1891 using rabbit embryos (Heape, 1891). The next successful embryo transfer pregnancies resulted in rabbit (Pincus, 1930) and rat (Nicholas, 1933). The first ET calf was born following the surgical transfer of a slaughterhouse-derived 5-day old embryo (Willett et al., 1951). Commercial ET in cattle started in the early 1970's, primarily as a result of the high prices paid for breeders for "exotic" beef cattle that had been imported in small numbers from Europe (Betteridge, 2000). This technique was done by mid-ventral surgical exposure of the uterus and ovaries with the donor under general anesthesia. Surgical ET was only possible in "in-house" programs with suitable facilities and in beef cows due to the fact that the udder of dairy cows hindered mid ventral access to the reproductive tract. However, the introduction of non-surgical embryo recovery (flushing) in the mid-1970's (Elsden et al., 1976) and non-surgical embryo transfer in the late 1970's (Bowen et al., 1978) allowed ET to be practiced on the farm and in dairy animals. Prior to the early 1980's, the inability to freeze and thaw bovine embryos made it necessary to synchronize a sufficient number of recipient animals so that all recovered embryos could be transferred fresh. Fortunately, development of freezing protocols utilizing dimethyl sulfoxide or glycerol as cryoprotectants (Wilmut and Rowson, 1973) made ET more appealing to producers and a much more efficient technologicalley.

21. Efficiency and limitations of embryo transfer

Efficiency of embryo recovery is probably between 70 to 90% (Seidel, 1984) and if no complications occur during uterine flushing (bleeding, clotting of the filter, cows going down in the chute, etc.) the procedure takes no longer than 30 min per cow. When performing ET, it is necessary to synchronize the estrous cycle of recipient cows with the estrous cycle of donor cows (Rowson et al., 1972; Lawson et al., 1975; Hasler, 2001). Asynchrony between donor and recipient estrous cycles greater that ± 24h resulted in poor pregnancy rates following ET (Hasler et al., 1987; Hasler, 2001). Moreover, if asynchrony between donor and recipient estrous cycles exists, then a synchrony between embryo stage with the day of estrous cycle of recipient cows should be made (Wright, 1981; Lester and McNew, 1999). For example, morula stage embryos should be transferred to recipients on day 6 ± 12h after estrous detection and blastocyst stage embryos should be transferred to recipients on day 7 ± 12h after estrous detection. Today, pregnancy rates in beef cattle after non-surgical embryo transfer of *in vivo*-derived frozen-thawed (ethylene glycol) embryos is about 40 to 55% (Leibo, 1986; Malayer et al., 1990; Nibart and Humblot, 1997; Lester and McNew, 1999), whereas pregnancy rate of those transferred fresh is about 55% to 80% (Hasler, 2001).

The biggest problem in the embryo transfer industry is the variability in superovulatory response of donor cows (Seidel, 1984). The average of transferable embryos recovered per cow after a superovulatory treatment is in the range of 5 to 7 (Donaldson and Perry, 1983; Hasler et al., 1983). However, many donors yield no transferable embryos or only a few, and in rare cases more than 30 transferable embryos are recovered (Seidel, 1984). In addition, administration of the same dose of FSH to the

same group of cows can result in cows not responding at all and presenting small ovaries, cows responding accordingly to the expectations, and cows overstimulated with very large ovaries and often yielding no transferable embryos (Seidel, 1981). Superovulation can be performed every 60 days after allowing donor cows to have at least two estrous cycles between superovulations (Hasler, 2003). Recently, Hasler (2003) described a superovulation protocol called "donor quick turn around" that significantly reduces this interval. The protocol consists of injecting a dose of $PGF_{2\alpha}$ and the insertion of a progesterone implant immediately after embryo recovery. Ten days later, the implant is removed and superovulation initiated following the next estrus with an average of 40 days between superovulations. A disadvantage of superovulation programs is that the donor response to this treatment sometimes decreases after repeated applications (Seidel, 1981; Donaldson and Perry, 1983). Several other factors influencing superovulatory treatments and embryo transfer were recently investigated by Stroud and Hasler (2006). In this review, the total number of ova/embryos recovered, the number of transferable embryos, and pregnancy rates after embryo transfer, were all positively correlated to good farm management (nutrition, recipient selection, synchronization protocols, estrous detection, and synchrony between donor and recipient) and semen evaluation before insemination of donor cows. Schrick et al. (2003) demonstrated a highly significant relationship between semen quality and the percentage of fertilized ova and transferable embryos in superovulated donors. Moreover, semen quality was also positively related to the percentage of excellent-quality embryos.

Embryo mortality after natural mating or AI from day 24 to term is only between 3 and 5% (Paisley et al., 1978; Sreenan and Diskin, 1986). These losses are in agreement

with data reported by King et al. (1985) who investigated losses associated to fresh embryo transfers. In this study, 3.15% and 2.14% pregnancies resulted in abortion between 2 and 3 months and 3 to 7 month of gestation, respectively. On the other hand, pregnancy rates from day 60 to term after transfer of frozen-thawed embryos resulted in abortion rates of 9.6 and 12.8% (Dochi et al., 1998). McMillan and Donnison (1999), in an attempt to understand maternal contributions to fertility in recipient cows, transferred *in vitro*-produced blastocyst stage embryo into contemporary beef heifers. After pregnancy diagnosis, pregnancies were terminated with a luteolytic dose of $PGF_{2\alpha}$ and the process repeated 6 more times over a period of 26 months. Based on pregnancy data obtained at 60 days following each round of transfers, heifers were ranked as high and low fertility. Pregnancy rates for the High and Low sub-herds were 76% and 11%, respectively, with 55% and 37% of this differences due to losses before day 25 (return to estrous) and losses between day 25 and 35, respectively. Moreover, when other reproductive parameters related to fertility were evaluated (site of ovulation, incidence of standing estrus, interval to end of estrus to standing estrus, and progesterone profiles during the estrous cycle), no differences between these two groups were observed, indicating no differences in fertility at the level of the ovary. The authors suggested that the mechanism of maternal recognition of pregnancy (MRP) is a major cause of differences between these two groups; however, losses during MRP were not observed. Dunne et al. (2000) reported that embryo loss following artificial insemination in beef heifers occurred by day 14 after mating with little loss after this time.

22. Implications of prostaglandin $F_{2\alpha}$ release during embryo transfer in cows

The bovine endometrium contains relatively large amounts of arachidonic acid that can be rapidly converted to different products such as $PGF_{2\alpha}$ (Salamonsen and Findlay, 1990). In fact, manipulation of the reproductive tract during embryo transfer resulted in $PGF_{2\alpha}$ release from the uterine endometrium (Scenna et al., 2005). Other studies have also reported a release of $PGF_{2\alpha}$ following uterine manipulation in mares (Kask and Odensvik, 1995), sows (Kunavongkrit et al., 1984) and cows (Wann and Randel, 1990). On the other hand, Odensvik et al. (1993) did not report an increase in concentrations of plasma $PGF_{2\alpha}$ after non-surgical embryo transfer in heifers. Differences among these studies may be attributed to differences in breed, handling of blood samples, and in the methodology used for determining $PGF_{2\alpha}$ concentrations (indirect determination of $PGF_{2\alpha}$ through measuring PGFM vs. direct determination).

Manipulation of the reproductive tract causes uterine trauma followed by an inflammatory process with release of several chemical mediators such as cytokines and prostaglandins. One of the most important cytokines, tumor necrosis factor α (TNF-α), is capable of inducing $PGF_{2\alpha}$ release by luteal cells (Nothnick and Pate, 1990) and epithelial cells in the uterine endometrium via activation of PLA_2 and nitric oxide synthase (Skarzynski et al., 2000). Moreover, Okuda et al. (2002) suggested that endometrial TNF-α may be a trigger for the production of $PGF_{2\alpha}$ from the uterine endometrium during luteolysis. Since treatment of luteal cells with $PGF_{2\alpha}$ induced further production of $PGF_{2\alpha}$ by this cell type in sheep (Rexroad and Guthrie, 1979; Wade and Lewis, 1996) and pigs (Guthrie et al., 1979), $PGF_{2\alpha}$ release after embryo transfer in cows may be able to stimulate its own production from the reproductive tract.

Even though release of $PGF_{2\alpha}$ during embryo transfer may not result in luteolysis, embryonic survival may be compromised by the presence of small concentrations of $PGF_{2\alpha}$ in the uterine lumen, creating a "hostile environment" for embryonic development. Scenna et al. (2004) demonstrated that development of *in vitro*-produced 16- to 32-cell embryos to blastocyst stage was reduced by addition of $PGF_{2\alpha}$ in the culture media. In addition, culture of *in vivo*-derived compact morulae in medium containing $PGF_{2\alpha}$ did not affect development to blastocyst, but reduced hatching rates (Scenna et al., 2004). This is in agreement with Maurer and Beier (1976) who reported that addition of $PGF_{2\alpha}$ to culture media directly decreased the ability of 8-cell rabbit embryos to form expanded and hatched blastocysts, but had no effect on development to early blastocyst. Successful hatching of the embryo is thought to be a key event for further embryonic development and proper implantation in humans (Petersen et al., 2004). In fact, assisted hatching of human embryos (consisting in creating a complete or partial hole in the zona pellucida) has been shown to result in better implantation rate after embryo transfer in humans (Ali et al., 2003; Kung et al., 2003; Wong et al., 2003).

Administration of flunixin meglumine (FM), an inhibitor of $PGF_{2\alpha}$ synthesis, at the time of embryo transfer resulted in increased pregnancy rates at the time of embryo transfer (Scenna et al., 2005). The same study showed no differences on pregnancy rate of quality 1 embryos following administration of FM when compared to control recipients. However, transfer of quality 2 embryos to recipient animals receiving FM resulted in higher pregnancy rates than control animals, indicating that developmentally compromised embryos (quality 2) are most susceptible to deleterious effects of $PGF_{2\alpha}$ than those of better quality (quality 1).

In agreement with these findings, Elli et al. (2001), McNaughtan et al. (2002), Pugh et al. (2004) and Purcell et al. (2004) reported beneficial effects of prostaglandin synthesis inhibitors on pregnancy rates in recipient cows after embryo transfer. In humans, women undergoing *in vitro* fertilization and receiving a daily oral dose of aspirin (an inhibitor of prostaglandin synthesis) had better ovarian responsiveness (greater number of follicles and oocytes retrieved), uterine and ovarian blood flow velocity, implantation, and pregnancy rates than women receiving placebo (Rubinstein et al., 1999). Similarly, Waldenstrom et al. (2004) reported higher pregnancy rates in women undergoing IVF and receiving a daily dose of aspirin from the day of embryo transfer until pregnancy diagnosis when compared to women not receiving a treatment.

In conclusion, $PGF_{2\alpha}$ synthesis and release at the time of embryo transfer may result in reduced pregnancy rate of recipient cows by altering/inhibiting the normal process of hatching. Moreover, developmentally-compromised embryos (those of lower quality) may be more susceptible to the effects of $PGF_{2\alpha}$ than good quality embryos. New therapeutic techniques aimed at inhibiting synthesis or binding of $PGF_{2\alpha}$ to its receptor in bovine embryos will likely improve reproduction in cattle, as well as in humans undergoing assisted reproduction programs.

CHAPTER 3

RECEPTOR-MEDIATED EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ ON BOVINE EMBRYOS

1. Abstract

Prostaglandin $F_{2\alpha}$ has been shown to have detrimental effects on embryonic development, quality and hatching ability of embryos and pregnancy rates in cows. In recipient cattle, administration of flunixin meglumine (a cyclooxygenase-1 and -2 inhibitor) at embryo transfer reduced detrimental effects associated with $PGF_{2\alpha}$ actions on establishment of pregnancy. Information regarding the presence of prostaglandin $F_{2\alpha}$ receptor (FPr) mRNA and protein in bovine embryos is absent in the literature. The objectives of the current study were 1) to identify the period of time where *in vitro*-derived embryos are most susceptible to $PGF_{2\alpha}$ and 2) to determine the presence of prostaglandin $F_{2\alpha}$ receptor (FPr) mRNA and protein in bovine embryos. Results indicated that culture of pre-compacted embryos for 48 h in medium containing 1 ng/mL of $PGF_{2\alpha}$ had decreased development to compact morula when compared to control embryos (PGF 1=55%C vs. CON=64%; $P<0.05$). On the other hand, culture of compacted embryos in medium containing 1 ng/mL of $PGF_{2\alpha}$ did not decrease development to blastocyst (PGF 1=57 vs. CON=59%). In addition, expression of FPr mRNA and protein in bovine embryos was confirmed by real time PCR and Western Blot analysis, respectively. In conclusion, $PGF_{2\alpha}$ decreases embryonic development of bovine embryos to compact morula, but once compaction occurred, $PGF_{2\alpha}$ does not appear to affect further

embryonic development *in vitro*. Furthermore, $PGF_{2\alpha}$ can have a direct negative effect on development of bovine embryos by activating its own cell membrane receptor.

2. Introduction

Prostaglandins, also called prostanoids, are ubiquitously produced compounds that mediate many physiological processes, including many reproductive functions (reviewed by Weems and coworkers, (2006). Receptors for tromboxane A_2 (TP), prostaglandin D_2 (DP), prostaglandin E_2 (EP1-4), prostaglandin I_2 (IP), and prostaglandin $F_{2\alpha}$ (FP), are integral membrane proteins that present seven transmembrane domains and are coupled to specific G proteins mediating the formation of second messengers such as cAMP, DAG and inositol triphosphate (Findlay et al., 1993; Hata and Breyer, 2004). The prostaglandin $F_{2\alpha}$ receptor (FPr) has been cloned from bovine (Sakamoto et al., 1994), ovine (Graves et al., 1995), mice (Sugimoto et al., 1994), rat (Kitanaka et al., 1994) and human (Abramovitz et al., 1994) cDNA libraries. In addition, several isoforms of this receptor have been cloned from cDNA libraries of ovine and bovine corpus luteum (Sakamoto et al., 1994; Pierce et al., 1997; Ishii and Sakamoto, 2001; Sakamoto et al., 2002).

Embryonic losses in bovine appear to occur soon after the embryo enters the uterus (5 to 8 days after mating or insemination), when the morula stage embryo is transitioning to blastocyst (Ayalon, 1978; Maurer and Chenault, 1983; Wiebold, 1988; Dunne et al., 2000). Previous studies have shown a detrimental effect of $PGF_{2\alpha}$ on *in vitro* development of rat, rabbit and bovine embryos (Maurer and Beier, 1976; Breuel et al., 1993; Scenna et al., 2004). Furthermore, administration of $PGF_{2\alpha}$ on days 5 through 8

after artificial insemination or mating has also been shown to decrease embryonic development, quality and pregnancy rates in progesterone-supplemented cows (Buford et al., 1996; Hockett et al., 2004; Sales et al., 2004). In addition, administration of prostaglandin synthesis inhibitors increased pregnancy rates after transfer in cows (Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005).

The objectives of this study were 1) to identify the period of time during *in vitro* embryo development that embryos are most susceptible to detrimental effects of $PGF_{2\alpha}$, and 2) to determine the presence of FPr gene and protein expression in bovine embryos.

3. Experimental procedures

3.1 Materials

Polyvinyl alcohol (PVA), bovine serum albumin (BSA), percoll, penicillamine, hypotaurine, epinephrine and the majority of reagents used for *in vitro* production of bovine embryos were purchased from Sigma Chemical, Inc. (St. Louis, MO). Tissue culture medium-199, gentamicin, and penicillin-streptomycin were purchased from Specialty Media, Inc. (Phillipsburg, NJ). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Folltropin-V and Vigro holding Plus were purchased from Bioniche Life Sciences, Inc. (Belleville, ON). Ovaries were purchased from a local abattoir. Media (HEPES-TALP, IVF-TALP, AND SPERM-TALP (Parrish et al., 1988)) and KSOM (Edwards et al., 2005), were prepared in the laboratory. Sperm characteristics (percent motile sperm and concentration) were analyzed using a computerized sperm analyzer (CASA; Hamilton Thorne Biosciences; Beverly, MA). The PicoPure RNA

isolation Kit was purchased from Arcturus (Mountain View, CA). DNase I enzyme and RNA*later* were purchased from Ambion (Austin, TX). Trizol Reagent was obtained from Invitrogen (Carlsbad, CA). All reagents for the RT reaction (5X RT Buffer, 25 mM Magnesium Chloride, Random Primers, RNasin Ribonuclease Inhibitor, dNTP Mix, and M-MLV Reverse Transcriptase) were purchased from Promega (Madison, WI). iQ™ SYBR Green Supermix was purchased from Bio-Rad (Hercules, CA).

3.2. General procedures for in vitro production of bovine embryos

The procedures utilized for *in vitro* production of embryos (IVP) were modifications of procedures previously described by Lawrence et al. (2004). Ovaries obtained from an abattoir were packaged in thermoses and contained within a cooler during air transport to the laboratory. Upon arrival to the laboratory, ovaries were immediately washed with warm tap water equilibrated to arrival temperature of ovaries (generally between 28 to 30°C). Excess tissue surrounding ovaries was removed with scissors and ovaries were washed with warm tap water an additional time.

For oocyte recovery, ovaries were held firmly by clamping the base of the ovary with a hemostat and checkerboard incisions were made across follicles (approximately 3-8 mm in diameter) using a scalpel blade. Ovaries were vigorously washed in oocyte collection medium (OCM) in order to remove cumulus oocyte complexes (COCs) contained within the follicles. Collection medium was filtered and rinsed using an Emcon Filter unit (Vet Concepts, Spring Valley, WI) until medium was clear. Medium containing COCs was poured into a gridded culture dish where searching of oocytes was accomplished. Cumulus oocyte complexes were transferred to an "X" plate containing

OCM and washed four times to eliminate cellular debris. Cumulus oocyte complexes of good quality (even cytoplasm, at least 3-4 layers of tight cumulus cells) were washed in oocyte maturation medium (OMM; M-199 with Earle's salts, 10% fetal bovine serum, 50 μg/mL gentamicin, 5 μg/mL LH, 0.2 m*M* sodium pyruvate, and 2 m*M* L-glutamine) and placed in groups of approximately 50 COCs per well in a 4-well plate containing 500 μL OMM. Maturation of oocytes was performed in an incubator at 5.5% CO_2 in air at 38.5°C until time of fertilization (approximately 24 h after placement in OMM). Maturation medium was equilibrated in the incubator (5.5% CO_2 in air at 38.5°C) the day before oocyte collection.

3.2.1. Sperm preparation and in vitro fertilization

After oocyte maturation (22-24 h in OMM in an incubator at 5.5% CO_2 in air at 38.5°C), OMM from each well was carefully removed and 25 μL of penicillamine/hypotaurine/epinephrine (PHE) and 500 μL of fertilization medium (IVF-TALP) were added to each well. Two straws of semen from two bulls (140 Se5, 140 Se55, 30 SM002, CG30, SM 101), known to have high motility as well as producing high cleavage and blastocyst percentages were used to fertilize oocytes for every replicate of the study. Briefly, semen straws were removed from liquid nitrogen tank and placed in water at 36.7°C for 45 sec. Each straw was then emptied on top of a discontinuous percoll density gradient (3 mL 45% percoll over 3 mL 90% percoll contained in a 15 mL conical tube) and sperm was centrifuged at 760 x *g* for 15 min in order to remove excess extender, debris, and nonmotile sperm prior to fertilization. Sperm pellet present at the bottom of the 90% fraction was collected and transferred to 10 mL SPERM-TALP and

centrifuged at 200 x *g* for 8 min. Supernatant was removed and sperm pellet re-suspended in 100 μL of modified IVF-TALP. Sperm concentration and motility were determined using a CASA and sperm was added to each well at a final concentration of 500,000 motile sperm per well. Lastly, oocytes and sperm were incubated at 5.5% CO_2 in air at 38.5°C for 18-22 h.

3.2.2. In vitro culture

Approximately 18-22 h post fertilization, putative zygotes (PZ) were denuded of cumulus cells by vortexing. Putative zygotes were transferred to a 2 mL centrifuge tube containing 50 μL of HEPES-TALP and vortexed for 5 min. Recovered putative zygotes were washed 4 times in HEPES-TALP before transferring groups of approximately 40-50 zygotes to each well of a 4-well plate containing 500 μL of KSOM culture medium per well. Zygotes were placed in a humidified atmosphere of 5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C until they were collected at 16-32 cells (approximately 97 h post IVF), compact morula (approximately 145 h post IVF) or blastocyst stage (approximately 193 h post IVF) to perform each experiment.

3.3. Time-specific effects of $PGF_{2\alpha}$ during in vitro embryo development

Previous data in our laboratory have shown that addition of $PGF_{2\alpha}$ to the culture medium of pre-compacted *in vitro* produced embryos (during days 4 to 9 of embryonic development) results in decreased embryonic development when compared to embryos cultured in control medium (Scenna et al., 2004). In order to investigate time-specific effects of $PGF_{2\alpha}$ during *in vitro* embryo development, embryonic development of pre-

compacted (Experiment 1) and compacted embryos (Experiment 2) cultured in medium containing $PGF_{2\alpha}$ was compared to embryos cultured in control medium.

3.3.1. Preparation of $PGF_{2\alpha}$ stock and treatment wells

Prior to the beginning of each experiment, prostaglandin $F_{2\alpha}$ (1 mg; Cayman Chemical Inc., Ann Arbor, MI, catalog#) was solubilized in 10 mL of DMSO (Stock #1 100,000 ng/mL). Then, 30 µL aliquots were stored at -20C° until needed. For each replicate of the experiment, serial 10-fold dilutions of $PGF_{2\alpha}$ stock #1 in KSOM-PVA (0.3%) were made to yield 1 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA culture medium. Control experimental wells were prepared by adding a DMSO (0.001%) concentration equal to the one use to prepare 1 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA. Four-well plates containing treatment wells (500 µl KSOM-PVA without the addition of $PGF_{2\alpha}$ or with the addition of 1 ng/mL $PGF_{2\alpha}$) were equilibrated in an incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for at least 12 h before placing embryos in their respective treatments. Addition of 1 ng/mL $PGF_{2\alpha}$ has been previously shown to result in lowered embryonic development of *in vitro* produced bovine embryos (Scenna et al., 2004).

3.3.2. Experiment 1: Effects of $PGF_{2\alpha}$ on pre-compacted embryos

Procedures for oocyte collection, maturation, fertilization and embryo culture were previously described under general methods. Pre-compacted (16- to 32-cell stage; approximately at 97 h post IVF) embryos were rapidly transferred from KSOM culture medium to pre-warmed HEPES-TALP for evaluation. Embryos were evaluated for quality (normal shape, defined blastomeres, extruded blastomeres, cytoplasmic

fragmentation, even cytoplasm) and evenly sorted according to their quality. Embryos were rapidly washed at least twice in KSOM-PVA and transferred to their respective treatments in a 4-well plate. Experimental plates were placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for 2 additional days when embryo development to compact morula stage was determined using IETS guidelines for classification of bovine embryos (Stringfellow and Seidel, 1998). Data for development to compact morula were analyzed using a randomized block design model with replicate (a 4 well plate with each well containing a treatment and a group of embryos considered an experimental unit) as random effect and treatment as fixed effect performing mixed procedures of SAS software (SAS 9.1, SAS Institute Inc., Cary, NC).

A total of 6 replicates were utilized to investigate the effects of $PGF_{2\alpha}$ on pre compacted *in vitro*-derived embryos. In each of these replicates, pre-compacted embryos received one of the following treatments: 1) CON (KSOM-PVA; n= 183), or 2) PGF 1 (1 ng/ml $PGF_{2\alpha}$ in KSOM-PVA; n= 184).

3.3.3. Experiment 2: Effects of $PGF_{2\alpha}$ on compacted embryos

Procedures for oocyte collection, maturation, fertilization and embryo culture were previously described under general methods. Compact morula stage embryos (collected at approximately 145 h post IVF) were rapidly transferred from KSOM culture medium to pre-warmed HEPES-TALP for evaluation. Embryos were evaluated for quality (normal shape, defined blastomeres, extruded blastomeres, cytoplasmic fragmentation, even cytoplasm) and evenly sorted according to their quality. Embryos were rapidly washed at least twice in KSOM-PVA and transferred to their respective

treatments in a 4-well plate. Experimental plates were placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for 2 additional days when embryo development to blastocyst stage was determined using IETS guidelines for classification of bovine embryos (Stringfellow and Seidel, 1998). Data for development to blastocyst were analyzed using a randomized block design model with replicate (a 4-well plate with each of its wells containing one treatment; a group of embryos in each well was considered an experimental unit) as random effect and treatment as fixed effect performing mixed procedures of SAS software (SAS 9.1, SAS Institute Inc., Cary, NC).

A total of 11 replicates were utilized to investigate the effects of $PGF_{2\alpha}$ on compacted *in vitro*-derived embryos. In each of these replicates, compact morula stage embryos received one of the following treatments: 1) CON (KSOM-PVA; n= 268), or 2) PGF 1 (1 ng/ml $PGF_{2\alpha}$ in KSOM-PVA; n= 278).

3.4. Determination of FPr mRNA and protein in bovine embryos

3.4.1. Preparation of embryo and semen samples for reverse transcription-real time-PCR

Due to a concern regarding the amplification of $PGF_{2\alpha}$ transcripts associated with the accessory sperm present in the zona pellucida (ZP) of bovine embryo (Nadir et al., 1993), PCR was performed in ZP-intact and ZP-free *in vitro*-derived compact morula and blastocyst stage embryos. Mechanical removal of ZPs from embryos was the preferred method over pronase treatment because it also allowed PCR analysis of isolated ZPs. Mechanical removal of ZPs was performed following procedures described by Edwards et al. (2003) to remove ZPs of bovine embryos. Briefly, embryos were removed from culture medium and washed 3 times in HEPES-TALP-PVA (0.3% PVA). Groups of 5

embryos were transferred to 10 μL drops of 0.3M sucrose (Van Soom et al., 1996) covered in mineral oil to induce shrinkage of the embryo mass. Following shrinkage of embryos, the majority of the 0.3 M sucrose solution was removed, and as a result, the weight of the mineral oil maintained embryos in place forming a tight group of embryos. Next, a 27-gauge tuberculin needle associated with its corresponding syringe was used to produce a hole in the ZP of each embryo. Each group of embryos was resuspended in HEPES-TALP-PVA and transferred to HEPES-TALP-PVA in a 4-well plate. Using a glass pipette just slightly thinner than the embryo's size each embryo was liberated from the ZP by gently pipetting. Zona pellucida-intact, ZP-free embryos and their corresponding ZPs (in groups of 20-30 embryos or ZPs) were washed in HEPES-TALP-PVA loaded in 2.5 μL of HEPES-TALP-PVA, lysed in 25 μL of RNA lysis buffer (Arcturus, Mountain View, CA) and stored at -80°C until use.

Semen straws from the same bull (BN 354) utilized for *in vitro* production of embryos used for detection of FPr protein were removed from liquid nitrogen tank and placed in water at 36.7°C for 45 sec. Straw were then emptied on top of a discontinuous percoll density gradient (3 mL 45% percoll over 3 mL 90% percoll contained in a 15 mL conical tube) and sperm centrifuged at 760 x *g* for 15 min. Sperm pellet present at the bottom of the 90% fraction was transferred to 10 mL HEPES-TALP-PVA column and centrifuged at 200 x *g* for 8 min. Supernatant was removed and sperm pellet re-suspended in 100 μL of modified IVF-TALP for determination of sperm motility and concentration. Approximately 2,500,500 sperm were then lysed in 50 μL of RNA lysis buffer and stored at -80°C until use.

3.4.2. Isolation of RNA

Total RNA was isolated using the PicoPure RNA isolation Kit (Arcturus, Mountain View, CA) as per the manufacturer's instructions. Briefly, RNA was mixed with 70% ethanol by gently pipetting and the mixture was added to an RNA purification column. DNAse treatment was performed by addition and incubation of DNase I enzyme (Qiagen, Valencia, CA) 15 min at room temperature in the RNA purification columns. Several washes of the columns were performed previous to elution of RNA from the column by using 12 μL elution buffer.

As a positive control, total RNA from bovine tongue epithelium was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). Bovine tongue tissue was obtained from a fresh tongue in a local abattoir. Epithelial cells were obtained by slicing of bovine tongue epithelium in sections less than 0.5 mm thick. Collected tissue was immediately placed in RNA later (Qiagen, Valencia, CA). Tissue was left at 4°C overnight and kept at -80°C until use. To isolate RNA, 100 mg of tongue epithelium was placed in a 17 x 100 mm polypropylene tube containing 1 mL of Trizol and homogenized with a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH). Homogenized samples were incubated for 5 min at room temperature and then mixed with 0.2 mL of chloroform. Tubes were vigorously shaken by hand for 15 sec and incubated at room temperature for 15 min. Samples were centrifuged at 12,000 x *g* for 10 min at 4°C. Next, the aqueous phase was transferred to a new tube, and in which RNA was precipitated by the addition of 0.5 mL isopropyl alcohol and incubation of the samples at room temperature for 15 min. Samples were centrifuged at 12,000 x *g* for 10 min at 4°C, the supernatant removed and the

resulting RNA pellet washed once using 75% ethanol. Lastly, the RNA pellet was air-dried for 5 min, dissolved in 50 µL of nuclease free water and stored at -80°C until used.

3.4.3. Reverse transcription

Isolated RNA from bovine embryos and bovine tongue epithelium was DNAase treated and reversed transcribed into complementary DNA in a total volume of 20 µL. The reaction mixture consisted of 1X RT buffer, 5 mM $MgCl_2$, 1 mM of each dNTP, 0.25 nM random primers, 20 IU RNase inhibitor, and 100 IU moleney murine leukemia virus reverse transcriptase (MMLV-RT). The RT reaction was carried out at 25°C for 10 min, 42°C for 1 h followed by a denaturation step at 99°C for 5 min and flash cooling to 4°C. Controls for genomic DNA contamination were prepared by omitting reverse transcriptase enzyme in the RT reaction.

3.4.4. Real time PCR

Polymerase chain reaction was performed using the iCycler iQ™ Real-Time PCR detection system (Bio-Rad, Hercules, CA) and the iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA). The PCR reaction mixture consisted of 25 µL 1X iQ Supermix (100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, iTaq DNA Polymerase 50 units/ml, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers), 1 µM concentration of forward and reverse primers for FPr and β-actin (Table 1), nuclease free water, and cDNA template from each RT reaction. Negative control for each primer set consisted of PCR reaction mixture without the inclusion of cDNA (no template). Amplification of housekeeping gene actin (Rambeaud et al., 2006), were used as standard

Table 1. Details of primers used for RT-RT-PCR.

Gene	GenBank Accesion Number	Sense	Sequence	Location	Fragment Size (bp)
$PGF_{2\alpha}$ receptor	NM181025	Forward Reverse	5'-gcagaccaagcacagtgaaa-3' 5'-ctgacagccaaccacgtatg-3'	1487-1506 1618-1637	151
β-Actin	AY141970.1	Forward Reverse	5'-cggcattcacgaaactacct -3' 5'-gggcagtgatctctttctg-3'	855-875 978-997	143

positive controls for the PCR reaction. The Real Time PCR protocol included an initial step of 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Fluorescence data was acquired during the elongation step. Since the melting curve of PCR products is sequence specific, it is used to identify PCR products. Melting protocol was performed by holding temperature at 45°C for 60 sec and then heating from 45 to 94°C, holding at each temperature for 5 sec while monitoring fluorescence. Product identity was also confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis in 1X TBE (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) visualized using a gel doc apparatus (Bio-Rad, Hercules, CA) and by target sequencing (University of Tennessee Molecular Biology Core Facility). At least three replicates containing ZP-intact and ZP-free embryos were utilized in this experiment.

3.4.5. Tissue collection for Western blot

In vitro-derived embryos were sorted according to their developmental stage in compact morulae and blastocysts, whereas *in vivo*-derived (ethylene glycol or glycerol frozen-thawed embryos) embryos at compact morula and blastocyst stage were pooled together into one group. To avoid foreign protein contamination, embryos were washed at least 3 times in HEPES-TALP supplemented with 0.3% PVA instead of BSA. Embryos were lysed in 2% SDS Extraction Buffer as described by Morgan et al. (1993) with the addition of a protease inhibitor cocktail (Roche, Indianapolis, IN). Lysate was heated at 94°C for 3 min and sonicated for 1 min. Lastly, samples were stored at -80°C until use.

Tissue from corpus luteum (CL) was removed from ovaries obtained at a local abattoir. Tissue was cut in thin slices (less than 1 mm) with a scalpel blade and placed in

a 17x100 mm polypropylene tube (BD Biosciences, San Jose, CA). Extraction buffer with protease inhibitors was added to the tube and tissue homogenized in Tekmar's Tissumizer for approximately for 5 min or until no gross debris were observed. Homogenate was centrifuged through a BD Falcon cell strainer (BD Biosciences Discovery Labware, Bedford, MA) attached to a 50 mL conicle tube for 2 min at 700 x *g*, aliquoted and stored at -80°C until use.

Two straws of frozen-thawed semen from two bulls (same bulls used for in vitro production of embryos) were centrifuged at 760 x *g* for 15 min using a percoll density gradient (3 mL 45% percoll over 3 mL 90% percoll contained in a 15 mL conical tube). Sperm pellet at the bottom of the tube was transferred to 10 mL HEPES-TALP-PVA (0.3%) and centrifuged at 200 x *g* for 8 min. Supernatant was removed and sperm pellet re-suspended in 100 µL of HEPES-TALP-PVA (0.3%). A total of 100 µL from the sperm mixture was combined with 100 µL of extraction buffer, heated at 94°C for 5 min and stored at -80°C until use.

3.4.6. Western blot

Protein concentration from corpus luteum and embryos was determined using the NanoOrange Protein Quantitation Kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA) in a TBS-380 minifluorometer (Turner Biosystems, Sunnyvale, CA). Proteins in each sample were loaded and electrophoresed on a 12% SDS polyacrylamide gel using 1X SDS-Running Buffer (3.02 g Tris, 18.8 g Glycine, 10 ml 10% SDS, Qs to 1 lt) at 20 mAmp/100 V for 2.5 h. Electrophoresed proteins were electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA) using a semi dry

apparatus (Bio-Rad, Hercules, CA) at 12 constant volts for 25 min. Prestained SDS standards (Bio-Rad, Hercules, CA) and chemiluminescence standards (MagicMark XP Standards, Invitrogen, Carlsbad, CA) were loaded to confirm transfer to PVDF membrane and to serve as molecular weight standards when developing the western blot, respectively. As positive control, a crude protein extract from bovine corpus luteum was used to determine the presence of FPr protein. After electrotransfer, SDS gels were stained with 0.1% Coomassie Blue solution to evaluate protein transfer efficiency, whereas PVDF membranes were dried overnight at 4°C. Membranes were then re-hydrated in 100% methanol for a few seconds, washed twice in water and blotted. Proteins were blocked for 1 h at room temperature with 10% non-fat dried milk (NFDM) in PBS. Blots were washed 3 times at 10 min intervals in PBS with 0.05% Tween-20 (PBS-T). Blots were incubated with primary antibody (rabbit polyclonal IgG raised against mouse FP receptor, Cayman Chemical Inc, Ann Arbor, MI) for 1 h at room temperature at a dilution of 1:1000 in 2% NFDM in PBS and washed 3 times with PBS-T. Blots were then incubated with secondary antibody (Goat anti-rabbit IgG, Pierce, Rockford, IL) for 1 h at room temperature at a dilution of 1:1000 in 1% NFDM in PBS and washed 3 times with PBS-T. Blots were developed using a chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL) following manufacturer's instructions. In this study, at least 3 replicates of *in vitro*-derived morulae, 3 replicates of in vitro-derived blastocysts, and 2 replicates of *in vivo*-derived embryos were utilized to study FPr protein expression in bovine embryos.

4. Results

4.1. Time-specific effects of $PGF_{2\alpha}$ during in vitro embryo development

Incubation of pre-compacted embryos with 1 ng/ml of $PGF_{2\alpha}$ in the culture medium decreased the percentage of embryos reaching compaction 48 h later (PGF 1=55 ± 7.7 vs. CON=64 ± 7.7%; *P<0.05*; Figure 2). Whereas, culture of compacted embryos in medium containing 1 ng/ml of $PGF_{2\alpha}$ did not result in lowered blastocyst formation (PGF 1=57 ± 7.3% vs. CON=59 ± 7.3%; *P>0.05*; Figure 2).

4.2. FPr gene

Prostaglandin $F_{2\alpha}$ receptor transcripts were successfully amplified from ZP-intact and ZP-free compact morula and blastocyst stage bovine embryos (Figure 3 and 4), as well as from tongue epithelium (the positive control; Figure 4) and bovine sperm (Figure 4). In contrast, transcripts for FPr were not observed in mechanically removed ZPs. In addition, amplification of the housekeeping gene β-actin was observed in all samples including mechanically removed ZPs. DNA contamination was not evident as negative controls did not result in any of the genes used being amplified (no template and RT negative reactions; Figure 3 and 4). Amplification products were positively identified by melting curve profiles and ethidium-bromide gel electrophoresis (Figure 3 and 4).

4.3. FPr protein expression

Because gene expression does not always correlate with protein expression, western blots were performed on *in vitro* and *in vivo*-derived embryos. Western blot analysis confirmed the expression of FPr protein in *in vitro*-derived blastocysts stage

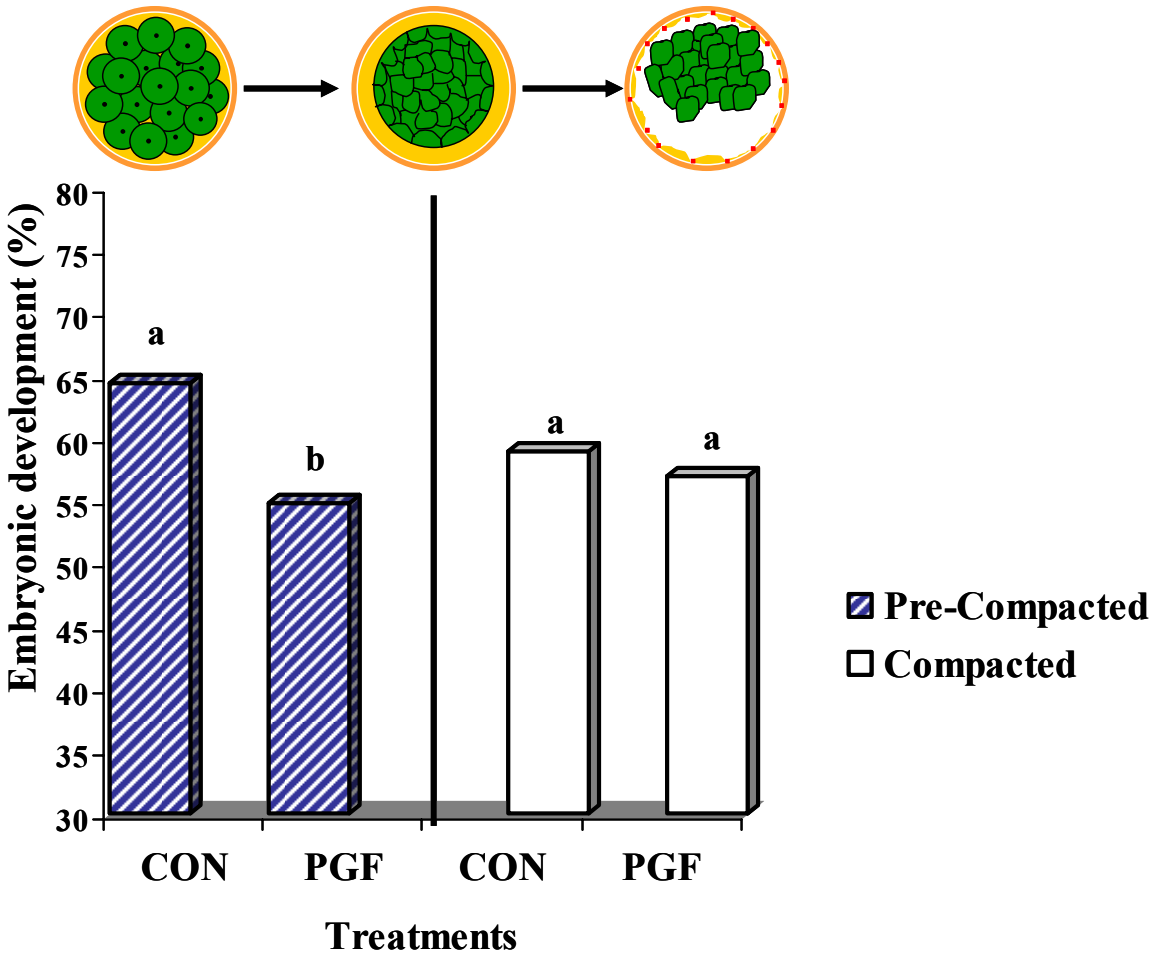


Figure 2. Embryonic development of pre-compacted (Experiment 1) and compacted (Experiment 2) embryos after 48 h culture in medium containing 1 ng/ml of prostaglandin $F_{2\alpha}$ or in control medium. Embryonic development of pre-compacted, but not compacted embryos was inhibited by addition of prostaglandin $F_{2\alpha}$ to the culture medium. [a.b]Values differ within treatments ($P<0.05$). Pooled standard errors were ± 7.7.

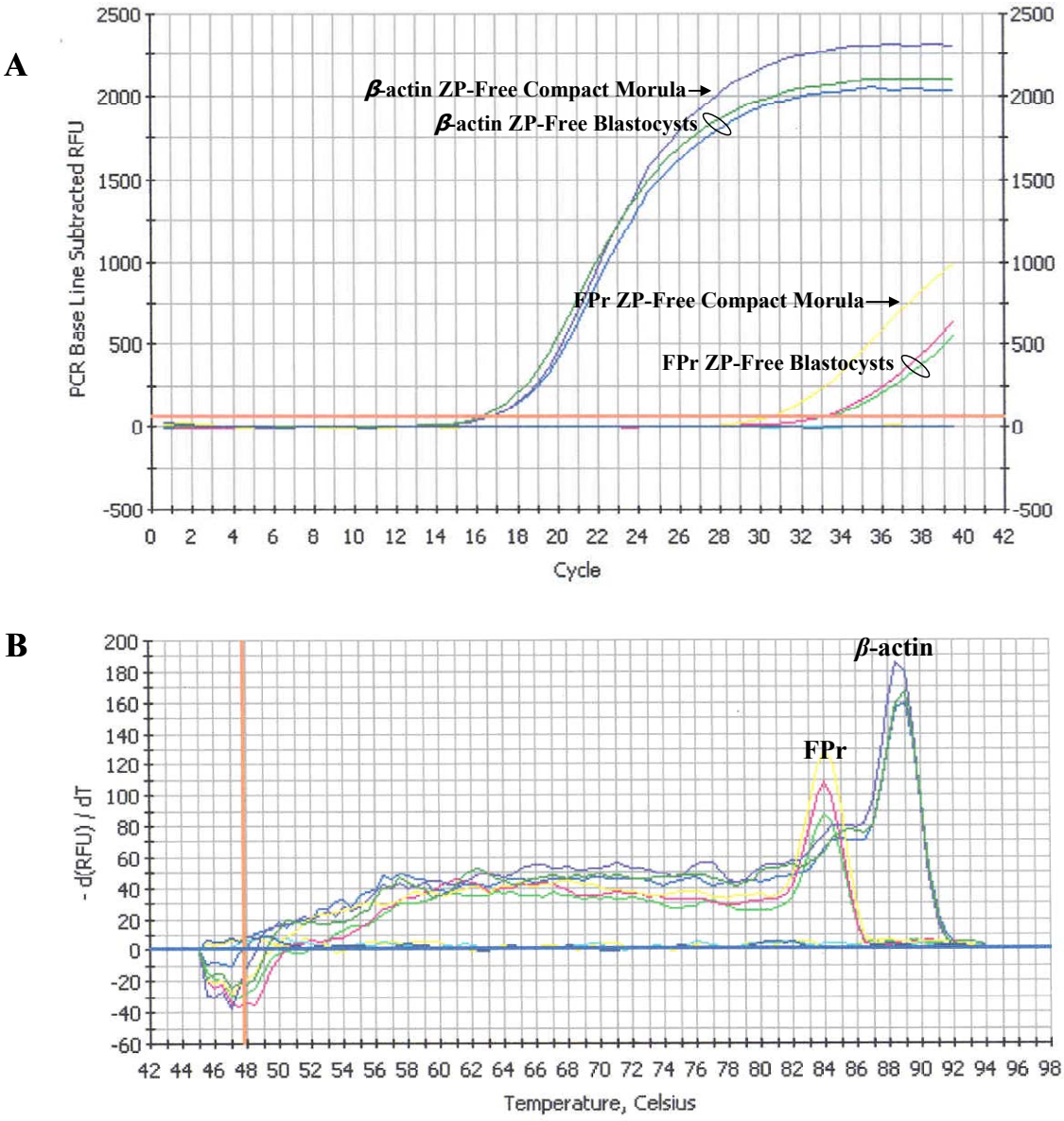


Figure 3. Real time RT PCR using SYBR Green I. A) Amplification curves for prostaglandin $F_{2\alpha}$ receptor (FPr) and β-actin transcripts in ZP-free and ZP-intact blastocysts and ZP-free compact morulae. Each embryo sample was used for amplification of FPr and β-actin transcripts; therefore each line represents an embryo sample. Controls for genomic DNA contamination for each of these embryo samples (omission of reverse transcriptase enzyme) showed no FPr amplification. B) Amplification products were positively identified by melting curve profile. Three replicates for ZP-intact and 3 replicates for ZP-free embryos at compact morula or blastocyst stage were utilized for determination of FPr transcripts in *in vitro*-derived embryos.

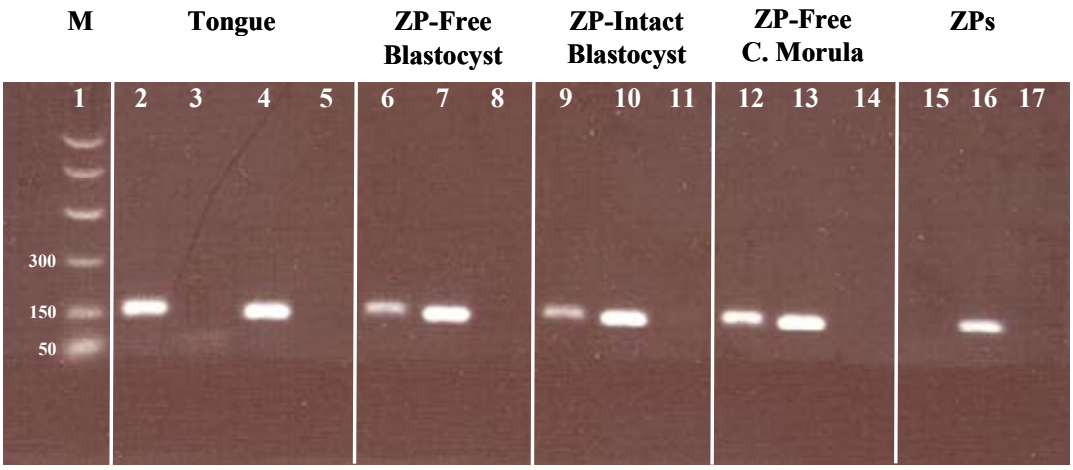


Figure 4. Ethidium bromide gel electrophoresis: Lane 1: PCR markers; lane 2: FPr in tongue epithelium (positive control); lane 3: FPr no template (negative control); lane 4: β-actin in tongue epithelium; lane 5: β-actin no template (negative control); lane 6: FPr in ZP-free blastocysts; lane 7: β-actin in ZP-free blastocysts; lane 8: FPr with RT negative reaction ZP-free blastocyst; lane 9: FPr in ZP-intact blastocyst; lane 10: β-actin in ZP-intact blastocyst; lane 11: FPr with RT negative reaction from ZP-intact blastocyst; lane 12: FPr in ZP-free compact morulae; lane 13: β-actin in ZP-free compact morulae; lane 14: FPr in RT negative from ZP-free compact morulae; lane 15: FPr in mechanically isolated zona pellucidas; lane 16: β-actin in mechanically isolated zona pellucidas; lane 17: FPr in RT negative from mechanically isolated zona pellucidas.

embryos, *in vivo*-derived (compact morulae and blastocysts) embryos, sperm, and corpus luteum samples (Figure 5). In addition, FPr was identified as approximately a 64 kDa protein. Pre-absorption of the primary antibody with its corresponding blocking peptide resulted in negative western blots which confirmed the specificity of the primary antibody to the FPr. Detection of FPr in *in vitro*-derived compact morulae was not evident.

5. Discussion

Several studies have indicated a negative effect of $PGF_{2\alpha}$ on embryonic development, hatching ability and embryo quality in different species (Maurer and Chenault, 1983; Breuel et al., 1993; Buford et al., 1996; Lemaster et al., 1999; Hockett et al., 2004). Moreover, Scenna et al., (2004) indicated that culture of pre-compacted *in vitro*-derived bovine embryos in medium containing $PGF_{2\alpha}$ decreased development to blastocyst. In the later study, time specific effects of $PGF_{2\alpha}$ on pre-compacted or compacted *in vitro*-derived embryos could not be determined because pre-compacted embryos were treated with $PGF_{2\alpha}$ until assessment of development to blastocyst. The objective of our initial experiment was to identify the period of time where *in vitro*-derived embryos are most susceptible to detrimental effects of $PGF_{2\alpha}$. In this study, addition of $PGF_{2\alpha}$ to the culture medium of pre-compacted bovine embryos reduced the ability of the embryos to undergo compaction. To the contrary, addition of $PGF_{2\alpha}$ to the culture medium of compacted embryos did not cause a reduction in blastocyst formation.

In a second experiment, the hypothesis that $PGF_{2\alpha}$ has a direct negative effect on embryos was strengthened by identification of FPr mRNA in ZP-free compact morula

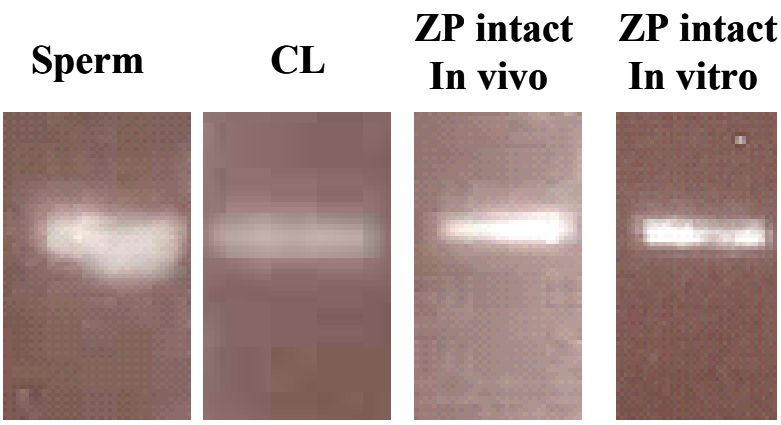


Figure 5. Western blot for FPr protein in bovine sperm, bovine corpus luteum (CL), zona pellucida (ZP) intact *in vivo*-derived bovine embryos, and zona pellucida (ZP) intact *in vitro*-derived bovine embryos.

and blastocyst stage embryos. Mechanical removal of the embryo from the zona pellucida was performed to avoid PCR amplification of $PGF_{2\alpha}$ transcripts associated with accessory sperm present in the zona pellucida (ZP) of the embryo (Nadir et al., 1993). In addition, Western blot analysis indicated that i*n vitro*-derived blastocysts and *in vivo*-derived (compact morulae and blastocysts) bovine embryos, semen samples, and corpus luteum (used as positive control) expressed FPr protein.

It is important to mention that amplification of FPr and β-actin transcripts were observed in bovine sperm samples. Furthermore, β-actin transcripts, but not FPr transcripts were amplified from mechanically isolated ZPs. These results suggest that amplification of β-actin transcripts from ZPs are a consequence of amplification of transcripts from accessory sperm present in the ZPs of *in vitro*-derived embryos. Lack of FPr mRNA from ZP samples may be explained by a lower amount of FPr transcripts compared to β-actin transcripts (a housekeeping gene) in the accessory sperm associated with ZP. The presence of mRNA in sperm was also correlated with the presence of FPr protein by Western blot.

Prostaglandin $F_{2\alpha}$ receptor protein expression in *in vitro*-derived compact morulae was not detectable; therefore, FPr gene expression did not correlate with its protein expression in *in vitro*-derived compact morulae. The fact that we did not detect FPr protein expression in compact morulae does not rule out the possibility that pre-compacted and compacted embryos express small amounts of FPr that under our experimental conditions were unable to be detected, but were sufficient enough to cause an effect of $PGF_{2\alpha}$ on the embryo. In addition, due to the lipophilic characteristics of prostaglandins (Bito, 1975) and the presence of prostaglandin transporters mediating

influx and efflux of prostaglandins to cells (Lu et al., 1996; Chan et al., 1998), negative effects of $PGF_{2\alpha}$ in pre-compacted embryos may also occur via none receptor-mediated events. This hypothesis agrees with a study performed by Scenna et al. (2004) in which culture of pre-compacted bovine embryos in medium containing 1, 10 or 100 ng/ml of $PGF_{2\alpha}$ decreased development to blastocyst; however, 1 ng/mL of $PGF_{2\alpha}$ had a more detrimental effect than addition of 10 ng/mL (Scenna et al., 2004), suggesting that effects of $PGF_{2\alpha}$ did not involve a receptor-mediated event. Therefore, non receptor-mediated effects of $PGF_{2\alpha}$ on the embryo may explain in part the negative effects of $PGF_{2\alpha}$ on *in vitro* development of pre-compacted embryos observed in our initial experiment.

Moreover, lack of $PGF_{2\alpha}$ effects on development of compact morulae to blastocyst stage suggests that once the embryo compacts, $PGF_{2\alpha}$ effects on the embryo ceases. However, due to the fact that previous studies have indicated a reduction on hatching rates of compacted bovine embryos with $PGF_{2\alpha}$ (Scenna et al., 2004) and that only a very low number of *in vitro*-derived embryos usually hatch from the zona pellucida, negative effects of $PGF_{2\alpha}$ on hatching may still be possible.

Until now, two isoforms for the FPr protein have been identified in ruminants; the original FP isoform named FP_A and a newer isoform generated by alternative mRNA splicing named FP_B (Pierce et al., 1997; Iwamoto et al., 1999). The molecular weight of FPr protein presented in this study was approximately 64 kDA, which agrees with several other studies showing expression of a 64 kDA FPr protein in bovine ocular tissues (Ansari et al., 2004; Kaddour-Djebbar et al., 2005; Thieme et al., 2006). However, the FPr molecular weight reported in our study differs from other studies in humans (Abramovitz et al., 1994) and bovines (Sakamoto et al., 1995) after cloning of FPr in

those species. The aforementioned studies estimated a molecular weight of 40,060 and 40,983 Daltons for the human and bovine FPr, respectively. In agreement with these authors, Al-Matubsi et al. (2001) demonstrated the presence of a 43 kDa FPr in rat myometrium by Western blotting.

In conclusion, our results indicated that direct effects of $PGF_{2\alpha}$ on *in vitr*o-derived embryos occur before or during compaction, but the possibility of $PGF_{2\alpha}$ affecting hatching of blastocyst from the zona pellucida still remains. In addition, to our knowledge, we have demonstrated for the first time the existence of FPr mRNA (in *in vitro*-derived compact morulae and blastocyst) and protein (in *in vitro*-derived blastocysts and *in vivo*-derived pools of morulae and blastocysts) in bovine embryos. These findings suggest that $PGF_{2\alpha}$ can have a direct negative effect on development of bovine embryos by receptor and non receptor-mediated events.

CHAPTER 4

DETRIMENTAL EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ ON *IN VITRO* EMBRYO DEVELOPMENT IN BOVINE ARE INHIBITED BY A RECEPTOR ANTAGONIST

1. Abstract

Numerous studies have demonstrated negative effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on bovine reproduction. Moreover, previous data from our laboratory indicated that *in vitro*-derived pre-compacted embryos are more susceptible to the effects of $PGF_{2\alpha}$ than compacted embryos. Presence of prostaglandin $F_{2\alpha}$ receptors (FPr) in bovine embryos will allow for new therapeutic strategies aimed at improving reproduction in bovines. Therefore, in Experiment 1, two studies were performed to investigate any toxic or negative effect of AL-8810, an FPr antagonist, on *in vitro* development of bovine embryos. In this study, pre-compacted embryos were culture in: 1) 25 AL (25 nM AL-8810 in KSOM-PVA; n= 94); 2) 50 AL (50 nM AL-8810 in KSOM-PVA; n= 94); 3) 100 AL (100 nM AL-8810 in KSOM-PVA; n= 94); and 4) CON (KSOM-PVA; n= 95). Subsequently, pre-compacted embryos were cultured in: 1) 250 AL (250 nM AL-8810 in KSOM-PVA; n=274); 2) 500 AL (500 nM AL-8810 in KSOM-PVA; n=274); 3) 1000 AL (1000 nM AL-8810 in KSOM-PVA; n= 282); and 4) CON (KSOM-PVA; n= 278). In Experiment 1, embryos remained in their treatments until blastocyst assessment. Next, in Experiment 2, the efficacy of AL-8810 on preventing detrimental effects of $PGF_{2\alpha}$ on pre-compacted embryos was investigated. In this experiment, pre-compacted embryos

were cultured in: 1) 100 AL (100 nM AL-8810 in KSOM-PVA; n= 121); 2) 10 PGF (10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=91); 3) 100 AL + PGF (100 nM AL-8810 and 10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=116); and 4) CON (KSOM-PVA; n= 96). Subsequently, pre-compacted embryos were cultured in: 1) 1000 AL (1000 nM AL-8810 in KSOM-PVA; n= 87); 2) 10 PGF (10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=87); 3) 1000 AL + PGF (1000 nM AL-8810 and 10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=84); and 4) CON (KSOM-PVA; n= 84). In Experiment 2, embryos remained in their treatments for approximately 48 h when development to morula was assessed. Results showed that addition of 25, 50, 100 nM did not compromise embryonic development to blastocyst compared to controls (61%, 56%, 55%, and 50% respectively). In addition, orthogonal contrasts indicated that 100 nM AL-8810 improved development to blastocyst (100 AL= 61% vs. CON= 50%, *P=0.01*). Culture of embryos with 250, 500, 1000 nM AL-8810 did not affect development to blastocyst (35%, 39% and 40%, respectively) when compared to controls (38%). Finally, addition of 1000 nM AL-8810, but not 100 nM, to culture medium of pre-compacted embryos exposed to $PGF_{2\alpha}$ increased the ability of embryos to undergo compaction 48 h later (1000 AL + PGF=51% vs. PGF=40%; *P=0.05*). In conclusion, AL-8810 at a concentration of 1000 nM inhibits detrimental effects of $PGF_{2\alpha}$ on development of pre-compacted bovine embryos and may prove beneficial for other assisted reproductive techniques in cattle.

2. Introduction

Inadequate reproductive efficiency has a tremendous impact in the economy of dairy and beef producers. Moreover, early embryonic death in cattle represents 75 to 80%

of all reproductive losses between fertilization and calving (Sreenan and Diskin, 1983). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a derivate of arachidonic acid (Flint et al., 1986) and the primary luteolytic hormone in ruminants (Goding, 1974; Braden et al., 1988; Okuda et al., 2002) has been shown to decrease embryo quality and development and to lower pregnancy rates in cows (Seals et al., 1998; Lemaster et al., 1999; Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005). In addition, $PGF_{2\alpha}$ decrease embryonic development of rabbit, rat and bovine embryos *in vitro* (Maurer and Beier, 1976; Breuel et al., 1993; Scenna et al., 2004).

The FPr is a seven transmembrane protein receptor coupled to a specific G protein (Gq) mediating the formation of inositol triphospate (IP_3) and diacylglycerol (DAG), second messengers (Coleman et al., 1994) leading to an increase in intracellular calcium levels and activation of protein kinase C (Wiltbank et al., 1989; Wiltbank et al., 1989; Wiltbank et al., 1990). Downstream signaling are not well understood, but an involvement of a Raf/MEK/MAPK signaling cascade followed by regulation of gene transcription has been suggested by Chen et al. (1998). Moreover, several isoforms of the FPr receptor have been characterized in bovine and ovine luteal cells (Ezashi et al., 1997; Pierce et al., 1997; Ishii and Sakamoto, 2001; Sakamoto et al., 2002). A selective antagonist for the FPr, AL-8810, has been well characterized by Griffin et al. (1999). This antagonist has been shown to inhibit calcium mobilization and MAP kinase activation induced after stimulation with several $PGF_{2\alpha}$ agonists (Sharif et al., 2001; Kelly et al., 2003; Sharif et al., 2003).

Recent studies from our laboratory have demonstrated the presence of prostaglandin $F_{2\alpha}$ receptor (FPr) mRNA and protein in bovine embryos (Scenna et al.

2006). Presence of FPr in bovine embryos allows for the development of new therapeutic strategies to improve embryo quality, development and survival in cows, which will result in better reproductive efficiency and higher monetary earnings to the cattle industry. Therefore, the objectives of this study were to 1) determine if AL-8810 possesses any toxic effect for *in vitro* culture of bovine embryos, and 2) to inhibit detrimental effects of $PGF_{2\alpha}$ on *in vitro* development of bovine embryos.

3. Experimental procedures

3.1. Materials

Dimethyl sulphoxide (DMSO), polyvinyl alcohol (PVA), bovine serum albumin (BSA), percoll, penicillamine, hypotaurine, epinephrine and the majority of reagents used for *in vitro* production of bovine embryos were purchased from Sigma Chemical, Inc. (St. Louis, MO). Tissue culture medium-199, gentamicin, and penicillin-streptomycin were purchased from Specialty Media, Inc. (Phillipsburg, NJ). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Folltropin-V and Vigro holding Plus were purchased from Bioniche Life Sciences, Inc. (Belleville, ON). Ovaries were purchased from Brown's Packing Plant (Gaffney, SC). Media (HEPES-TALP, IVF-TALP, and SPERM-TALP; Parrish et al., 1988) and KSOM (Edwards et al., 2005), were prepared in the laboratory. Sperm characteristics were analyzed using a computerized sperm analyzer (CASA) from Hamilton Thorne Biosciences (Beverly, MA).

3.2. General procedures for in vitro production of bovine embryos

3.2.1. Oocyte collection

The procedures utilized for *in vitro* production of embryos (IVP) were modifications of procedures previously described by Lawrence et al. (2004). Ovaries obtained from an abattoir were packaged in thermoses and contained within a cooler during air transport to the laboratory. Upon arrival to the laboratory, ovaries were immediately washed with warm tap water equilibrated to arrival temperature of ovaries (generally between 28 to 30 C°). Excess of tissue surrounding ovaries was removed utilizing a scissor and ovaries were washed with warm tap water an additional time.

For oocyte recovery, ovaries were held firmly by clamping the base of the ovary with a hemostat and checkerboard incisions were made across follicles (approximately 3-8 mm in diameter) using a scalpel blade. Ovaries were vigorously washed in oocyte collection medium (OCM) in order to remove cumulus oocyte complexes (COCs) contained within the follicles. Collection medium was filtered and rinsed using an Emcon Filter unit (Vet Concepts, Spring Valley, WI) until medium was clear. Medium containing COCs was poured into a gridded culture dish where searching of oocytes was accomplished. Cumulus oocyte complexes were transferred to an "X" plate containing OCM and washed four times to eliminate cellular debris. Cumulus oocyte complexes of good quality were washed in oocyte maturation medium (OMM) and placed in groups of approximately 50 COCs per well in a 4-well plate containing 500 µL OMM. Maturation of oocytes was performed in an incubator at 5.5% CO_2 in air at 38.5° C until time of fertilization (approximately 24 h after placement in OMM). Maturation medium was equilibrated in the incubator (5.5% CO_2 in air at 38.5°C) the day before oocyte collection.

3.2.2. Sperm preparation and in vitro fertilization

After oocyte maturation (22-24 h in OMM in an incubator at 5.5% CO_2 in air at 38.5°C), OMM from each well was carefully removed and 25 µl of penicillamine/hypotaurine/epinephrine (PHE) and 500 µL of fertilization medium (IVF-TALP) were added to each well. Two straws of semen from two bulls (140 Se5, 140 Se55, 30 SM002, CG30, SM 101), known to have high motility as well as producing high cleavage and blastocyst percentages were used to fertilize oocytes for every replicate of the study. Briefly, semen straws were removed from liquid nitrogen tank and placed in water at 36.7°C for 45 sec. Each straw was then emptied on top of a discontinuous Percoll density gradient (3 mL 45% percoll over 3 mL 90% percoll contained in a 15 mL conical tube) and sperm was centrifuged at 760 g for 15 min in order to remove excess extender, debris, and nonmotile sperm prior to fertilization. Sperm pellet present at the bottom of the 90% fraction was collected and transferred to 10 mL SPERM-TALP and centrifuged at 200 g for 8 min. Supernatant was removed and sperm pellet re-suspended in 100 µL of modified IVF-TALP. Sperm concentration and motility were determined using a CASA and sperm was added to each well at a final concentration of 500,000 motile sperm per well. Lastly, oocytes and sperm were incubated at 5.5% CO_2 in air at 38.5°C for 18-22 h.

3.2.3 In vitro culture

Approximately 18-22 h post fertilization, putative zygotes (PZ) were denuded of cumulus cells by vortexing. Putative zygotes were transferred to a 2 mL centrifuge tube

containing 50 μL of HEPES-TALP and vortexed for 5 min. Recovered putative zygotes were washed 4 times in HEPES-TALP before transferring groups of approximately 40-50 zygotes to each well of a 4-well plate containing 500 μL of KSOM culture medium per well. Zygotes were placed in a humidified atmosphere of 5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C until desired stage of development and initiation of respective treatments.

3.3. Experiment 1: Determination of AL-8810 dose toxicity on in vitro embryo development

AL-8810 is an 11β-fluoro analog of $PGF_{2\alpha}$ which acts as a potent and selective antagonist at the FP receptor. This compound has weak intrinsic activity on FPr in the 200-300 nM range. However, it fully antagonizes the activity of the potent FPr agonist fluprostenol at these concentrations (Griffin et al., 1999). At a concentration of 1 to 2 μM, AL-8810 inhibits several potent agonists at the cloned human ciliary body (Sharif et al., 2002). In addition, AL-8810 does not inhibit the activity of prostaglandin E, D, I receptors (Crider et al., 1998; Crider et al., 1998; Crider et al., 1999) suggesting a selective antagonism at the FP receptor.

The effects of AL-8810 during *in vitro* embryo development in cattle have not been studied. Therefore, two preliminary experiments were performed to determine possible negative effects (or toxicity) of AL-8810 on embryonic development of *in vitro*-produced embryos cultured in KSOM-PVA (3 mg/mL, 0.3%) alone or in KSM-PVA with the addition of 6 different concentrations of AL-8810.

3.3.1. Preparation of AL-8810 stock and addition to the culture medium

Prior to the beginning of each experiment, the content of a vial containing 10 mg of AL-8810 (Cayman Chemical Inc., Ann Arbor, MI; catalog# 16735) was reconstituted and solubilized in DMSO (Primary Stock, 1000 μM AL-8810). Aliquots of 1 ml of primary stock solution were stored at -80C° until needed. For each replicate, the primary stock solution was diluted 1:10 in KSOM-PVA (Secondary Stock, 100 μM AL-8810) and added to each experimental well (500 μl of equilibrated KSOM-PVA) to obtain final concentrations of 25, 50, 100, 250, 500, and 1000 nM AL-8810. Control experimental wells were prepared by adding the same concentration of DMSO (0.1%) used to prepare the highest concentration of AL-8810 in KSOM-PVA.

3.3.2. Experimental design for Experiment 1

Procedures for oocyte collection, maturation, fertilization and embryo culture were previously described under general methods. Pre-compacted 16- to 32-cell stage embryos (approximately 97 h post IVF) were rapidly transferred to HEPES-TALP, evaluated for quality (normal shape, defined blastomeres, extruded blastomeres, cytoplasmic fragmentation, even cytoplasm), evenly sorted according to their quality, washed rapidly in KSOM-PVA, and transferred to experimental treatments in a 4-well plate. Experimental plates were placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for 3 additional days when embryo development was determined using IETS guidelines for classification of bovine embryos (Stringfellow and Seidel, 1998). Data for development to blastocyst were analyzed using a randomized block design model with replicate (a 4-well plate with each of its wells containing one treatment; a group of

embryos in each well was considered an experimental unit) as random effect and treatment as fixed effect performing mixed procedures of SAS software (SAS 9.1, SAS Institute Inc., Cary, NC). In addition, orthogonal contrasts were performed to determine differences on development to blastocyst among treatments.

A total of 5 replicates were utilized to investigate the effects of 25, 50, 100, and 0 nM of AL-8810 on *in vitro* embryo development. In each of these replicates, pre-compacted embryos at 16- to 32-cell stage received one of the following culture treatments: 1) 25 AL (25 nM AL-8810 in KSOM-PVA; n= 94); 2) 50 AL (50 nM AL-8810 in KSOM-PVA; n= 94); 3) 100 AL (100 nM AL-8810 in KSOM-PVA; n= 94); and 4) 0 CON (KSOM-PVA; n= 95; Figure 6).

A total of 11 replicates were used to investigate the effects of 250, 500, 1000, and 0 nM of AL-8810 on *in vitro* embryo development. In each of these replicates, pre-compacted embryos at 16- to 32-cell stage received one of the following culture treatments: 1) 250 AL (250 nM AL-8810 in KSOM-PVA; n=274); 2) 500 AL (500 nM AL-8810 in KSOM-PVA; n=274); 3) 1000 AL (1000 nM AL-8810 in KSOM-PVA; n= 282); 0 CON (KSOM-PVA; n= 278; Figure 7).

3.4. Experiment 2: Does AL-8810 prevent detrimental effects of $PGF_{2\alpha}$ on in vitro produced embryos?

3.4.1 Preparation of prostaglandin $F_{2\alpha}$ stocks

Prior to the beginning of each experiment, prostaglandin $F_{2\alpha}$ (1 mg; Cayman Chemical Inc., Ann Arbor, MI; catalog# 16010) was solubilized in 10 mL of DMSO each

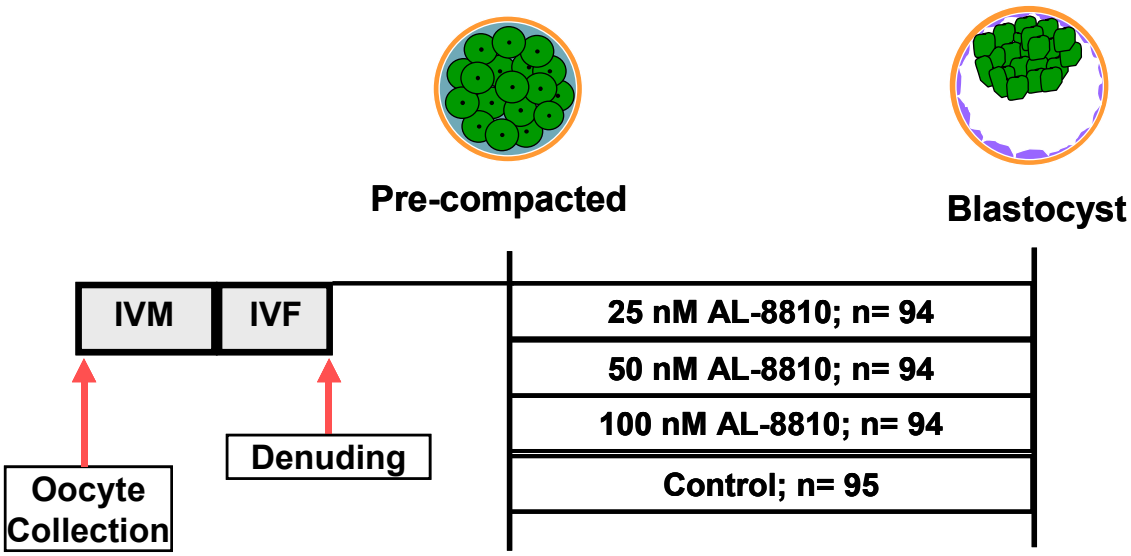


Figure 6. Experimental design for determination of 25, 50, and 100 nM AL-8810 toxicity on *in vitro* embryo development of bovine embryos. Treatments were 25 nM AL-8810 (25 AL), 50 nM AL-8810 (50 AL), 100 nM AL-8810 (100 AL), and Control.

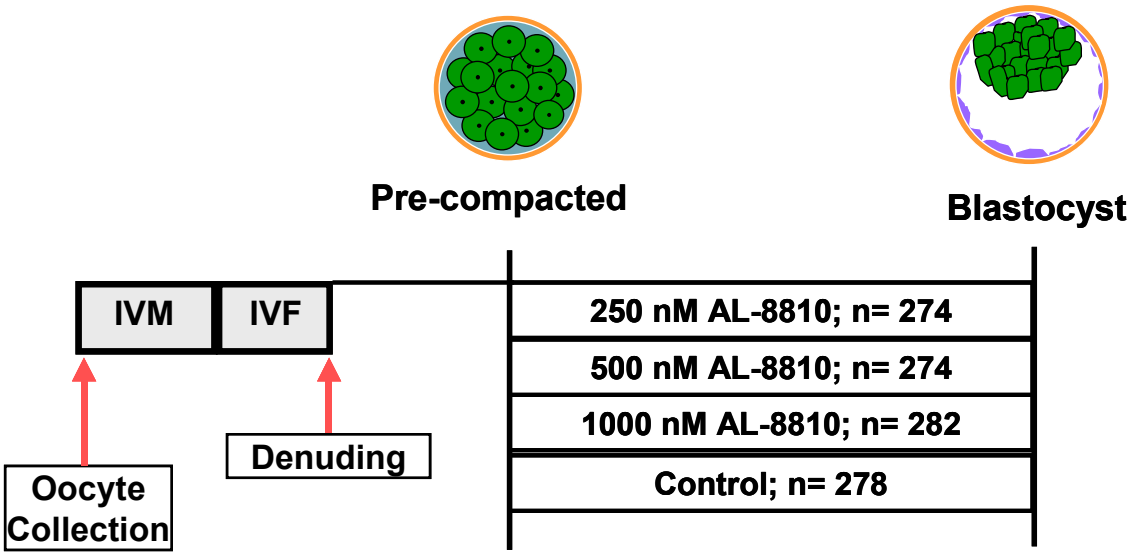


Figure 7. Experimental design for determination of 250, 500, and 1000 nM AL-8810 toxicity on *in vitro* embryo development of bovine embryos. Treatments were 250 nM AL-8810 (250 AL), 500 nM AL-8810 (500 AL), 1000 nM AL-8810 (1000 AL), and Control.

(Stock #1; 100,000 ng/mL). Then, 30 µL aliquots were stored at -80C° until needed. Based on manufacturer's instructions, once $PGF_{2\alpha}$ has been reconstituted in DMSO, the storage life of $PGF_{2\alpha}$ at -80°C is 6 months. For each replicate of the experiment, 12.5 µL of $PGF_{2\alpha}$ stock #1 were combined with 987.5 µL of KSOM-PVA (stock #2; 1.25 ng of $PGF_{2\alpha}$ per µL). Next, 4 µL of stock #1 were added to each experimental well (500 µL of equilibrated KSOM-PVA or 500 µl of 100 nM AL-8810) to obtain a final concentration of 10 ng/mL of $PGF_{2\alpha}$ in the culture medium. It is important to mention that $PGF_{2\alpha}$ was added at least 30 min after the embryos were placed in 100 nM or 1000 nM AL-8810 to allow the interaction of the FPr antagonist with its receptors at the plasma membrane. Four-well plates containing 500 µL of KSOM-PVA in each well were equilibrated in an incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for at least 12 h before initiation of treatments.

3.4.2. Experimental design for Experiment 2

Previous data from our laboratory demonstrated that addition of $PGF_{2\alpha}$ to the culture medium of pre-compacted *in vitro* produced embryos reduced the ability of embryos to undergo compaction. On the other hand, exposure of compacted *in vitro* produced embryos to $PGF_{2\alpha}$ did not compromise the ability of embryos to reach blastocyst stage (Scenna et al., 2006). Therefore, two experiments were performed to determine the ability of AL-8810 on preventing detrimental effects of $PGF_{2\alpha}$ on *in vitro* produced pre-compacted embryos.

Procedures for oocyte collection, maturation, fertilization and embryo culture were previously described under general methods. Embryos at the 16- to 32-cell were

rapidly transferred to HEPES-TALP, evaluated for quality (normal shape, defined blastomeres, extruded blastomeres, cytoplasmic fragmentation, even cytoplasm), evenly sorted according to their quality, washed rapidly in KSOM-PVA, and transferred to experimental treatments in a 4-well plate.

A total of 5 replicates were used to evaluate effects of 100 nM AL-8810 on embryonic development of embryos cultured in medium containing $PGF_{2\alpha}$. Pre-compacted embryos at 16- to 32-cell embryos received one of the following culture treatments: 1) 100 AL (100 nM AL-8810 in KSOM-PVA; n= 121); 2) PGF-10 (10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=91); 3) 100 AL + PGF-10 (100 nM AL-8810 and 10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=116); 4) CON (KSOM-PVA; n= 96; Figure 8). Subsequently, a total of 3 replicates were performed to determine the effects of 1000 nM AL-8810 on embryonic development of pre-compacted embryos exposed to $PGF_{2\alpha}$. In this study, 16-32-cell embryos were cultured in: 1) 1000 AL (1000 nM AL-8810 in KSOM-PVA; n= 87); 3) PGF-10 (10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=87); 3) 1000 AL + PGF-10 (1000 nM AL-8810 and 10 ng/mL of $PGF_{2\alpha}$ in KSOM-PVA; n=84); 4) CON (KSOM-PVA; n= 84; Figure 9). It is important to mention that $PGF_{2\alpha}$ was added at least 30 min after the embryos were placed in 1000 nM AL-8810 to allow the interaction of the FPr antagonist with its receptors at the plasma membrane.

In each of these two experiments, experimental plates were placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for 48 h and embryo development to compact morula was determined using IETS guidelines for classification of bovine embryos. Collected data were analyzed by using mixed procedures of SAS software (SAS 9.1, Cary, NC). Data for development to compact morula were analyzed using a

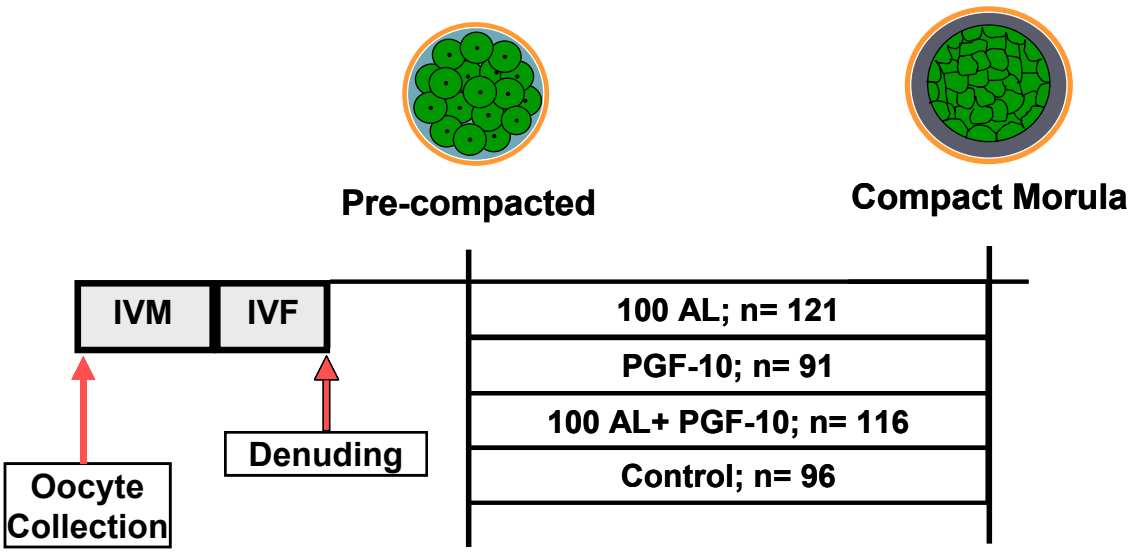


Figure 8. Experimental design for evaluation of AL-8810 efficacy on inhibiting prostaglandin $F_{2\alpha}$ effects on pre-compacted bovine embryos. Treatments were 100 nM AL-8810 (100 AL), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF-10), 100 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$, (100 AL + PGF-10) and control.

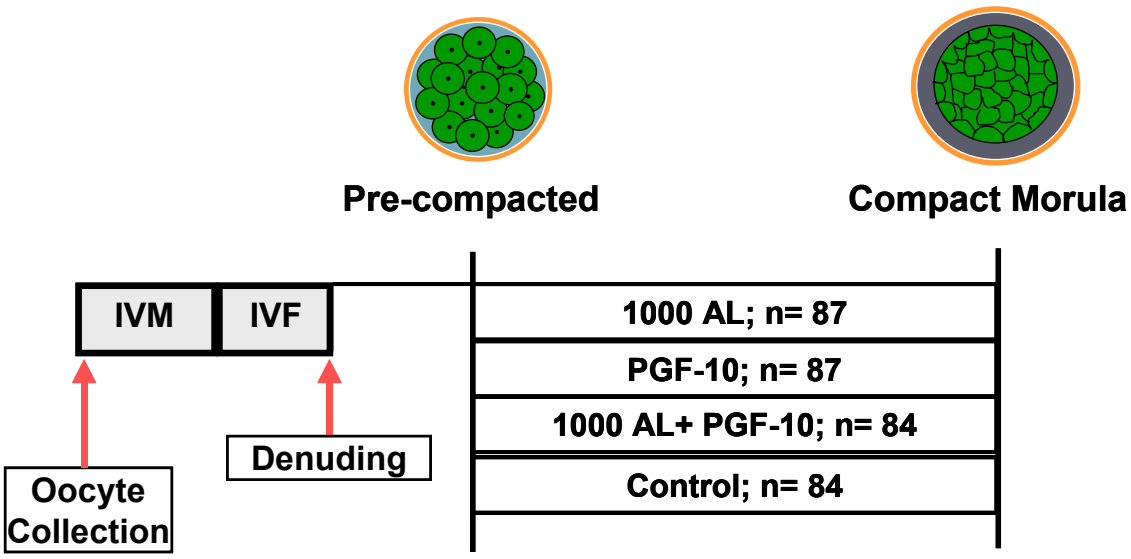


Figure 9. Experimental design for evaluation of AL-8810 efficacy on inhibiting prostaglandin $F_{2\alpha}$ effects on pre-compacted bovine embryos. Treatments were 1000 nM AL-8810 (1000 AL), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF-10), 1000 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$ (1000 AL + PGF-10) and control.

randomized block design model with replicate (a 4-well plate with each of its wells containing one treatment; a group of embryos in each well was considered an experimental unit) as random effect and treatment as fixed effect performing mixed procedures of SAS software (SAS 9.1, SAS Institute Inc., Cary, NC). In addition, orthogonal contrasts were performed to determine differences on development to compact morula stage among treatments.

4. Results

Data from Experiment 1 indicated that culture of pre-compacted *in vitro* produced embryos in medium containing 25, 50, and 100 nM of the specific FPr antagonist AL-8810, did not have a negative (or toxic) effect on embryonic development when compared to embryos cultured in control medium (61%, 56 %, 55%, and 50%, respectively; Figure 10). Moreover, addition of AL-8810 at a concentration of 100 nM to the culture medium increased blastocyst formation when compared to control medium (61% vs. 50%, respectively, *P=0.01*). Similarly, addition of AL-8810 to the culture medium at a concentration of 250, 500, and 1000 nM did not cause any detrimental effects on the ability of embryos to reach blastocyst stage when compared to embryos cultured in control medium (40%, 39%, 35% and 38%, respectively, Figure 11).

In Experiment 2, no differences in embryonic development of pre-compacted embryos to morula stage between 100 AL, PGF-10, 100 AL+PGF and CON groups were observed (55%, 44%, 55%, and 51%; respectively; Figure 12). In addition, orthogonal contrast showed a strong tendency of 100 AL + PGF-10 and 100 AL to increase development to compact morula when compared to embryos treated with $PGF_{2\alpha}$ (55% for

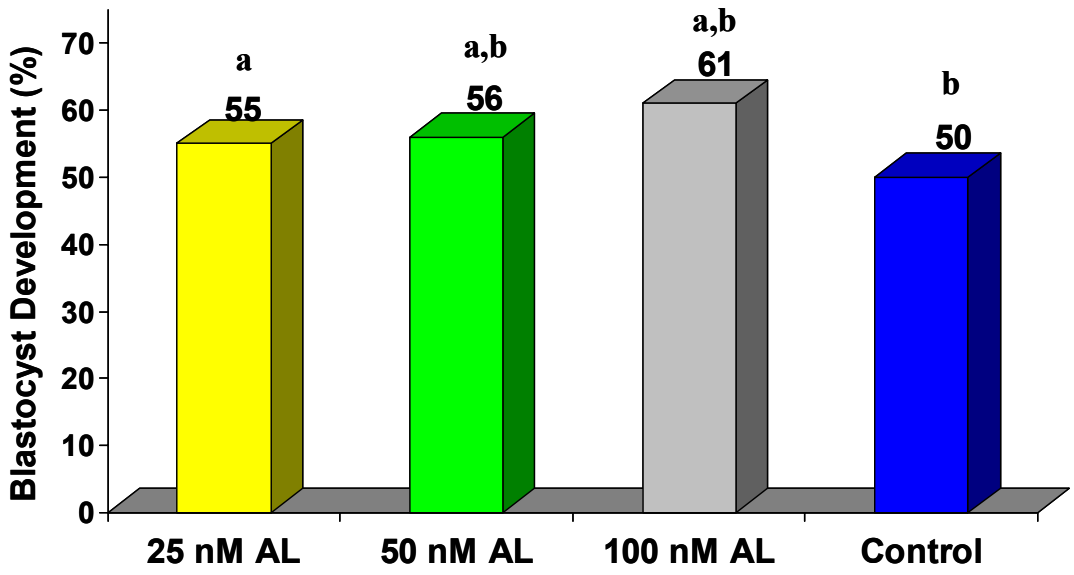


Figure 10. Percentage of pre-compacted embryos that continued development to blastocyst in Experiment 1 after culture in medium containing 25, 50, 100 nM AL-8810 or control medium. [a,b] Values with different superscripts differ; *P<0.005*. Standard errors were ± 5.2 100 nM AL-8810, 25 nM AL-8810 and control, and ± 5.34 for 50 nM AL-8810.

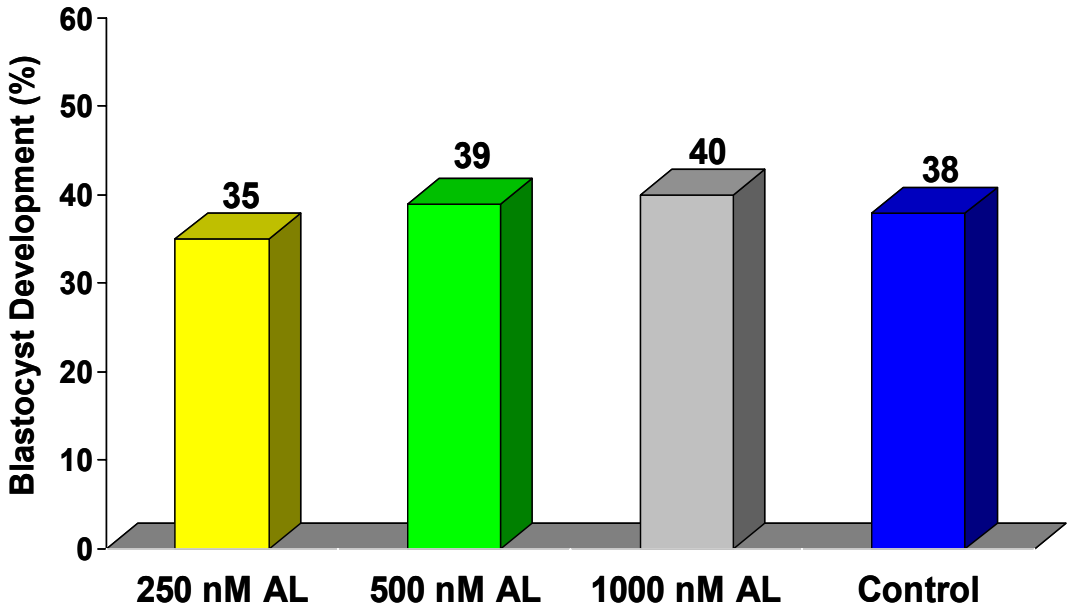


Figure 11. Percentage of pre-compacted embryos that continued development to blastocyst in Experiment 1 after culture in medium containing 250, 500, 1000 nM AL-8810 or control medium. Standard errors were ± 3.63 for each treatment.

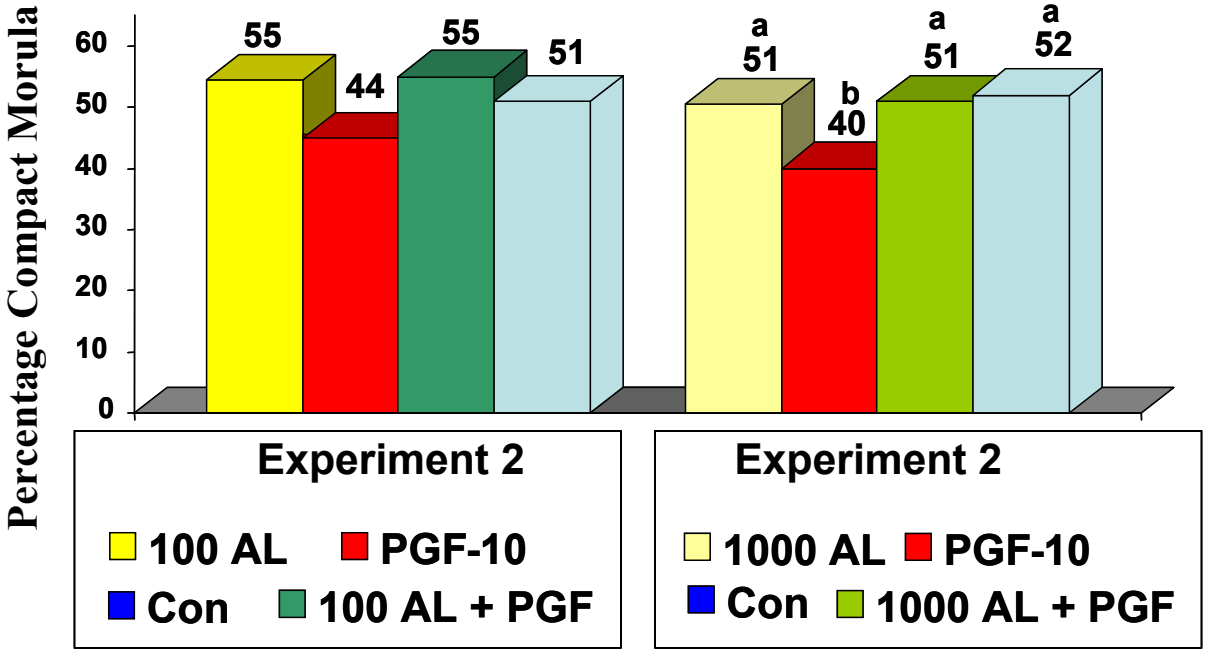


Figure 12. Percentage of pre compacted embryos that continued development to compact morula in Experiment 2. Treatments in Experiment 2a included: 100 nM AL-8810 (100 AL), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF-10), 100 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$ (100 AL + PGF-10), and control (Con). Treatments in Experiment 2b were: 1000 nM AL-8810 (1000 AL), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF-10), 1000 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$ (1000 AL + PGF-10), and control (Con). ^{a,b}Values differ between treatments; *P=0.05*. Standard errors in Experiment 2a were ± 4.8, 4.8, 4.8, and 5.3 for 100 AL, PGF-10, 100 AL + PGF-10, and Con, respectively. Standard errors in Experiment 2b were ± 4.6, 4.6, 5, and 4.6 for 1000 AL, PGF-10, 1000 AL + PGF-10 and Con, respectively.

both treatments vs. 44% in PGF-10). In Experiment 2, no differences on development to morula stage were observed between 1000 AL, 1000 AL+PGF and CON (51%, 51% and 52%, respectively; *P>0.05*; Figure 12). However, previous exposure of embryos to AL-8810 before addition of $PGF_{2\alpha}$ to the treatment well improved embryonic development to compact morula (1000 AL+PGF=51% vs. PGF=40%; *P<0.05*; Figure 12).

5. Discussion

Experiments 1 indicated that addition of various concentrations of an FPr antagonist, AL-8810, to the culture medium of *in vitro*-produced embryos did not result in lowered embryonic development. Furthermore, AL-8810 at a concentration of 100 nM in the culture medium improved the percentage of pre-compacted embryos reaching blastocyst stage. Since our culture system consisted in modified KSOM medium without the addition of animal proteins (bovine serum albumin), the positive effects of 100 nM of AL-8810 on embryonic development when compared to control medium was relatively unexpected. Several studies have indicated that embryos from different species are able to synthesize a wide range of prostaglandins (Shemesh et al., 1979; Hyland et al., 1982; Stone et al., 1986; Harper et al., 1989; Sayre and Lewis, 1993). Even though the presence of cyclooxygenase-1 (COX-1) cyclooxygenase-2 (COX-2) enzymes in bovine embryos has not been determined, studies in other species indicated that early stage embryos express these enzymes and are able to produce prostaglandins. For example, human embryos at early stage of development predominantly express COX-1, whereas 8-cell to blastocyst stage embryos express mostly COX-2 (Wang et al., 2002). Similarly, Parr et al. (1988) and Tan et al. (2005) demonstrated the presence of COX-1 and COX-2 in rat and

mouse embryos, respectively. *In vitro*-produced bovine embryos may be able to secrete discrete amounts of $PGF_{2\alpha}$ to the culture medium, which in turn could have a negative effect on embryonic development. This may explain in part the beneficial effects observed on embryonic development after culturing embryos in medium containing 100 nM of AL-8810 when compared to control medium.

In Experiment 2, previous exposure of pre-compacted embryos to the FPr antagonist AL-8810 at a concentration of 1000 nM in the culture medium inhibited detrimental effects of $PGF_{2\alpha}$ on embryonic development to morula stage. Similarly, several *in vitro* studies showed the efficacy and inhibitory actions of AL-8810 on inhibiting $PGF_{2\alpha}$ effects after binding to its receptors. For example, previous exposure of mouse fibroblast to AL-8810 abolished the production of IP_3 associated with FPr stimulation by FPr agonist (fluprostenol) or its endogenous ligand ($PGF_{2\alpha}$) (1999). Furthermore, several other studies also demonstrated the efficacy of AL-8810 on inhibiting the activation of FPr by several $PGF_{2\alpha}$ agonists (Sharif et al., 2002; Kelly et al., 2003; Sharif et al., 2003; Sharif et al., 2003).

To date, the mechanism/s of action of $PGF_{2\alpha}$ on bovine embryos has/have not been documented in the literature. However, based on the recent discovery of FPr in bovine embryos (Scenna et al., 2006), we can hypothesize that downstream signaling events occurring after $PGF_{2\alpha}$ binds to its receptor are interfering with several mechanisms leading to compaction of bovine embryos. Compaction represents a key critical event for continued embryonic development. During this process, formation of junctional apical complexes (formed by E-cadherin and tight junctions) between outer blastomeres allows polarization and differentiation of these blastomeres into trophectoderm cells. On the

other hand, lack of junctional complexes and polarization of inner blastomeres allows these blastomeres to differentiate into the inner cell mass (Fleming et al., 2001). The junctional complexes between trophectoderm cells facilitate fluid accumulation within the blastocoele cavity and directly contributes to the formation of the blastocyst and its subsequent hatching from the zona pellucida (Watson et al., 1992; Fleming et al., 2001).

It is tempting to hypothesize that $PGF_{2\alpha}$ may impair the ability of pre-compacted embryos to undergo compaction by disassembling, destructing, or decreasing synthesis of cell-cell adhesion molecules (E-cadherin and tight junctions) between blastomeres. Indeed, studies have indicated that overexpression of COX-2 and therefore, an increase in prostaglandin synthesis, were associated with a disruption of cell-cell adhesion molecules, tumor formation, tumor invasion and metastasis (Hida et al., 1998; Takahashi et al., 2002). On the other hand, treatment of cancer cell lines with COX-2 specific inhibitors resulted in increased E-cadherin expression and inhibition of cell growth (Noda et al., 2002; Erdogru et al., 2005; Dohadwala et al., 2006). In addition, upregulation of intracellular Ca^{++} (an effect associated with $PGF_{2\alpha}$ actions on target cells) leads to contraction of Ca^{++} calmodulin-dependent actin-myosin filaments which in turn alters cellular shape, opens up TJ's and increases paracellular permeability (Lindmark et al., 1998). Moreover, protein kinase C (a protein activated by $PGF_{2\alpha}$ on target cells) and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) have been shown to increase cellular permeability by opening of tight junctions (Sjo et al., 2003; Angelow et al., 2005). Furthermore, it has been shown that $PGF_{2\alpha}$ stimulates the synthesis of matrix metalloproteinases (MMP's; (Lindsey et al., 1996; Weinreb et al., 1997) which can in

turn induce proteolytic degradation of tight junction proteins and result in increase epithelial permeability (Wachtel et al., 1999; Lohmann et al., 2004; Gurney et al., 2006).

Alterations in E-cadherin or tight junctions caused by the effects of $PGF_{2\alpha}$ on the embryo may result in an impairment of the embryo to compact and form a blastocoele cavity, two critical steps for continued development. In fact, homozygous null mutants mouse embryos for the E-cadherin gene initially compacted (an event attributed to the presence of maternal or oogenetic E-cadherin proteins) but then proceeded to decompact, with cells becoming apolar due to interference in the formation of apical junctional complexes (Laure et al., 1994; Riethmacher et al., 1995). Moreover, these embryos failed to develop a normal blastocoel cavity and did not hatch from the zona pellucida (Riethmacher et al., 1995). Further evidence of the importance of E-cadherin on compaction was observed when mouse blastomeres decompacted after treatment with antibodies against Ca^{++}-dependent molecules (Reima, 1990). Recently, targeted disruption of the E-cadherin gene in bovine pre-attachment embryos by RNA interference technology resulted in less embryos reaching compact morula or blastocyst stage (Nganvongpanit et al., 2006). These findings indicate a critical role of E-cadherin not only during compaction, but on further embryonic development in bovine embryos.

In conclusion, addition of a selective FPr antagonist, AL-8810, to the culture medium of *in vitro* produced embryos inhibited detrimental effects of $PGF_{2\alpha}$ on embryo development without toxicity. These findings suggest that the effects of $PGF_{2\alpha}$ on bovine embryos are in part caused by receptor-mediated events and downstream signaling cascades. We hypothesized that binding of $PGF_{2\alpha}$ to its receptor causes downstream signaling events (mobilization of intracellular Ca^{++}, activation of PKC and MAPK's) that

may decrease the embryos' ability to compact by causing disruption, destruction or inhibition of synthesis of adherens and tight junctions between blastomeres. Moreover, addition of AL-8810 to the culture medium may become a standard procedure for enhancing development of *in vitro* produced mammalian embryos.

CHAPTER 5

PREGNANCY RATES OF RECIPIENT ANIMALS FOLLOWING APPLICATION OF A SELECTIVE PROSTAGLANDIN F_{2A} RECEPTOR ANTAGONIST DURING EMBRYO RECOVERY

1. Abstract

In the presence of supplemental progesterone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to directly reduce embryo quality, development and pregnancy rates in cattle. The aim of this study was to evaluate addition of a $PGF_{2\alpha}$ receptor (FPr) antagonist to collection medium on pregnancy rates after transfer of embryos to recipient animals. An initial experiment was performed to determine embryonic development and gene expression of *in vivo*-derived frozen-thawed embryos cultured in KSOM-PVA medium with 1000 nM AL-8810 (AL, n= 94), 1000 nM AL-8810 and 10 ng/mL $PGF_{2\alpha}$ (AL+PGF, n= 94), 10 ng/mL $PGF_{2\alpha}$ (PGF, n= 94), or serving as controls (CON, n= 91). Embryos remained in their treatment for a 30-h period, at the end of which embryo development per treatment was recorded. Embryos from each treatment group were then processed for analysis of Na^+/K^+ ATPase α1 and ZO-1 transcripts by reverse transcription real time PCR. In the subsequent study, embryos were recovered from superovulated donor cows on day 7 after artificial insemination with medium containing 1000 nM AL-8810 (Flush-AL) or without (Flush-Con). Following collection in their respective treatment, embryos were classified by stage and quality and maintained in holding medium with 1000 nM AL-8810 or without until freezing, then transferred following thawing to suitable

recipients. Results from the initial experiment indicated that culture of *in vivo*-derived bovine embryos in medium containing AL-8810 improved blastocyst development compared to PGF (58% vs. 46%; *P=0.05*). In addition, a strong tendency on embryo development was observed in AL+PGF compared to PGF treatment group (57% vs. 46%; *P=0.07*). Blastocyst development of embryos in CON did not differ when compared to embryos in PGF (55% vs. 46%; *P=0.12*). Relative abundance of Na^+/K^+ ATPase α1 and ZO-1 transcripts among treatments did not differ. Recovery of embryos with medium containing AL-8810 increased pregnancy rates following embryo transfer when compared to embryos recovered without AL-8810 (Flush-AL= 46% ± 0.06 vs. Flush-Con= 36% ± 0.06; *P=0.04*). In conclusion, the addition of an FPr antagonist prevented detrimental effects of $PGF_{2\alpha}$ on *in vitro* development of *in vivo*-derived bovine embryos. Moreover, recovery of embryos with flushing medium containing an FPr antagonist improved pregnancy rates after transfer of embryos to recipient cows.

2. Introduction

Multiple ovulation and embryo transfer (MOET) programs in livestock production are utilized as a means for rapid acquisition of superior genetics and to increase productive life of elite donor cows by increasing the number of valuable progeny. Currently, MOET programs in cattle are used around the world, with more than 500,000 embryos being transferred annually (Thibier, 2000). Efficiency of such programs in cattle depends on several factors associated with donor's response to superovulation (Seidel, 1984), number of viable embryos recovered per collection (Donaldson and Perry, 1983; Hasler et al., 1983; Seidel, 1984; Stroud and Hasler, 2006), and the percentage of

recipient animals establishing a pregnancy after transfer (Leibo, 1986; Hasler, 1992; Nibart and Humblot, 1997; Lester and McNew, 1999; Hasler, 2001, 2003).

Manipulation of the reproductive tract during embryo transfer results in $PGF_{2\alpha}$ release from the uterine endometrium (Scenna et al., 2005). Other studies have also reported a release of $PGF_{2\alpha}$ following uterine manipulation in mares (Kask and Odensvik, 1995), sows (Kunavongkrit et al., 1984) and cows (Wann and Randel, 1990). Moreover, several studies have shown detrimental effects of elevated $PGF_{2\alpha}$ on *in vitro* development of rat, rabbit and bovine embryos (Maurer and Beier, 1976; Breuel et al., 1993; Scenna et al., 2004). Elevated $PGF_{2\alpha}$ has also been shown to have detrimental effects on embryonic development, quality and hatching ability of embryos and pregnancy rates in cows, even in the presence of supplemental progesterone (Schrick et al., 1993; Buford et al., 1996; Lemaster et al., 1999; Elli et al., 2001; Hockett et al., 2004; Sales et al., 2004). Furthermore, administration of inhibitors of $PGF_{2\alpha}$ synthesis at the time of embryo transfer to recipient animals increased pregnancy rates (Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005).

The objectives of this study were 1) to determine *in vitro* development and gene expression of two relevant genes involved in blastocyst formation (Na^+/K^+ ATPase α1 and ZO-1) of *in vivo*-derived bovine embryos cultured with a selective FPr antagonist; and 2) to determine if addition of a FPr antagonist to collection medium during recovery of bovine embryos increased pregnancy rates following transfer of these embryos to recipient cows. Improving pregnancy rates after embryo transfer in cows using an FPr antagonist will allow for development of new therapeutic techniques in other species, including humans, to increase reproduction and success rates.

3. Materials and Methods

3.1. Experimental procedures to determine the effects of $PGF_{2\alpha}$ and AL-8810 on development and relative abundance of genes associated with compaction

3.1.1. Preparation of treatments

3.1.1.1. Preparation of AL-8810

Prior to the beginning of each experiment, the content of a vial containing 10 mg of AL-8810 (Cayman Chemical Inc., Ann Arbor, MI; catalog# 16735) was reconstituted and solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO; Primary Stock, 1000 μM AL-8810) to obtain a primary Stock solution of 1000 μM AL-8810. Aliquots containing 1 mL of primary stock solution were stored at -80°C until needed. For each replicate, the primary stock solution was diluted 1:10 in KSOM-PVA (0.3%; Secondary Stock, 100 μM AL-8810) and added to the experimental wells (495 μl of equilibrated KSOM-PVA) to obtain a final concentration of 1000 nM AL-8810 in the culture medium. Control experimental wells were prepared by adding the same concentration of DMSO (0.1%) used to prepare AL-8810 in KSOM-PVA.

3.1.1.2 Preparation of prostaglandin $F_{2\alpha}$

Prior to the beginning of each experiment, prostaglandin $F_{2\alpha}$ (5 mg; Cayman Chemical Inc., Ann Arbor, MI; catalog# 16010) was solubilized in 1 mL of DMSO each (Stock #1; 5,000 μg/mL). Then, 20 μL aliquots were stored at -80°C until needed. For each replicate of the experiment, 10 μL of $PGF_{2\alpha}$ stock #1 were combined with 990 μL of KSOM-PVA (stock #2: 50 μg/mL). Next, 10 μL of stock #2 were combined with 490 μL of KSOM-PVA (stock #3: 1 μg/mL or 1 ng/μL). Finally, 5 μL of stock#3 were added to

each experimental well (495 µL of equilibrated KSOM-PVA) to obtain a final concentration of 10 ng/mL of $PGF_{2\alpha}$ in the culture medium. Four-well plates with KSOM-PVA in each well were equilibrated in an incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for at least 12 h before preparation of treatments.

3.1.2. Assignment of treatments

In vivo-derived bovine embryos were thawed and sorted by stage of development and quality according to the IETS guidelines for classification of bovine embryos (Stringfellow and Seidel, 1998). Similar groups of embryos at the morula and early blastocyst stage of quality grade 1, 2 or 3 were washed three times in HEPES-TALP-PVA (0.3%) and once in KSOM-PVA (0.3%) and randomly transferred to one of four treatments: 1) 1000 AL (1000 nM AL-8810 in KSOM-PVA, n=94); 2) 1000 AL + PGF-10 (1000 nM AL-8810 + 10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA, n=94); 3) PGF-10 (10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA, n=94); and 4) CON (KSOM-PVA, n= 91; Figure 1) in a 4-well plate and placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C). Three replicates were utilized for this experiment. It is important to mention that $PGF_{2\alpha}$ was added at least 30 min after the embryos were placed in 1000 nM AL-8810 to allow the interaction of the FPr antagonist with its receptors before addition of $PGF_{2\alpha}$.

After culturing embryos for 30 h, embryos from each treatment were placed in HEPES-TALP-PVA (0.1%) and development to blastocyst stage was recorded. Next, each treatment group (approximately 30 embryos) was washed 3 times in HEPES-TALP-PVA (0.1%), loaded in 2.5 µL of HEPES-TALP-PVA (0.1%) and transferred to 25 µL of RNA lysis buffer (Arcturus, Mountain View, CA) and stored at -80° C until use.

3.1.3. Isolation of RNA

Total RNA from each treatment group was isolated using the PicoPure RNA isolation Kit (Arcturus, Mountain View, CA) as per the manufacturer's instructions. Briefly, RNA was mixed with 70% ethanol by gently pipetting and the mixture was added to an RNA purification column. DNAse treatment was performed by addition and incubation of DNase I enzyme (Qiagen, Valencia, CA) for 15 min at room temperature in the RNA purification columns. Several washes of the columns were performed previous to elution of RNA from the column by using 12 μL elution buffer.

3.1.4. Reverse transcription

Isolated RNA from bovine embryos and bovine tongue epithelium was reversed transcribed into complementary DNA in a total volume of 20 μL. The reaction mixture consisted of 1X RT buffer, 5 mM $MgCl_2$, 1 mM of each dNTP, 0.25 nM random primers, 20 iu RNase inhibitor, and 100 IU moleney murine leukaemia virus reverse transcriptase (MMLV-RT). The RT reaction was carried out at 25°C for 10 min, 42°C for 1 h followed by a denaturation step at 99°C for 5 min and flash cooling to 4°C. Controls for genomic DNA contamination were prepared by emitting reverse transcriptase enzyme in the RT reaction.

3.1.5. Real time PCR

Polymerase chain reaction was performed using the iCycler iQ™ Real-Time PCR detection system (Bio-Rad, Hercules, CA) and the iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA). The PCR reaction mixture consisted of 25 μL 1X iQ Supermix (100

mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, iTaq DNA Polymerase 50 units/ml, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers), 1 μM concentration of forward and reverse primers (Table 2) for β-actin (Rambeaud et al., 2006), Na^+/K^+ ATPase α1 (Wrenzycki et al., 2003), and ZO-1, nuclease free water, and cDNA template from each RT reaction. Negative control for each embryo sample consisted of PCR amplification of RT negative reactions or mix without cDNA (no template). The real time PCR protocol included an initial step of 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Fluorescence data was acquired during the elongation step. Since the melting curve of PCR products is sequence specific, it is used to identify PCR products. Melting protocol was performed by holding temperature at 45°C for 60 sec and then heating from 45 to 94°C, holding at each temperature for 10 sec while monitoring fluorescence. Product identity was also confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis in 1X TBE (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) visualized using a gel doc apparatus (Bio-Rad, Hercules, CA) and by target sequencing (University of Tennessee Molecular Biology Core Facility).

3.1.6. Statistical analysis

Collected data for ZO-1, and Na^+/K^+ ATPase α1 gene expression in each treatment were computed using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) and expressed as fold change in gene expression relative to control embryos samples after normalizing for β-actin gene expression. Data for mRNA expression and

Table 2. Details of primers used for RT-RT-PCR.

Gene	GenBank Accesion Number	Sequence	Location	Fragment size (bp)
Na^+/K^+ ATPase α1	NM_012504	5'-acctgttgggcatccgagagac-3' 5'-agggaaggcacagaaccacca-3'	2882–2903 3196–3217	336
ZO-1	L14837	5'-gtctgccattacacggtcct-3' 5'-ggcttaaatccaggggagtc-3'	3803-3822 3921-3940	138
β-Actin	AY141970.1	5'-cggcattcacgaaactacct -3' 5'-gggcagtgatctctttctg-3'	855-875 978-997	143

development to blastocyst from a total of 3 replicates were analyzed using a randomized block design model with replicate (a 4-well plate with each of its wells containing one treatment; a group of embryos in each well was considered an experimental unit) as random effect and treatment as fixed effect performing mixed procedures of SAS software (SAS 9.1, SAS Institute Inc., Cary, NC). In addition, orthogonal contrasts were performed to determine differences on development to blastocyst among treatments.

3.2. In vivo production of embryos

3.2.1. Preparation of AL-8810

AL-8810 (Cayman Chemical Inc., Ann Arbor, MI; 10 mg) was reconstituted and solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO; Primary Stock, 1000 μM AL-8810) and aliquots containing 1 mL of primary stock solution were stored at -80°C until needed. For each liter of flushing medium, 1 mL of primary stock solution was added to obtain a final AL-8810 concentration of 1000 nM (Flush-AL1000). Similarly, 1 mL of DMSO was added to each liter of flushing medium (Flush-Con) to obtain the same amount of diluent (0.1%) as in the treatment flushing medium. In addition, an aliquot containing 1 mL of a ten fold dilution of the primary stock solution in DMSO was added to each liter of flushing medium to obtain a final AL-8810 concentration of 100 nM (Flush-AL100).

3.2.2. Estrous cycle synchronization and superovulation

On day 5 after CIDR insertion (day 1; Eazi-Breed CIDR; Pfizer Animal Health, Kalamazoo, MI), embryo donors started a superovulation program consisting of

administration of pFSH (range of 162-400 mg i.m.; Folltropin-V, Bioniche, Belleville, Ontario, Canada) in decreasing doses twice daily for 4 days. Luteolysis was induced by administration of two doses of prostaglandin $F_{2\alpha}$ (500 μg cloprostenol; Estrumate, Schering-Plough, Kenilworth, NJ; 12 h before and at the time of CIDR removal, day 9). Cows were fitted with Heatwatch® detectors (DDX, Boulder, CO) for estrous detection in addition to visual surveillance twice daily for 30 min. Cows were artificially inseminated with 1 unit of frozen-thawed semen at 12 h after standing estrus and again 12 h later.

3.2.3. Embryo recovery and freezing

Collection of embryos from donor cows (Angus breed) was performed 7 days after detection of estrus by placing a silicone catheter (Bioniche, Belleville, Ontario, Canada) through the cervix and up to the uterus body (Drost et al., 1976). The uterus was flushed several times with Vigro Complete Flush Solution (Bioniche, Belleville, Ontario, Canada), containing either 100 nM AL-8810 (Flush-AL100), 1000 nM AL-8810 (Flush-AL1000) or dimethyl sulfoxide (DMSO; Flush-Con). Collected flush medium was filtered through a 0.22 μm EM-Con filter (Veterinary Concepts Incorporated, Spring Valley, WI) and searched for the presence of embryos. Embryos collected with Flush-AL100, Flush-AL1000 or Flush-Con were placed into holding medium (Vigro Holding Plus; Bioniche, Belleville, Ontario, Canada) with either100 nM AL-8810, 1000 nM AL-8810 or DMSO, respectively, and scored for quality and stage accordingly to the International Embryo Transfer Society guidelines (Stringfellow and Seidel, 1998). Following a 3X wash procedure in holding medium, embryos were frozen using 1.5 M ethylene glycol (Vigro Ethylene Glycol Freeze Plus; Bioniche, Belleville, Ontario,

Canada). Briefly, after scoring for stage and quality, embryos were placed for 5 min in 1.5 M ethylene glycol. Embryos were then individually loaded into 0.25 mL straw and placed at -6°C in an automatic freezing unit (CL 5500; Cryologic, Mulgrave, Victoria, Australia). The freezing program consisted in holding embryos at -6°C for 10 min (seeding induced after 2 min at -6°C) followed by a descending freezing curve of 0.5°C/min until reaching -32°C. Embryos remained at -32°C for 10 min and then were plunged into liquid nitrogen for final storage. Statistical analysis did not show differences in pregnancy rates following transfer between Flush-AL100 and Flush-AL1000; therefore, both treatment groups were combined as Flush-AL.

3.2.4. Embryo transfer

A total of 619 transfers into recipient cows (Registered Angus and Commercial [Angus X British, Continental, or Bos Indicus]) were performed in a two year period from February to May at four different locations in three states (Arkansas, Tennessee, and Texas). Recipients were in moderate body condition, multiparous, and provided *ad libitum* access to minerals, water and supplemental feed. Due to donor X semen interactions determined after transfer (Schrick & Saxton, unpublished data), only 425 recipients were used for statistical analysis. Transfer data were included from the May 2006 Arkansas study in which recipients were improperly vaccinated with a modified-live vaccine during synchronization. Even though pregnancy rates were reduced in all animals across treatments for this replicate, the trend in pregnancy rates was similar to all other locations and thus, data were included in the final analyses. Animals that responded to estrus synchronization and had an acceptable corpus luteum were used as embryo

recipients 7 days (range 6-7.5 days) after detection of estrous. Briefly, animals were restrained in a cattle handling facility and location of the corpus luteum was determined by palpation per rectum or ultrasonography (Aloka 500 ultrasound unit equipped with a 7.5MHz linear transducer; Corometrics Medical System, Wallingford, CT). Prior to embryo transfer, animals received an epidural injection of 2% lidocaine (2.5 ml; Lidoject; Burns Veterinary Supply, Inc., Westbury, NY) and the perineal region was washed with povidone solution. Embryos were thawed and non-surgically transferred to the upper third of the uterine horn ipsilateral to the CL (Schrick et al., 1993). Pregnancy rates were determined 30 to 60 days following transfer by ultrasonography or by palpation per rectum.

3.2.5. Statistical analysis

Data were arranged in a randomized block design. Pregnancy rates (%), embryo quality and stage variables were evaluated. Data pertaining to pregnancy rates were analyzed using generalized linear mixed models (Proc Glimmix; SAS version 9.1, 2003; SAS Institute Inc., Cary, NC). Data are presented as least squares means (± SEM). A model procedure that included flush treatment (Flush-AL or Flush-Con), quality (1 or 2), and stage (morula or blastocyst) were used to compare differences among treatments. Due to a possible direct effect on pregnancy rates, embryo quality and stage were used as covariates and location was included as a random effect. Differences in individual least squares means were protected by using Tukey-Kramer method. A *P* value less or equal to 0.05 was considered statistically significant.

4. Results

Results from the initial experiment indicated that culture of *in vivo*-derived bovine embryos in medium containing AL-8810 improved blastocyst development when compared to PGF (58% vs. 46%, respectively; *P=0.05*; Figure 13). In addition, a strong tendency on improving embryo development was observed in embryos cultured in 1000 AL + PGF-10 compared to those cultured in PGF-10 (57% vs. 46%, respectively; *P=0.07*; Figure 13). Blastocyst development of embryos cultured in CON tended to be lower when compared to embryos in PGF-10 (55% vs. 46%, respectively; *P=0.12*; Figure 13). Relative abundance of Na^+/K^+ ATPase α1 (Figure 14) and ZO-1 (Figure 15) transcripts did not differ between treatments. Moreover, pregnancy rates after transfer of embryos recovered with medium containing AL-8810 were higher than those obtained after transfer of embryos recovered with flushing medium without AL-8810 (46% vs. 36%; *P=0.04*; Figures 16). No differences on pregnancy rates by quality or stage or by treatment interactions on pregnancy rates were observed (Figures 16 and 17; respectively).

5. Discussion

Currently, MOET programs in beef and dairy cows are utilized worldwide for rapid acquisition of superior animals. However, the costs of superovulation treatment, labor, and the variability in superovulatory response and pregnancy rates after embryo transfer make this technique economically unappealing to the great majority of producers. Several factors such as recipient management, age of recipient animal (reviewed by (Stroud and Hasler, 2006), synchrony between estrous cycles of donor and recipient cows

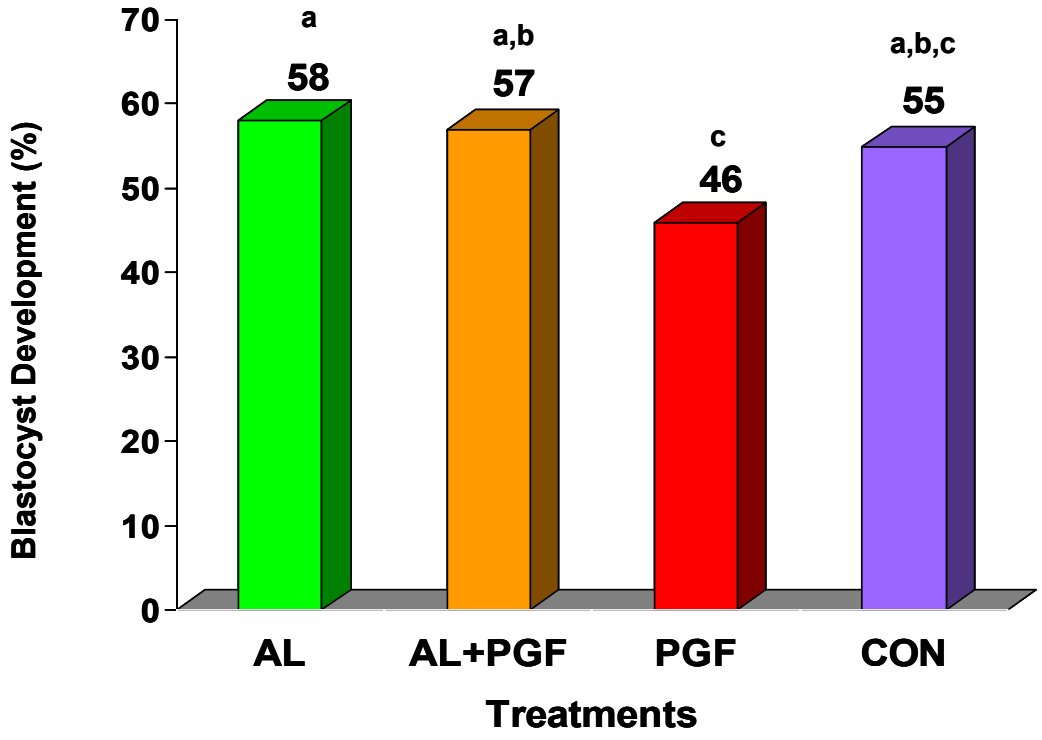


Figure 13. *In vitro* blastocyst development of frozen-thawed *in vivo*-derived embryos cultured in KSOM medium with 1000 nM AL-8810 (AL, n= 94), 1000 nM AL-8810 + 10 ng/mL prostaglandin F2α (AL+PGF, n= 94), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF, n= 94), and Control (CON, n= 91). Standard errors were ± 3.7.

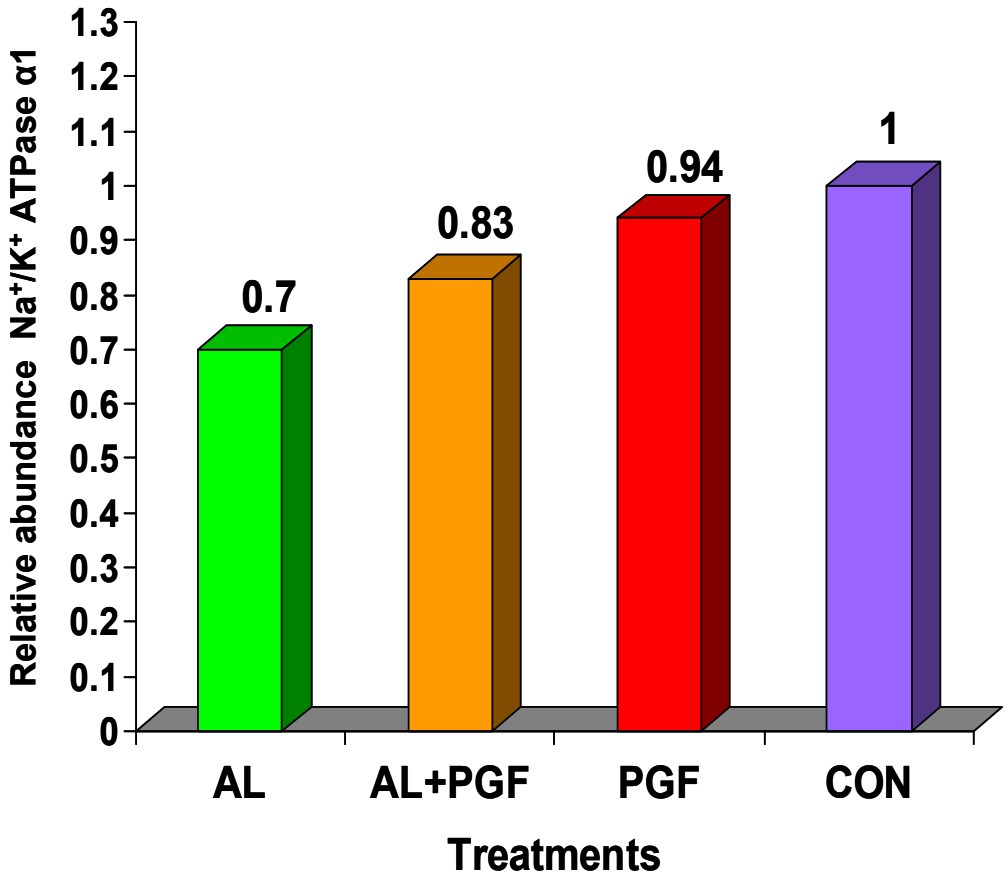


Figure 14. Relative abundance of Na^+/K^+ ATPase mRNA expression of *in vivo*-derived embryos cultured in KSOM medium with 1000 nM AL-8810 (AL), 1000 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$ (AL+PGF), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF), and Control (CON). Standard errors were ± 0.14 for each treatment.

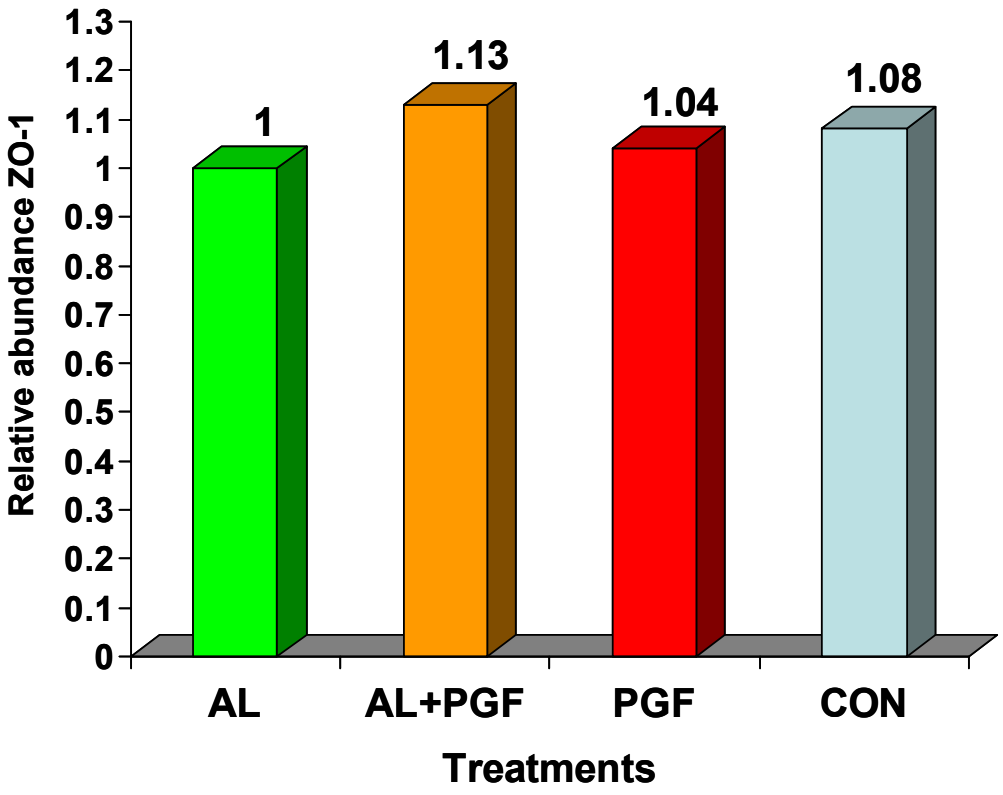


Figure 15. Relative abundance of ZO-1 mRNA expression of *in vivo*-derived embryos cultured in KSOM medium with 1000 nM AL-8810 (AL), 1000 nM AL-8810 + 10 ng/mL prostaglandin $F_{2\alpha}$ (AL+PGF), 10 ng/mL prostaglandin $F_{2\alpha}$ (PGF), and Control (CON). Standard errors were ± 0.25 for each treatment.

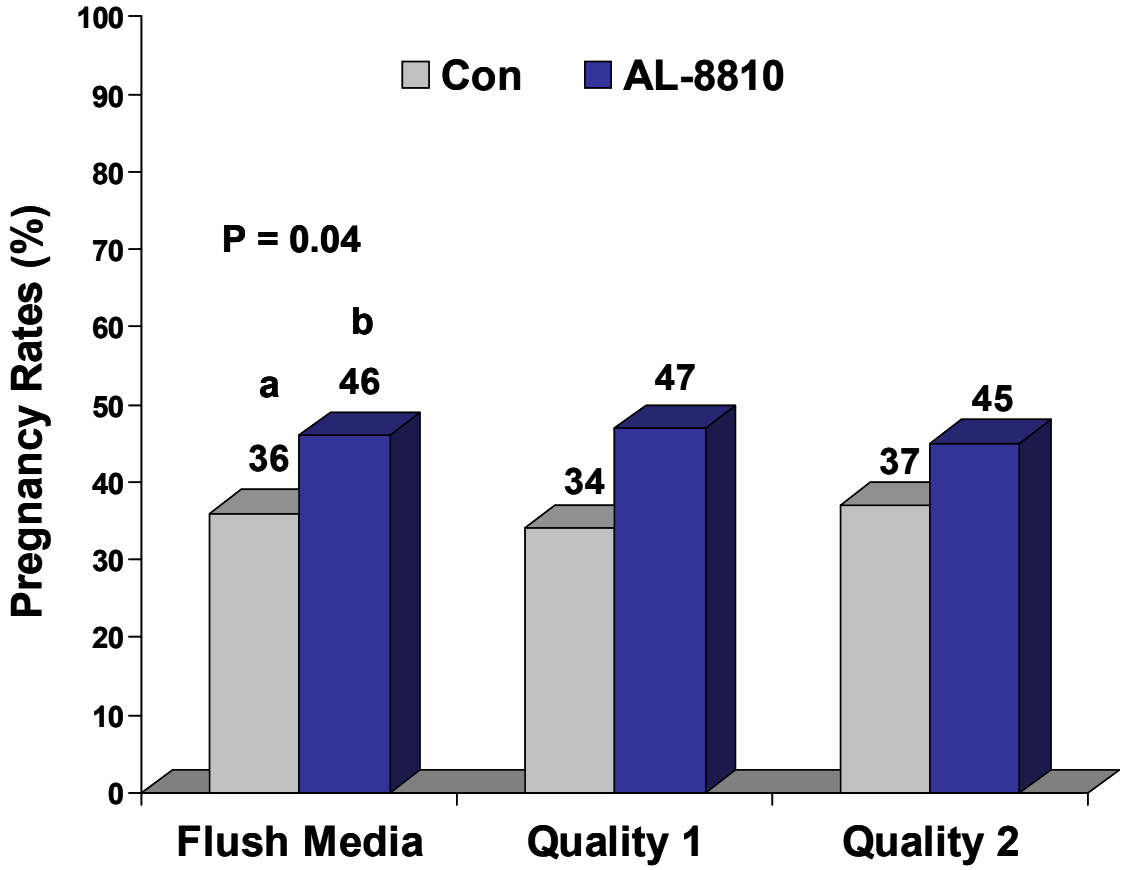


Figure 16. Pregnancy rates following transfer of embryos collected in medium containing AL-8810 were increased compared to controls. The interaction of collection medium treatment with embryo quality 1 or 2 did not differ. [a,b] Values differ between treatments; *P<0.05*. Standard errors were ± 0.06 for AL-8810 and Con flush medium, and ± 0.06, ±0.08, ±0.06, ±0.09 for the interaction of AL-8810 with quality 1, AL-8810 with quality 2, Con with quality 1, and Con with quality 2.

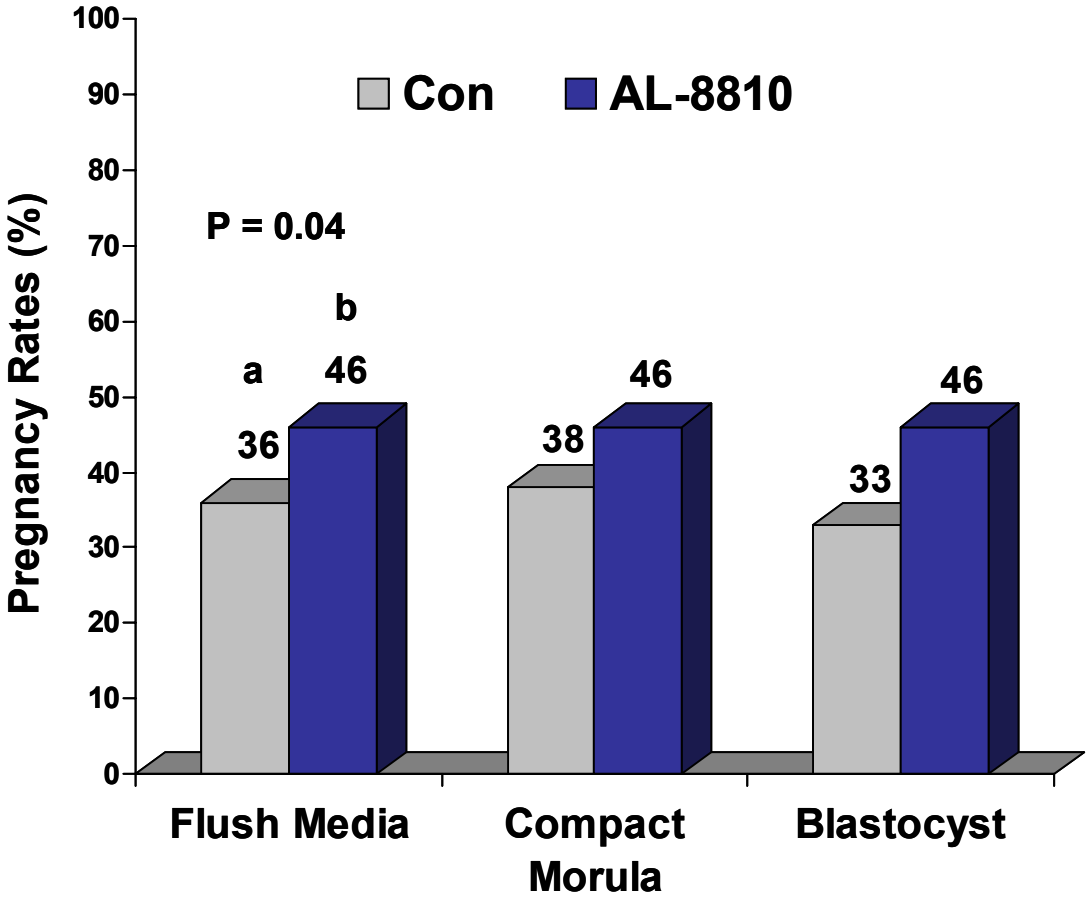


Figure 17. Pregnancy rates following transfer of embryos collected in medium containing AL-8810 were increased compared to controls. The interaction of collection medium treatment with stage of the embryo (compact morula or blastocyst) did not differ. [a,b] Values differ between treatments; *P<0.05*. Standard errors were ± 0.06 for AL-8810 and Con flush medium, and ± 0.07, ±0.07, ±0.07, ±0.08 for the interaction of AL-8810 with compact morula, AL-8810 with blastocyst, Con with compact morula, and Con with blastocyst.

(Wright, 1981), infectious diseases (Vanroose et al., 2000), toxins (Woclawek-Potocka et al., 2005), uterine environment composition (elevated concentrations of urea and $PGF_{2\alpha}$ (Schrick et al., 1993; Butler, 1998; Scenna et al., 2005), and several others factors, can influence pregnancy rates following embryo transfer to recipient cows. Pregnancy rates after non-surgical embryo transfer of *in vivo*-derived frozen-thawed (ethylene glycol) embryos is about 40 to 55% (Leibo, 1986; Malayer et al., 1990; Nibart and Humblot, 1997; Lester and McNew, 1999), whereas those obtained after transfer of fresh embryos are about 55% to 80% (Hasler, 2001). Development of new strategies aimed at increasing pregnancy rates after embryo transfer will increase efficiency of this technique and lower costs to cattle owners. Therefore, the objective of this study was to reduce reproductive losses associated with negative effects of $PGF_{2\alpha}$ on the embryo after performing embryo transfer in cattle.

In the cow, hatching is a critical step for establishment of a successful pregnancy because it allows filamentous development of the embryo and posterior attachment of the embryo to the uterine endometrium. Similarly, low implantation rates of *in vitro*-fertilized human embryos are largely due to impaired development and hatching of blastocyst (Magli et al., 1998). In cattle, accumulation of fluid (between days 8 and 10 after fertilization) within the blastocoel cavity of the blastocyst distends the ZP until it ruptures allowing hatching of the blastocyst by protrusion through the opening in the ZP (Flechon and Renard, 1978; Massip and Mulnard, 1980). Accumulation of fluid within the blastocoel cavity is achieved by a sodium concentration gradient created by the presence and activity of Na^+/K^+-ATPase pump (Biggers et al., 1988) at the basolateral surface of the trophectoderm epithelium. Additionally, presence of a junctional apical

complex (formed by tight junction and adheren junctions at the apical part of the TE epithelium) prevents uncontrolled leakage of this fluid from the blastocoel cavity (Watson et al., 1992; Bavister, 1995).

Manipulation of the reproductive tract has been shown to increase release of $PGF_{2\alpha}$ from the uterine endometrium in the cow (Scenna et al., 2005), sow (Kunavongkrit et al., 1984), and mare (Kask and Odensvik, 1995). Elevated uterine concentrations of $PGF_{2\alpha}$ in the uterine lumen caused by mechanical manipulation of the uterus at the time of embryo transfer may create a "hostile environment" for embryonic development. In fact, Schrick et al. (1993) observed that quality of bovine embryos tended to be negatively correlated with concentrations of $PGF_{2\alpha}$ in the flushing media. Furthermore, Scenna et al. (2004) demonstrated that development of *in vitro*-produced pre-compacted embryos to blastocyst stage and hatching rates of *in vivo*-derived bovine embryos were directly reduced by $PGF_{2\alpha}$. Prostaglandin $F_{2\alpha}$ has also been shown to decrease pregnancy rates in cows provided supplemental progesterone (Buford et al., 1996; Seals et al., 1998; Lemaster et al., 1999). Further involvement of $PGF_{2\alpha}$ as an embryotoxic agent was observed on embryonic development of rat, mouse, and rabbit embryos *in vitro* (Harper and Skarnes, 1972; Maurer and Beier, 1976; Breuel et al., 1993).

In our initial study, orthogonal contrast indicated a strong tendency for improved *in vitro* development of embryos treated with AL-8810 before exposure to $PGF_{2\alpha}$ (1000 AL + PGF-10 group) when compared to embryos treated with $PGF_{2\alpha}$ alone (PGF-10 group). Moreover, evaluation of relative abundance of important genes participating in blastocyst formation and successful hatching (Na^+/K^+ ATPase α1 isoform and ZO-1, a protein constituent of tight junctions) of bovine embryos did not differ among treatments.

Therefore, negative effects of $PGF_{2\alpha}$ on embryonic development of *in vivo*-derived bovine embryos do not appear to be mediated by a reduction on gene expression of genes participating in blastocyst formation nor hatching. However, another explanation for the lack of effects of $PGF_{2\alpha}$ on gene expression in those two genes may be related to the presence of several other Na^+/K^+ ATPase isoforms (Betts et al., 1997) and several other proteins that contribute to tight junction assembly (Stevenson and Keon, 1998). Prostaglandin $F_{2\alpha}$ may also decrease embryonic development by altering the activity of the Na^+/K^+ ATPase pump and increasing permeability of tight junctions through post-translational modification (especially by activation of PKC) of these proteins.

It worth noting that the percent difference between 1000 AL + PGF-10 (57%) and PGF-10 (46%) on development to blastocyst *in vitro* was in the order of 11%. Similarly, a 10% difference on pregnancy rates after transfer of embryos previously exposed to AL-8810 (46%) or embryos not exposed to the receptor antagonist (36%) was observed *in vivo*. Since uterine manipulation induces synthesis and release of $PGF_{2\alpha}$ from the uterine endometrium to the uterine lumen, i*n vitro* development of embryos in 1000 AL + PGF-10 and PGF-10 would "mimic" our *in vivo* conditions when transferring embryos treated with (Flush-AL) or without AL-8810 (Flush-Con) into recipient cows. Therefore, our *in vitro* conditions appear to represent an excellent model for the study of $PGF_{2\alpha}$ effects on development of bovine embryos as well as for the development of therapeutic strategies that can later be applied *in vivo*.

The improvement on pregnancy rates observed in our study agreed with previous data from our laboratory in which administration of flunixin meglumine to recipient cows at the time of embryo transfer increased pregnancy rates (Scenna et al., 2005). In

addition, Elli et al. (2001), McNaughtan et al. (2002), Pugh et al. (2004) and Purcell et al. (2004) also reported beneficial effects of prostaglandin synthesis inhibitors on pregnancy rates after embryo transfer in recipient cows. The increase on pregnancy rates in the present study after utilization of a selective FPr antagonist (10%) was higher than the increase on pregnancy rates observed after administration of flunixin meglumine (5%) reported by Scenna et al. (2005). An explanation for differences on pregnancy rates between these two studies may be explained by 1) a more efficient means of inhibiting $PGF_{2\alpha}$ effects inside the uterus (readily available FPr antagonist in the collection medium vs. parenteral administration of flunixin meglumine), and/or 2) by only inhibiting the effects of $PGF_{2\alpha}$ on the embryo by AL-8810 vs. the inhibition of all prostaglandins synthesis with the administration of flunixin meglumine, a non-specific inhibitor of cyclooxygenase synthases-1 and -2 enzymes (Cheng et al., 1998; Campbell and Blikslager, 2000). As a consequence, flunixin meglumine inhibits synthesis of all prostaglandins, including those considered beneficial for embryo development (especially PGE_2; (Biggers et al., 1978; Gurevich et al., 1993).

Our results suggest that inability of $PGF_{2\alpha}$ to interact with its receptors (by the utilization of a $PGF_{2\alpha}$ receptor antagonist in the flushing medium) on bovine embryos at the time of embryo recovery improves pregnancy rates of those embryos transferred to recipient cows. These findings will increase the efficiency of embryo transfer programs in cattle and lower the costs associated with this procedure by increasing the number of pregnancies after transfer.

CHAPTER 6

SUMMARY

Reproductive losses associated with the beef industry in the United States are estimated to cost $500 million dollars annually (Bellows et al., 2002). Previous studies have shown detrimental effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on *in vitro* development of rat, rabbit and bovine embryos (Maurer and Beier, 1976; Breuel et al., 1993; Scenna et al., 2004). Moreover, elevated concentrations of $PGF_{2\alpha}$ decreased embryonic development, quality and hatching ability of embryos and pregnancy rate in cows, even in the presence of supplemental progesterone (Schrick et al., 1993; Buford et al., 1996; Lemaster et al., 1999; Elli et al., 2001; Hockett et al., 2004; Sales et al., 2004). Therefore, $PGF_{2\alpha}$ is a major cause of reproductive inefficiency in cows and results in vast economic losses for the cattle industry.

To date, the mechanisms through which $PGF_{2\alpha}$ reduces embryonic survival and development in the cow are still unclear. In addition, the presence of prostaglandin $PGF_{2\alpha}$ receptors (FPr) in early stage bovine embryos has not been documented in the literature. Lack of information regarding presence of FPr in bovine embryos did not allow for direct therapeutic strategies aimed at inhibiting $PGF_{2\alpha}$ effects associated with reproductive inefficiency in cattle. However, non-selective inhibitors of prostaglandin synthesis have been shown to increase pregnancy rates after embryo transfer to recipient animals in cattle (Elli et al., 2001; McNaughtan et al., 2002; Pugh et al., 2004; Purcell et al., 2004; Scenna et al., 2005).

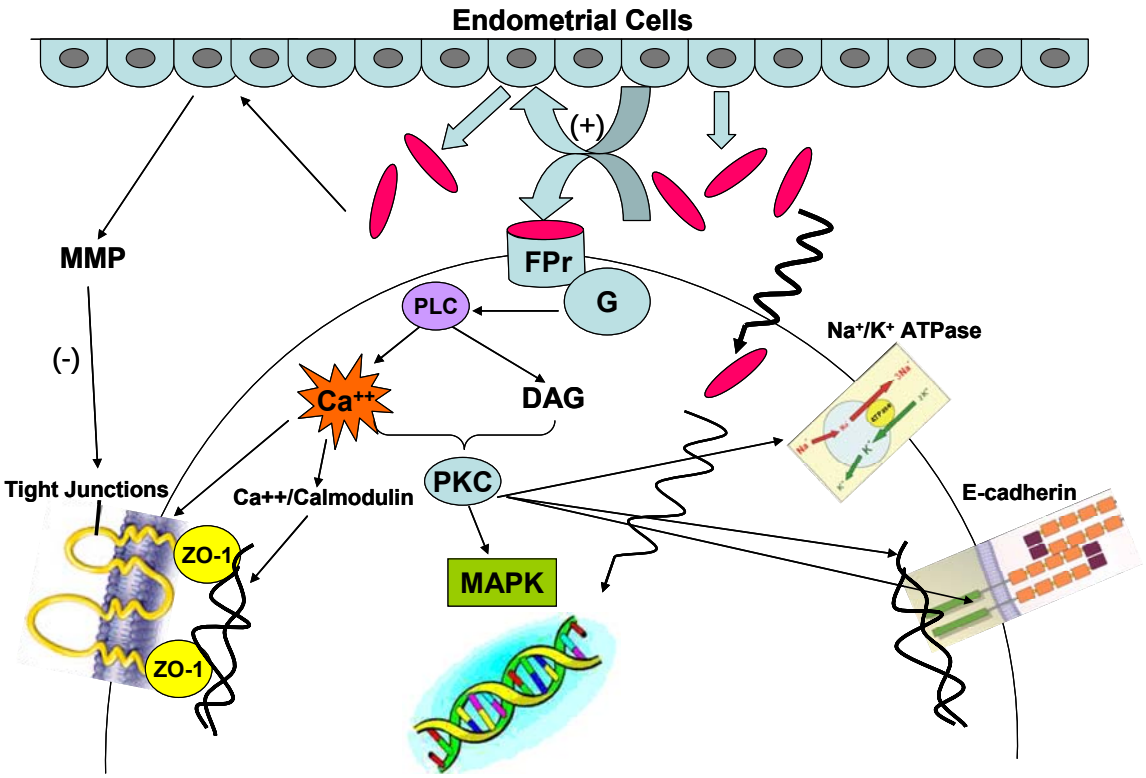
For the first time in the literature, we demonstrated the presence of FPr mRNA and protein in pre-attachment bovine embryos. Discovery of FPr in bovine embryos allowed us to develop new therapeutic strategies to improve embryo quality, development and survival in cows. Our initial experiments determined that the FPr antagonist AL-8810 had no negative (or toxic) effects on development of *in vitro*- and *in vivo*-derived bovine embryos. Secondly, we observed that AL-8810 inhibited detrimental effects of $PGF_{2\alpha}$ development of *in vitro*- and *in vivo*-derived bovine embryos. Finally, we demonstrated that addition of AL-8810 to the collection medium during recovery of bovine embryos in donor cows was able to increase pregnancy rates following transfer of these embryos to recipient cows. Thus, our results indicated that addition of AL-8810 inhibits direct negative effects of $PGF_{2\alpha}$ on *in vitro* (before compaction) and *in vivo* (after compaction) embryo development by preventing $PGF_{2\alpha}$ to interact with its cell surface receptor in bovine embryos.

During embryo transfer, manipulation of the reproductive tract may trigger an inflammatory process in the uterus with release of several chemical mediators. One of these mediators, TNF-α, has been shown to activate $cPLA_2$ in endometrial cells (Okuda et al., 2002). Activation of $cPLA_2$ in endometrial cells will stimulate $PGF_{2\alpha}$ synthesis and release into the uterine lumen. In fact, Scenna et al. (2005) reported increased plasma concentrations of $PGF_{2\alpha}$ following manipulation of the reproductive tract after embryo transfer. Prostaglandin $F_{2\alpha}$ released into the uterine lumen may also stimulate its own synthesis and release from endometrial cells (Wade and Lewis, 1996), and thus, increasing intralumenal concentrations of $PGF_{2\alpha}$. Based on our results, we hypothesize that $PGF_{2\alpha}$ alters embryonic development by binding to its own receptor and triggering a

cascade of events (Figure 18) leading to: 1) inability to compact of pre-compacted embryos, and 2) decrease hatching rates of compacted embryos. It can be speculated that the effects of $PGF_{2\alpha}$ are not all or none, but enough to cause a temporary decrease on embryonic development. These effects will result in reduced embryo development and hatching rates. As a consequence, an asynchrony between the uterus and the stage of the embryo will result in a retarded embryo not able to secrete interferon-τ at the moment of spontaneous luteolysis in the cow and resulting in embryonic loss.

We hypothesize that binding of $PGF_{2\alpha}$ to its receptor causes downstream signaling events (mobilization of intracellular Ca^{++}, activation of PKC and MAPK's) leading to disruption, destruction or synthesis inhibition (by decreasing gene expression) of cell-cell adhesion molecules, but especially E-cadherin and tight junctions. In addition, $PGF_{2\alpha}$–associated signaling events may cause post-translational regulations of several important proteins, such as Na^+/K^+ ATPase pump, and decrease their physiological activities (Kim and Yeoun, 1983). Therefore, effects of $PGF_{2\alpha}$ will result in a decrease on the ability of the embryo to either compact (especially by altering E-cadherin assembly or activity) or hatch from the zona pellucida (especially by altering tight junctions and Na^+/K^+ ATPase pump assembly or activity) (Figure 18).

Improving pregnancy rate after embryo transfer in cows using AL-8810 will allow for development of new therapeutic techniques in other species, including humans, to increase reproduction and success rates. These findings will also allow to increase the efficiency of embryo transfer programs in cattle and to lower the costs associated with this technique, making it more appealing to cattle owners. Lastly, inhibition of detrimental effects of $PGF_{2\alpha}$ on bovine reproduction will result in better reproductive



= prostaglandin $F_{2\alpha}$ G = G-protein PLC = phospholipase C

DAG = diacylglycerol MAPK = mitogen activated protein kinase

MMP = matrix metalloproteinases

Figure 18. Proposed mechanisms of action of $PGF_{2\alpha}$ on development of bovine embryos. Following synthesis and release of $PGF_{2\alpha}$ to the uterine lumen, $PGF_{2\alpha}$ can interact with its own receptor (FPr) at the cell membrane of blastomeres and trigger a cascade of signaling events (G-protein/Phospholipase C/Ca^{++}/DAG/PKC/MAPK) or diffuse to the cytoplasm of the blastomeres without interacting with its receptor. In addition, $PGF_{2\alpha}$ can stimulate its own production in the uterine endometrium increasing its concentrations in the uterine lumen and also inducing synthesis of matrix metalloproteinases with the ability to destruct tight junctions. These signaling events will modify assembly and/or functions of E-cadherin, tight junctions or Na^{+}/K^{+} ATPase proteins affecting compaction and hatching of the embryos.

efficiency (higher pregnancy rates) and higher monetary earnings to the cattle industry (increase on cattle sales).

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VITA

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