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I am submitting herewith a thesis written by Fernando Néstor Scenna entitled "Detrimental Effects of Prostaglandin $F_{2\alpha}$ on Preimplantation Bovine Embryos". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

F. Neal Schrick, Major Professor

We have read this thesis
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DETRIMENTAL EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ ON PREIMPLANTATION BOVINE EMBRYOS

A Thesis

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ABSTRACT

Two studies were performed to determine effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on continued embryonic development of pre-compacted (*in vitro*-produced) and compacted (*in vivo*-derived) bovine embryos. In Experiment 1, pre-compacted (16-32 cell) *in vitro*-produced embryos were randomly assigned to receive either: 1) control (KSOM-PVA; n= 168); 2) PGF-1 (1 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 143); 3) PGF-10 (10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 168); 4) PGF-100 (100 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 136), or 5) PGE-5 (5 ng/mL PGE_2 in KSOM-PVA; n= 62). Following four days of culture (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) in assigned treatments, development of embryos was determined by experienced technicians uninformed of treatments. Continued development of *in vitro*-produced 16- to 32-cell embryos (pre-compacted) to blastocyst was reduced by addition of $PGF_{2\alpha}$ in culture media (PGF-1, 30.4%; PGF-10, 41.4%; PGF-100, 33.3%, and control, 51.8%; $P=0.002$). In addition, culture of embryos in medium containing 1 ng/mL of $PGF_{2\alpha}$ had a more detrimental effect than addition of 10 ng/mL of $PGF_{2\alpha}$ ($P<0.05$). Furthermore, blastocyst development did not differ between control and PGE_2 treatments ($P>0.10$).

In Experiment 2, compacted (*in vivo*-derived) glycerol-frozen embryos were thawed and then allowed to regain normal morphology in holding medium for 30 min. Embryos were then sorted by stage of development and quality according with IETS guidelines for classification of bovine embryos. Compacted morula in the quality score of 1, 2 or 3 (n= 436) were randomly assigned to one of four treatments: 1) control (KSOM-PVA; n= 110); 2) PGF-0.1 (0.1 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n=108); 3) PGF-1 (1

ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n=109); 4) PGF-10 (10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n=109). After 24 h in culture (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C), embryos were washed and placed in KSOM containing 0.5% BSA without $PGF_{2\alpha}$ for an additional 48 h. Embryo development was evaluated by experienced technicians uninformed of treatments. Continued development of *in vivo*-derived compacted morula to blastocyst was not affected by addition of $PGF_{2\alpha}$ to the culture medium (control, 68.1%; PGF-0.1, 58.9%; PGF-1, 61%, and PGF-10, 60%; *P>010*). However, hatching rates of embryos cultured with 0.1, 1 and 10 ng/mL $PGF_{2\alpha}$ were significantly lower than controls (24, 29.1, 24.5, and 44.5, respectively; *P=0.05*). No significant differences in hatching rates between doses of $PGF_{2\alpha}$ were observed.

In conclusion, these studies suggest a direct negative effect of $PGF_{2\alpha}$ on continued embryonic development of pre-compacted and compacted bovine embryos. Responses to $PGF_{2\alpha}$ of pre-compacted (decreased blastocyst development) and compacted (decreased hatching rates) embryos may involve different mechanisms of action of $PGF_{2\alpha}$ depending on stage of development. In compacted embryos, $PGF_{2\alpha}$ may act through inhibiting completion of blastocoel formation and hatching; whereas, $PGF_{2\alpha}$ may have more dramatic effects by altering compaction in pre-compacted embryos.

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CHAPTER 1

INTRODUCTION

Inadequate reproductive performance in dairy and beef cattle reduces production efficiency and results in a profound economic impact to the cattle industry. Net calf crop (percent of beef cows weaning a calf) in the United States has been estimated to be between 65 to 85% (Bellows et al., 1979), which indicates a high incidence of embryonic or fetal wastage. Early embryonic death accounts for 75 to 80% of reproductive losses between fertilization and calving (Sreenan and Diskin, 1983). Embryonic losses appear to occur soon after the embryo enters the uterus (5-8 days after mating or insemination), when the morula stage embryo is developing into blastocyst (Ayalon, 1978; Maurer and Chenault, 1983; Wiebold, 1988; Dunne et al., 2000).

Numerous factors such as heat stress (Putney et al., 1989; Malayer et al., 1990), nutrition (Butler, 1998), mastitis (Hockett et al., 1998), manipulation of the reproductive tract during embryo transfer and artificial insemination (Wann and Randel, 1990; Velez et al., 1991), and plant toxins (tall fescue; J. W. Oliver, personal communication) may contribute to early embryonic losses through premature release of uterine luminal concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in cattle. Schrick and coworkers (1993) reported that embryo quality in beef cattle tended to be negatively correlated with elevated uterine luminal concentrations of $PGF_{2\alpha}$. Furthermore, addition of $PGF_{2\alpha}$ to culture medium has been shown to inhibit *in vitro* development of rabbit (Maurer and Beier, 1976) and rat embryos (Breuel et al., 1993a). In addition, several studies have shown that administration of $PGF_{2\alpha}$ on days 5 through 8 following artificial insemination in cows receiving supplemental progestogen resulted in reduced pregnancy rates and

retarded development of embryos (Buford et al., 1996; Hockett et al., 1998; Seals et al., 1998; Lemaster et al., 1999). These results suggest a direct and/or indirect effect of $PGF_{2\alpha}$ on embryonic survival in the cow.

The mechanisms by which $PGF_{2\alpha}$ reduces embryonic survival and development are unknown. Therefore, the objective of this thesis was to determine if direct effects of $PGF_{2\alpha}$ occur on embryonic development during culture of pre-compacted *(in vitro*-produced) or compacted (*in vivo*-derived) bovine embryos.

CHAPTER 2

LITERATURE REVIEW

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, estrus must be detected by the male, mating must be completed 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 $PGF_{2\alpha}$ of endometrial or exogenous origin), the dominant follicle increases 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 mature (progresses to metaphase of the second meiotic division) and ovulation to take place (Espey, 1994).

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

oocyte is captured by the infundibulum and transported to the ampullary-isthmus junction where capacitated sperm binds to the zona pellucida surrounding the oocyte, undergoes acrosome reaction and penetrates the oocyte's plasma membrane (reviewed by 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 2-1).

The first cleavage division generates a two-cell embryo, in which cells are called blastomeres. As the early embryo is transported through the oviduct (isthmus region), each blastomere undergoes subsequent divisions yielding 4-, 8-, 16- and 32-cell embryos (Figures 2-1; 2-2). The embryo reaches the uterine horn on day 5-6 after estrus; at which time, blastomeres flatten against each other forming a "solid ball" of cells called morula (Figure 2-2). The morula stage embryo begins to form a fluid filled cavity (the blastocoel) and the embryo becomes a blastocyst (Watson et al., 1992). Throughout all these developmental stages, blastomeres become smaller with no net increase in size of the embryo. As a result of additional fluid accumulation within the blastocoel, the embryo starts to expand (expanded blastocyst) and finally hatches from the zona pellucida (hatched blastocyst) on approximately day 9 after estrus (Figure 2-2; Van Soom et al., 1997).

For attachment to the uterine endometrium, the blastocyst must hatch (escape) from the zona pellucida (Figure 2-1). Thus, embryo hatching is a critical step for a successful pregnancy in cattle. Furthermore, low implantation rates of *in vitro*-fertilized human embryos are largely due to impaired development and hatching of blastocyst (Magli et al., 1998).

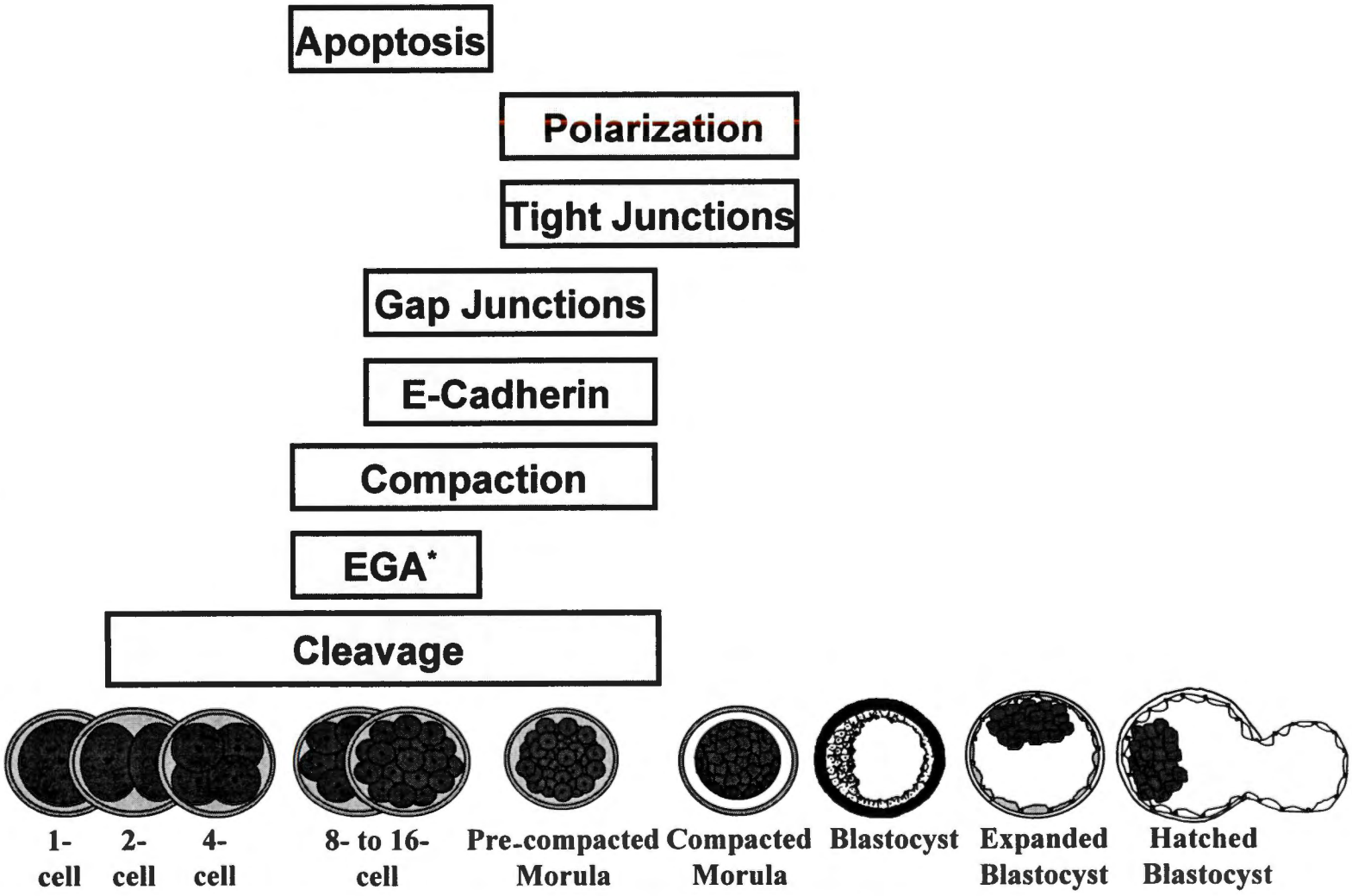

Figure 2-1. Onset of key regulatory events during development of bovine embryos. EGA= embryonic genome activation.

Location	Day	Development
Oviduct (Ampulla)	1	Oocyte
Oviduct (A-I Junction)	2	1 to 2 cell
Oviduct (Isthmus)	2-3	4 cell
Oviduct (Isthmus)	3-5	8 cell
Uterus	5	16 to 32 cell (pre-compacted)
Uterus	6	Morula, Compacted Morula
Uterus	7,8,9	Blastocyst Expanded Blastocyst, Hatched Blastocyst

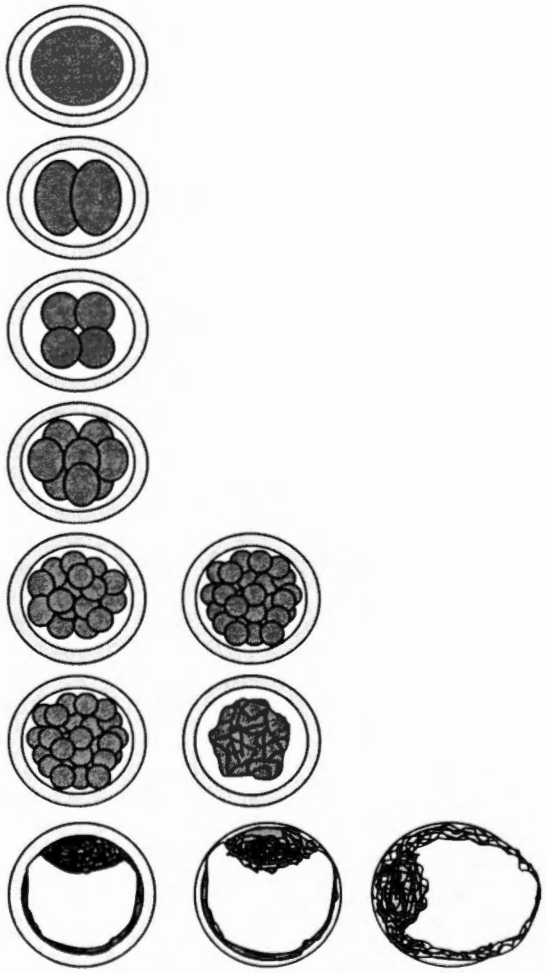

Figure 2-2. Location and timing of development in bovine embryos.

The process of hatching in bovine embryos has been well documented by Massip and Mulnard (1980). In cattle, accumulation of fluid in the blastocoel of *in vivo*-derived embryos distends the zona pellucida (ZP), which becomes gradually thinner. Then, the ZP breaks rapidly and hatching begins immediately by protrusion of the blastocyst through the opening in the ZP. The ZP does not show signs of lysis but is perforated in one location only. Moreover, zona-pellucida-intact embryos, as well as empty ZP, can be found in uterine flushings up to day 16 after ovulation (Betteridge et al., 1980) indicating that enzymatic lysis of the ZP does not occur during hatching in cattle.

The fully expanded bovine blastocyst must exert hydrostatic pressure to create a hole in the ZP through which the blastocyst egresses. Fluid accumulation in the blastocoel is achieved by the presence of a Na^+-K^+-ATPase pump and tight junctions in the trophectoderm epithelium. On the other hand, *in vitro* studies in hamsters and mouse indicated that blastocyst expansion is followed by a focal ZP thinning preceding a focal ZP rupture, protrusion of the embryo, and complete ZP dissolution. These findings indicate that enzymatic degradation of the ZP is essential for hatching of hamsters and mouse embryos (Kane and Bavister, 1988; Gonzalez and Bavister, 1995; O'Sullivan, et al., 2001). 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; Sawada et al., 1990).

Embryonic genome activation

Maternal mRNAs and proteins accumulated in the oocyte's cytoplasm during the growth phase within the follicle are able to support early embryonic divisions (Arends

and Willye, 1991). Further development is achieved by activation of the embryonic genome, which differs in time of initiation 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., 1984; Figure 2-1), sheep (Crosby et al., 1988), and rabbits (Manes, 1973). The majority of maternal proteins and mRNA are degraded during transition from maternal to embryonic genome activation (Latham et al., 1991). Memili and coworkers (1998) suggested minor genome activation between the 1- and the late 4-cell stages and a major gene activation at the 8-cell stage in bovine embryos. 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), connexins (Kidder, 1987) and Na^+-K^+ ATPase enzyme (Watson and Kidder, 1988).

Compaction process in embryos

Compaction represents one of the first key critical events important for continued 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 et al., 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; Figure 2-1) and rabbit (Koyama et al., 1994) at 32-cell stage.

During compaction, blastomeres adhere to and flatten against each other so boundaries cannot be recognized between cells (Van Soom et al., 1997). During this period, development of Ca^{++}-dependent cell adhesion molecules such as E-cadherin and establishment of gap and tight junctions lead to cellular and morphogenetic differentiation of blastomeres. Outer blastomeres polarize and form the trophectoderm (TE), while inner blastomeres remain apolar and produce the inner cell mass (ICM; Koyama et al., 1994). The inner cell mass forms the embryo proper and some extra-embryonic membranes (allantoids and amnion); whereas, trophectoderm cells will combine with the ICM-derived extra-embryonic membranes to form the fetal placenta (Schlafer et al., 2000).

The free or apical membrane surfaces of the TE 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 blastocoel (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). Even though all mammalian embryos undergo compaction, some differences exist between *in vivo*-derived and *in vitro*-produced bovine embryos (Van Soom et al., 1997). Both embryo types start compaction at 32-cell stage, but compaction in *in vitro*-produced embryos is shorter and less obvious than in *in vivo*-derived embryos.

In summary, development of cell adhesion molecules and gap junction-mediated interblastomeric cell communication induces cell polarization and differentiation of undifferentiated blastomeres into TE epithelium and ICM during compaction. Moreover, the trophectoderm develops the capacity to initiate and regulate blastocoel formation by the action of Na^+-K^+ ATPase enzyme (in the basolateral surface TE epithelium). Additionally, 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 blastocoel cavity (Watson et al., 1992).

Involvement of cadherins in embryonic development

Cell adhesion molecules play a critical role during compaction and blastocyst formation in the early embryo, and therefore in subsequent development and viability of the conceptus. Cadherins are a superfamily of Ca^{++}-dependent adhesion molecules that provide strong mechanical attachment between adjacent cells. Cadherin molecules have 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). Also, 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

et al., 2001). 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; Figure 2-1). 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). Moreover, mouse embryos lacking E-cadherin and alpha catenin failed to generate a normal trophectoderm and died before implantation (Laure et al., 1994; Riethmacher et al., 1995). 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 trophectoderm cells during morula stage, while maintaining apolar distributions in the ICM (Barcroft et al., 1998).

Role of gap junctions in embryonic development

Gap junctions are channels present in opposing plasma membranes of neighboring cells (Kidder and Winterghager, 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; 1999), and rat (Reuss et al., 2002) preimplantation embryos.

Intercellular communication through gap junctions is believed to be essential for cellular growth, cellular differentiation and development (Warner, 1992; Zhang and Thorgeirsson, 1994). Recently, a targeted gene inactivation approach has been used to explore the functional significance of connexin diversity. For example, connexin 26 is expressed in the labyrinth of the mouse placenta and its absence causes impaired metabolite transfer from maternal blood to the developing embryo, leading to death in mid-gestation (Gabriel et al., 1998). Female mice lacking connexin 37, which is present in the ovary, develop a block in ovarian follicular development and are sterile (Simon et al., 1997). Although connexin 43 is the most widely expressed connexin, offspring lacking connexin 43 survive until birth and die soon after as a result of morphological heart defects (Reaume et al., 1995), but they do not show any other abnormalities when compared to animals with normal expression of connexin 43. De Sousa et al. (1997) showed that 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. Researchers suggested that loss of connexin 43 can be compensated by other connexins such as connexin 45, a protein also assembled into membrane plaques at the time of compaction.

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 passage of ions and small molecules such as cyclic AMP, Ca^{++} and inositol tri-phosphate (IP_3) between cells and thereby coordinate metabolic and electrical activities (Lowenstein, 1981). Studies using Lucifer yellow have shown that blastomeres of *in vivo*- and *in vitro*-produced mouse embryos are coupled by

functional gap junctions (De Sousa et al., 1993). Boni et al. (1999) showed that diffusion of dye between ICM cells started at morula stage in *in vivo*-derived and at the blastocyst stage in *in vitro*-produced bovine embryos. In the same study, gap junctions were found in the ICM as well as between ICM and TE, but not between TE cells of *in vitro*-derived embryos. However, gap junctions were present through all cells and in a higher density in *in vivo*-derived embryos.

Although assembly of functional gap junctions in mammalian preimplantation embryos is a temporally regulated event occurring during compaction (Figure 2-1), both mRNA and protein corresponding to connexin 43 are being synthesized as early as the 4-cell stage in mouse embryos (one cell cycle before compaction; De Sousa et al., 1993). 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 stage 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).

In mouse embryos, De Sousa et al. (1993) demonstrated that connexin 43 shows different localization between blastomeres of morula and blastocyst embryos: a zonular distribution predominated between outside blastomeres and trophectoderm cells; whereas, plaque-like localizations predominated between inside blastomeres and cells of the inner cell mass. In the same study, treatment of uncompacted 8-cell mouse embryos with monensin or brefeldin-A (inhibitors of protein trafficking) prevented the appearance

of gap junction-like structures. In another study, inhibition of DNA synthesis with aphidicolin during the third and fourth cell cycles of mouse embryos had no affect on establishment of gap junctional coupling, but administration of aphidicolin for 10 h during the second cell cycle resulted in failure of gap junctional coupling (Valdimarsson and Kidder, 1995). Thus, timing of gap junctional coupling is linked to DNA replication in the 2-cell mouse embryo in the mouse.

Data on intercellular communication through gap junctions in early bovine embryos are limited. Utilizing polymerase chain reaction (PCR), Wrenzycki and colleagues (1996) reported that transcripts for connexin 43 were present in immature oocytes and all developmental stages of *in vitro*-produced embryos through morula stage; whereas, only *in vivo*-derived morula and blastocyst stages expressed connexin 43. This findings correlate with reports from Prather et al. (1993) reporting that *in vivo*-derived morula and blastocyst embryos showed dye transfer indicating the existence of gap junctions, while *in vitro*-produced morula and blastocyst embryos did not show spreading of dye.

In cultured vascular cells, gap junctional intercellular communication is inhibited by PKC-dependent excessive phosphorylation of connexin 43, which is the primary gap junction in vascular cells. In addition, excessive phosphorylation of the same protein induces impairment of ventricular conduction in the heart (Inoguchi et al., 2001). Furthermore, Criswell (1995) demonstrated that high intracellular levels of Ca^{++}, addition of Ca^{++} ionophore 4-br-A23187, increased PKC activity and inclusion of TPA (an activator of PKC) resulted in inhibition of gap junctional communication in cultured uterine smooth muscle cells.

Importance of Tight junctions in embryonic development

Tight junctions (zonula occludens) seal the trophectoderm epithelium in a narrow band just beneath their apical surfaces and right above zonula adherens junctions. Tight junctions represent a site of close intercellular adhesion including partial membrane fusion that forms a barrier against paracellular diffusion, generates a trans-epithelial resistance, and allows blastocoel formation (Biggers et al., 1988). Tight junctions comprise several proteins (both transmembrane and cytoplasmic) with the most important being zonula occludens 1 (ZO-1) and cingulin (Citi, 1993), which are only present within the trophectoderm. Following compaction, polar cells adhere to and gradually envelope nonpolar cells causing ZO-1 in the nonpolar cells to disappear. Thus, symmetrically 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 (Barcroft et al., 1998; Figure 2-1). In the blastocyst, ZO-1 is localized as a continuous ring at the apical points of trophectoderm cell contact, whereas it remains undetectable in the ICM.

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 eggs and preimplantation mouse embryos showed high levels of cingulin in eggs and early cleavage stages, a decline during later cleavage, and an increase in cingulin levels during the stage of expanding blastocyst (Javed et al., 1993). In cattle, ZO-1 is undetectable until morula stage when first appears as punctuate points between outer cells. In the blastocyst, ZO-1 is localized as a continuous ring at the apical points of

trophectoderm cell contact, whereas remains undetectable in the ICM (Barcroft et al., 1998).

Cell death during embryonic development

Cell death occurs as a normal feature of early embryonic development in both *in vivo* and *in vitro*-derived embryos (Jurisicova et al., 1996; Hardy, 1997; Matwee et al., 2000). Two forms of cell death can be distinguished based on morphological and molecular criteria (Willye et al., 1980; Granville et al., 1998).

Necrosis is characterized by nuclear disintegration, cellular swelling, rupture of internal and external membranes with release of lytic enzymes, and damage to surrounding cells by stimulation of an inflammatory reaction (Majno and Joris, 1995). Necrosis is considered to be an "accidental" cell death initiated by gross environmental insults such as severe hypoxia, heat shock and exposure to metabolic toxins (Willye et al., 1980). In contrast, apoptosis (also called programmed cell death) occurs in single rather than groups of cells and is thought to be a self-directed death or "cell suicide" to assure normal development and differentiation by removal of damaged or defective cells (Edwards, 1998).

Apoptosis is characterized by a series of morphological changes. First, chromatin aggregates into large compact granular masses on the inner nuclear membrane, the cytoplasm condenses and the nuclear and cytoplasmic membranes became indented. Then, DNA fragments and the nucleus separate into discrete masses. During the final phases of the apoptotic processes there is extensive blebbing of the plasma membrane

with the formation of membrane-bound apoptotic bodies, which can be phagocytosed by surrounding cells or extruded into an adjacent lumen (Willye et al., 1980).

In addition to morphological changes, biochemical alterations at the plasma membrane or at the DNA structure are also observed. In the earliest stages of apoptosis, phosphatidyl-serine, a membrane phospholipid generally associated to the inner cytoplasmic leaflet, is exposed to the outer leaflet and promotes phagocytosis by neighboring cells (Martin et al., 1995). This feature is useful in determining apoptosis by utilizing the anticoagulant, protein annexin V, which has a specific and high affinity for phosphatidyl-serine. Healthy cells incubated in fluorescein isothiocyanate (FITC)-conjugated annexin V remain unlabelled, as annexin V can not access to the inner leaflet of the plasma membrane. However, cells undergoing apoptosis show a marked increase in annexin V binding, indicating that phosphatidyl-serine is translocated to the outer leaflet (Martin et al., 1995).

Rounding and separation of apoptotic cells from neighboring cells is associated to a lack of gap junction communication between cells (Hardy et al., 1996). Deoxyribonucleic acid between nucleosomes is cleaved by endonucleases into oligonucleosomal fragments (Arends and Willye, 1991; Rueda et al., 1995). These fragments can be visualized as a typical "DNA ladder" with bands at 180-200 bp intervals by agarose gel electrophoresis.

Due to the small number of cells in preimplantation embryos, it is impossible to use electrophoretic techniques to observe for DNA laddering typical of apoptotic nucleus (Hardy, 1999). Fortunately, development of TdT-mediated dUTP nick-end labelling (TUNEL) enables assessment of nuclear DNA fragmentation *in situ* (Gavrieli et al.,

1992). Matwee et al. (2000) observed TUNEL labelling in bovine oocytes, 8- to 16-cell embryos, morula, and blastocyst stage embryos, but labelling was not observed in zygotes or early cleavage stage embryos. Similar results were observed in human (Jurisicova et al., 1996) and porcine embryos (Long et al., 1998) suggesting that occurrence of apoptosis is dependent on developmental stage of the embryo and early stage embryos (before compaction) seem to be more resistant to apoptosis than later stages.

Levels of transcripts involved in the apoptotic pathway might be below a specific threshold before embryonic genome activation and cellular differentiation into the trophectoderm and inner cell mass. Undifferentiated tissues during fetal development in humans possess an inhibitor of caspases activity (Adida et al., 1998). Early stage bovine embryos may possess a similar mechanism (maternally produced proteins and mRNA) to avoid apoptosis until morula stage. Apoptosis may also be an efficient mechanism to eliminate blastomeres that did not begin transcription of the embryonic genome after the 8-cell stage in cattle.

Prostaglandin synthesis and action

Prostaglandins are biochemical compounds derived from the polyunsaturated fatty acid, arachidonic acid. Prostaglandins are involved in several physiological processes such as inflammation, production of pain and fever, control of blood vessel tone and arterial blood pressure, luteolysis, and blood clotting (Nett et al., 1976; Seibert et al., 1994; Blatteis and Sehic, 1997). With the exemption 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 stimulation (Bito, 1975).

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid (AA), a precursor of eicosanoids including prostaglandins and leukotrienes. So far, at least 19 enzymes that possess PLA_2 activity have been identified in mammals (reviewed by Murakami and Kudo, 2002). Since arachidonic acid contains 20 carbon atoms, prostaglandins are referred to eicosanoids. Arachidonic acid is derived from membrane phospholipids primarily by the action of cytoplasmic phospholipase A_2 (Flint et al., 1986). This cleavage state is the rate-limiting step in prostaglandin synthesis (Figure 2-3). Free arachidonic acid is then converted to prostaglandin H_2 (PGH_2) by the action of cyclooxygenase enzymes at the membranes of the endoplasmic reticulum (Smith and DeWitt, 1996) or the nucleus (Parfenova et al., 2001).

There are two forms of cyclooxygenase: COX-1, a constitutive enzyme found in almost all cells; and COX-2, an inducible form in response to inflammatory reactions, hormonal and environmental stimulus (Smith and DeWitt, 1996). Cyclooxygenase-1 and –2 catalyze the conversion of free arachidonic acid to PGH_2 by 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).

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. Prostaglandin production can be blocked by administration of nonsteroidal anti-

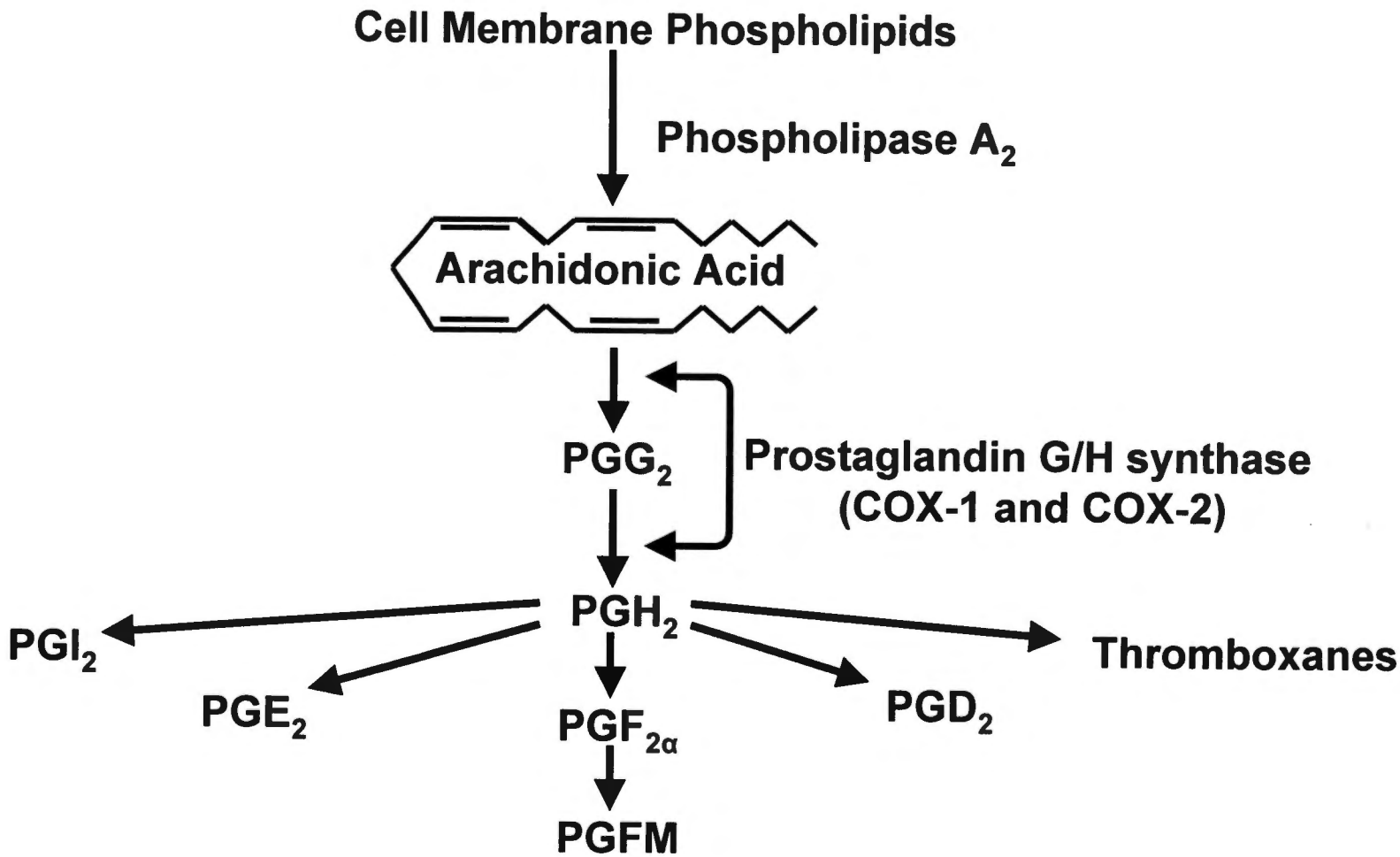

Figure 2-3. Schematic representation of prostaglandin $F_{2\alpha}$synthesis.

inflammatory drugs (NSAIDs) such as aspirin, 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).

Mechanism of action of $PGF_{2\alpha}$

In nonpregnant cows, $PGF_{2\alpha}$ is released from the uterus in a pulsatile manner to cause regression of the corpus luteum (Kindahl et al., 1976) and allows ovulation of the dominant follicle. Oxytocin stimulates uterine secretion of $PGF_{2\alpha}$ when administered *in vivo* (Silvia and Taylor, 1989) and *in vitro* (Lafrance and Goff, 1990). Oxytocin released from large luteal cells binds to its receptors in the uterus endometrium and stimulates uterine secretion of $PGF_{2\alpha}$ by activation of phoslipase A_2 and conversion of arachidonic acid to $PGF_{2\alpha}$. Then, uterine $PGF_{2\alpha}$ stimulates further oxytocin release from the corpus luteum (CL; Flint and Sheldrick, 1982). Thus, a positive feedback loop between $PGF_{2\alpha}$ and oxytocin seems responsible for the generation of each pulse of $PGF_{2\alpha}$ during luteolysis.

The $PGF_{2\alpha}$ receptor is a seven transmembrane G-protein coupled receptor (Sakamoto et al., 1994). After binding to its receptor, $PGF_{2\alpha}$ activates a membrane-bound phospholipase C, which causes hydrolysis of membrane phosphatidylinositol bi-phosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1987). Increased concentrations of IP_3 result in a release of free Ca^{++} from intracellular

stores to the cytosol, while DAG enhances the affinity of protein kinase C (PKC) for Ca^{++}, which results in PKC activation.

Although information regarding the presence of $PGF_{2\alpha}$ receptors in early stage embryos has not been found in the literature, 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 the 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 $F_{2\alpha}$ also 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).

Although $PGF_{2\alpha}$ signals cells through G-protein coupled receptors, other prostaglandins such as the PGJ_2 series are actively incorportated into the nucleus and associate with nuclear proteins by unknown mechanisms (Narumiya et al., 1987). 15-deoxy-12, 14- PGJ_2 is the final metabolite of PGD_2 degradation and is a potent ligand for a nuclear hormone receptor known as peroxisome proliferator-activated receptor γ (PPAR-γ). Peroxisome proliferator-activaded receptor-γ is a member of a superfamily of nuclear hormone receptors that function as ligand-dependent transcription factors (Schoonjans et al., 1996). Three receptor subtypes of PPARs (termed α, δ, and γ) have been identified. The transcriptional activity of PPAR subtypes is enhanced by numerous chemical compounds including fatty acids, selected prostaglandins, and peroxisome proliferators (Schoonjans et al., 1996).

After activation, PPARs control expression of genes implicated in extra- and intracellular lipid metabolism involved in peroxisomal β-oxidation pathways (Schoonjans, 1996). Padilla et al. (2000) reported that PPAR-γ agonist and PGJ_2 produced cytotoxicity and antiproliferative effects on normal and malignant B cells. Cytotoxicity was mediated via apoptosis as shown by positive annexin V staining and

confirmed by DNA fragmentation detected using TUNEL assay. Prostaglandin I_2, a key prostaglandin during mouse implantation, also mediates its actions through activation of PPARs (Hyunjung 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).

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., 1993b). Moreover, when good quality frozen-thawed embryos were transferred to progestogen-supplemented animals on day 7 after estrus, pregnancy was maintain 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 cycle 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., 1993a).

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 were injected to nonlactating 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 $PGF_{2\alpha}$ 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), and plant toxins (tall fescue; J. W. Oliver, personal communication) 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; Schrick et al., 2001). 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.

Summary of literature

Early embryonic wastage results in reduced reproductive performance of cattle and in a vast economic loss to beef and dairy producers. Several factors such as heat stress, nutrition, mastitis, plant toxins and manipulation of the reproductive tract may cause early embryonic death by induction of $PGF_{2\alpha}$ release from the uterine endometrium.

Presence of $PGF_{2\alpha}$ has been shown to be have direct and indirect negative effects on embryo development; however, the exact mechanisms of $PGF_{2\alpha}$ responsible of lowering embryo survival are still unclear. Possible explanations may involve: 1) stimulation of the apoptotic cascade (by up regulation of pro-apoptotic genes, activation of endonucleases and/or formation of free radicals); and/or 2) unknown downstream signals capable of phosphorylation/dephosphorylation of key regulatory proteins (such as PKC) during compaction, which may lead to failures to either compact or form a blastocoelic cavity.

The objective of this study was to determine any direct effect of $PGF_{2\alpha}$ on embryonic development of pre-compacted (*in vitro*-produced) and compacted (*in vivo*-derived) bovine embryos.

CHAPTER 3

EXPERIMENTAL PROCEDURES

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). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) were purchased from Cayman Chemical Company (Ann Arbor, MI). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Follitropin-V was provided by Vetrepharm Canada, Inc. (London, Ontario) and luteinizing hormone (LH) was obtained from the United States Department of Agriculture (Beltsville, MD). Vigro was purchased from AB Technology, Inc. (Pullman, WA). Embryos were generously donated by Trans Ova (Sioux City, IA), Em Tran (Elizabethtown, PA), and Harrogate Genetics Inc. (Harrogate, TN). Ovaries were purchased from Brown's Packing Plant (Gaffney, SC). Media (HEPES-TALP, IVF-TALP, and SPERM-TALP; Parrish et al., 1988) and KSOM (Biggers et al., 2000), with modifications generously provided by Dr. John Hasler, were prepared in the laboratory or purchased from Specialty Media.

Preliminary experiment

A preliminary experiment was performed to determine if differences in embryo development existed between KSOM media supplemented with either PVA (3 mg/mL,

0.3%) or BSA (5 mg/mL, 0.5%). This study was performed due to a concern regarding the possibility that $PGF_{2\alpha}$ might bind to BSA present in KSOM culture medium utilized during embryo development (Tanaka et al., 2000). A total of 187 *in vivo*-derived glycerol frozen bovine embryos were thawed using the three-step glycerol removal procedure (see below). Morula stage embryos were cultured for 3 days in either KSOM supplemented with PVA or BSA in a humidified atmosphere of 5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C. After 3 days of culture (corresponding to day 9 of embryo development; Figure 2-2), blastocyst development was assessed using IETS guidelines for classification of bovine embryos (see below).

Chi-square analysis was used to determine potential differences in development of embryos (SAS 8.02, SAS Institute Inc., Cary, NC). There were no differences ($P>0.10$) in proportion of morula continuing to blastocyst between KSOM-PVA (45.2%) or KSOM-BSA (54.2%) culture media. As such, PVA was added to KSOM media during $PGF_{2\alpha}$ treatments to avoid any possible binding of $PGF_{2\alpha}$ to BSA.

Preparation of prostaglandin stocks

Prostaglandin $F_{2\alpha}$ (1 mg) and PGE_2 (1 mg) were solubilized in 10 mL of DMSO each (Stock #1 of either $PGF_{2\alpha}$ or PGE_2, 1000 μg/mL). Then, 30 μL aliquots from each prostaglandin solution were stored at -20 C° until needed. For each replicate of Experiments 1 and 2, serial 10-fold dilutions of $PGF_{2\alpha}$ stock #1 in KSOM-PVA were made to yield desired concentrations of $PGF_{2\alpha}$. Likewise, stock #1 of PGE_2 was diluted to a final concentration of 5 ng/mL in 3 replicates of Experiment 1.

Experimental Methods for Experiment 1

Oocyte collection

The procedures utilized for *in vitro* production of embryos (IVP) were modifications of procedures previously described by Edwards and Hansen (1996). 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 water equilibrated to arrival temperature of ovaries (generally between 28 to 30 C°). Extraneous tissue surrounding the ovaries was removed and ovaries were washed with 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 clean. 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 with 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 22 h after placement in OMM). Maturation medium was

equilibrated in the incubator (5.5% CO_2 in air at 38.5°C) for a minimum of 5 h before oocyte collection.

Sperm preparation and in vitro fertilization

At 22-24 h following oocyte collection, maturation medium from each well was carefully removed and 25 µl of penicillamine/hypotaurine/epinephrine (PHE) and 500 µL of fertilization medium (IVF-Talp) were added per well. Two straws of semen from two bulls (140 Se5 and 140 Se55), 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 (2 mL 45% Percoll over 2 mL 90% Percoll contained in a 15 mL conical tube) and sperm was centrifuged at 2200 rpm 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 1100 rpm for 8 min. Supernatant was removed and sperm pellet re-suspended in 500 µL of modified IVF-Talp. Sperm concentration was determined using a hemocytometer and sperm was added to each well at a final concentration of 375,000 sperm per well. Lastly, oocytes and sperm were incubated at 5.5% CO_2 in air at 38.5°C for 18-22 h.

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 15 mL conical tube containing 500 μL of Hepes-Talp and vortexed for 4 min. Recovered putative zygotes were washed 4 times in Hepes-Talp and once in KSOM-BSA before transferring groups of approximately 50 zygotes to 4-well plates containg 500 μL of KSOM-BSA 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 for 5 days.

On day 5 of embryonic development, 16- to 32-cell stage embryos were rapidly washed in KSOM-PVA four times, evaluated for quality (normal shape, defined blastomeres, extruded blastomeres, cytoplasmic fragmentation, even cytoplasm), evenly sorted in four groups (formed by embryos of good quality), transferred to experimental treatments 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). Embryos remained in each treatment for 4 additional days (Figure 3-1). Embryo development was assessed using IETS guidelines for classification of bovine embryos.

Utilizing past studies on concentrations of $PGF_{2\alpha}$ in uterine flush media, dilutions of $PGF_{2\alpha}$ were prepared to obtain 0, 1, 10 and 100 ng/mL (Figure 3-1). Treatments were placed in each well of a 4-well plate (500 μL/well) and equilibrated in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for at least 5 h prior to embryo placement.

For each replicate, two media samples from each treatment were collected to determine concentrations of $PGF_{2\alpha}$ by radioimmunoassay. The first sample was collected

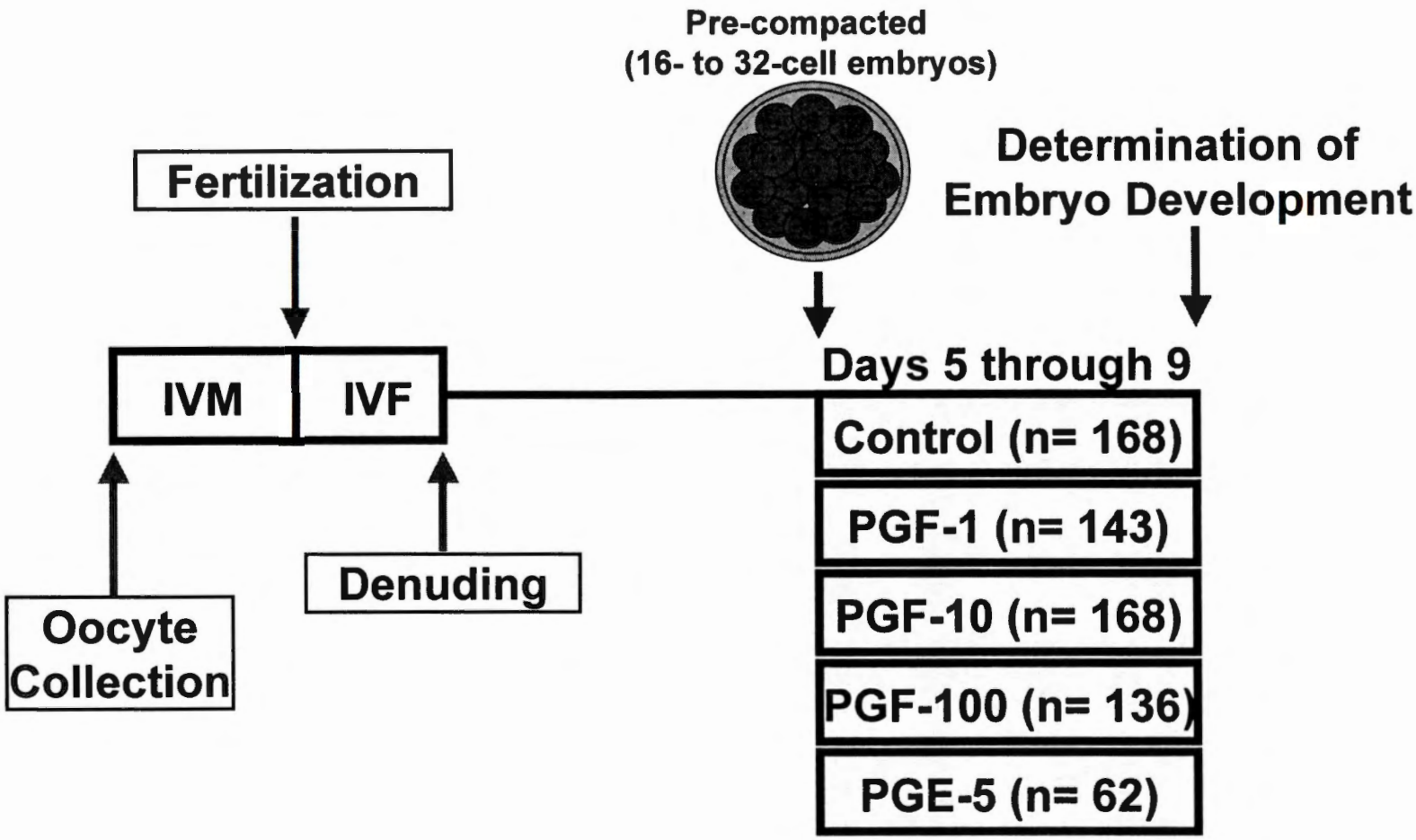

Figure 3-1. Schematic illustration of experimental design for Experiment1. Treatments were control, 1 ng/mL (PGF-1), 10 ng/mL (PGF-10), and 100 ng/mL of prostaglandin $F_{2\alpha}$(PGF-100), and 5 ng/mL of prostaglandin E_2 (PGE-5).

on the day in which treatments began (day 5 of culture) and the second sample obtained after determination of blastocyst development (day 9 of culture).

A total of 7 replicates were utilized for Experiment 1. In each replicate, embryos at 16- to 32-cell stage receive one of the following culture treatments: 1) control (KSOM-PVA; n= 168); 2) PGF-1 (1 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 143); 3) PGF-10 (10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 168); 4) PGF-100 (100 ng/mL $PGF_{2\alpha}$ in KSOM-PVA; n= 136), and 5) PGE-5 (5 ng/mL PGE_2 in KSOM-PVA; n= 62; Figure 3-1). Prostaglandin E_2 is thought to be a "beneficial" prostaglandin during embryo development (Biggers et al., 1978). Therefore, embryos cultured in media with addition of 5 ng/mL of PGE_2 were considered to be a positive control group (Gurevich et al., 1993).

Experimental Methods for Experiment 2

In vivo-derived glycerol-frozen bovine embryos were thawed utilizing the three-step glycerol removal procedure. Briefly, after removal from liquid nitrogen, straws containing one or more embryos were held in air for 10 sec and then placed in a water bath at 25°C for 45 sec. Straws were then emptied into an empty well of a 6-well plate. Embryos were subsequently held and washed 5 min in each of the following solutions (present in the same 6-well plate): 1) 1 mL of a solution made of 2 mL of 1M sucrose plus 4 mL 10% glycerol, 2) 1 mL of a solution made of 2 mL of 1M sucrose plus 2 mL 10% glycerol plus 2 mL PBS (with 0.4% BSA), 3) 1 mL of a solution made of 2 mL of 1M sucrose plus 4 mL PBS (with 0.4% BSA), and 4) 1 mL of PBS. After washing in the last solution, embryos were allowed to regain normal morphology for 30 min in Vigro

holding medium and sorted by stage of development and quality according with the IETS guidelines for classification of bovine embryos. For this study, a total of 4 replicates were used.

Schrick and coworkers (1993) reported 0.64 ng/mL of $PGF_{2\alpha}$ in flush media from short cycle cows. Based on these findings, concentrations of 0 (control, KSOM-PVA), 0.1, 1 and 10 ng/mL of $PGF_{2\alpha}$ in culture medium were selected as treatments. Each treatment was placed in a different well of a 4-well plate (500 µL/well) and then maintained in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C) for at least 5 h before placement of embryos to allow equilibration of medium. At the same time, media samples from each treatment were collected and stored at -20° C for determination of $PGF_{2\alpha}$ concentrations by radioimmunoassay.

Morula embryos (corresponding to day 6-7 of embryo development; Figure 2-2) of quality grade 1, 2 or 3 were rapidly washed four times in KSOM-PVA and randomly assigned to one of four treatments: 1) control (KSOM with PVA, n= 110); 2) PGF-0.1 (0.1 ng/mL $PGF_{2\alpha}$ in KSOM-PVA, n=108); 3) PGF-1 (1 ng/mL $PGF_{2\alpha}$ in KSOM-PVA, n=109), 4) PGF-10 (10 ng/mL $PGF_{2\alpha}$ in KSOM-PVA, n=109; Figure 3-2) and placed in the incubator (5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C).

After culturing embryos for 24 h in their respective treatment, embryos were washed and placed in KSOM-BSA without $PGF_{2\alpha}$ for an additional 48 h (Figure 3-2). Evaluation of embryo development using IETS guidelines for classification of bovine embryos was performed by experienced technicians uninformed of treatments.

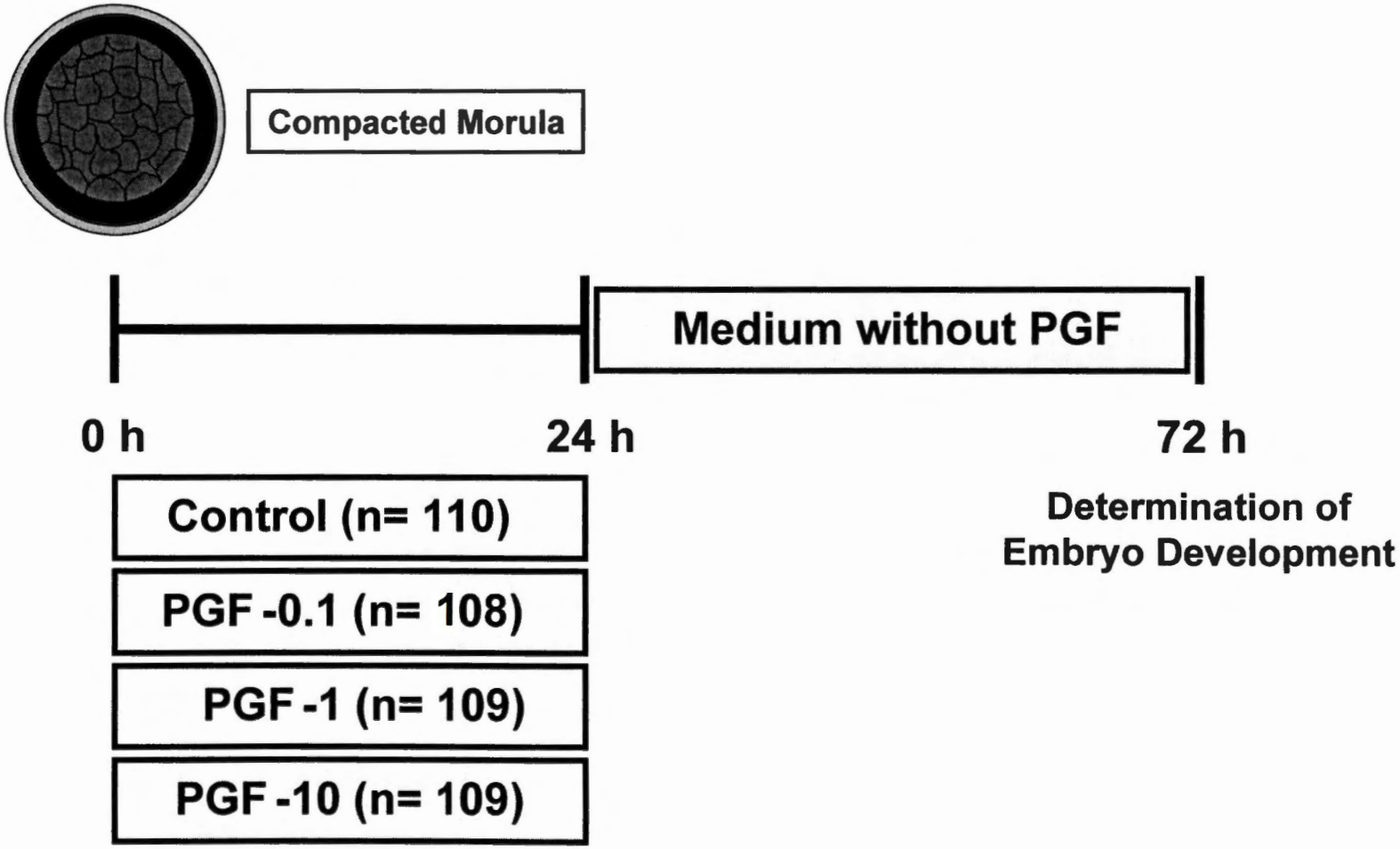

Figure 3-2. Schematic illustration of experimental design for Experiment 2. Treatments were control, 0.1 ng/mL (PGF-0.1), 1 ng/mL (PGF-1), and 10 ng/mL of prostaglandin $F_{2\alpha}$(PGF-10).

Radioimmunoassay for $PGF_{2\alpha}$ concentrations in culture media

Concentrations of prostaglandin $F_{2\alpha}$ in culture media samples were determined by the procedure of Cooper et al. (1991) as modified by Schrick et al. (1993) for flash samples. Inter- and intra-assay coefficients of variation for the $PGF_{2\alpha}$ assay were 14% and 24%, respectively. For Experiment 1, concentrations of $PGF_{2\alpha}$ in culture media at the beginning (day 5) and at the end of culture period (day 9) yielded: 0.3 and 0.4 ng/mL for control, 1.1 and 1.3 ng/mL for PGF-1, 9.6 and 11.0 ng/mL for PGF-10, and 80.9 and 103.5 ng/mL for PGF-100, respectively (Figure 3-3). Prostaglandin E_2 concentrations at the beginning of the treatments (0.27 ng/mL) were analyzed using the same RIA protocol than $PGF_{2\alpha}$ to verify absence of the latter during the culture period. For Experiment 2, concentrations of $PGF_{2\alpha}$ were 0.4, 0.5, 1.3, and 9.9 ng/mL for control, PGF-0.1, PGF-1, and PGF-10, respectively (Figure 3-4).

Guide for classification of bovine embryos

The International Embryo Transfer Society (IETS) has published a guide for classification of bovine embryos according to stage of development and quality (Manual of the International Embryo Transfer Society. 1998. Third Edition). As mentioned, these guidelines for development were used throughout both experiments.

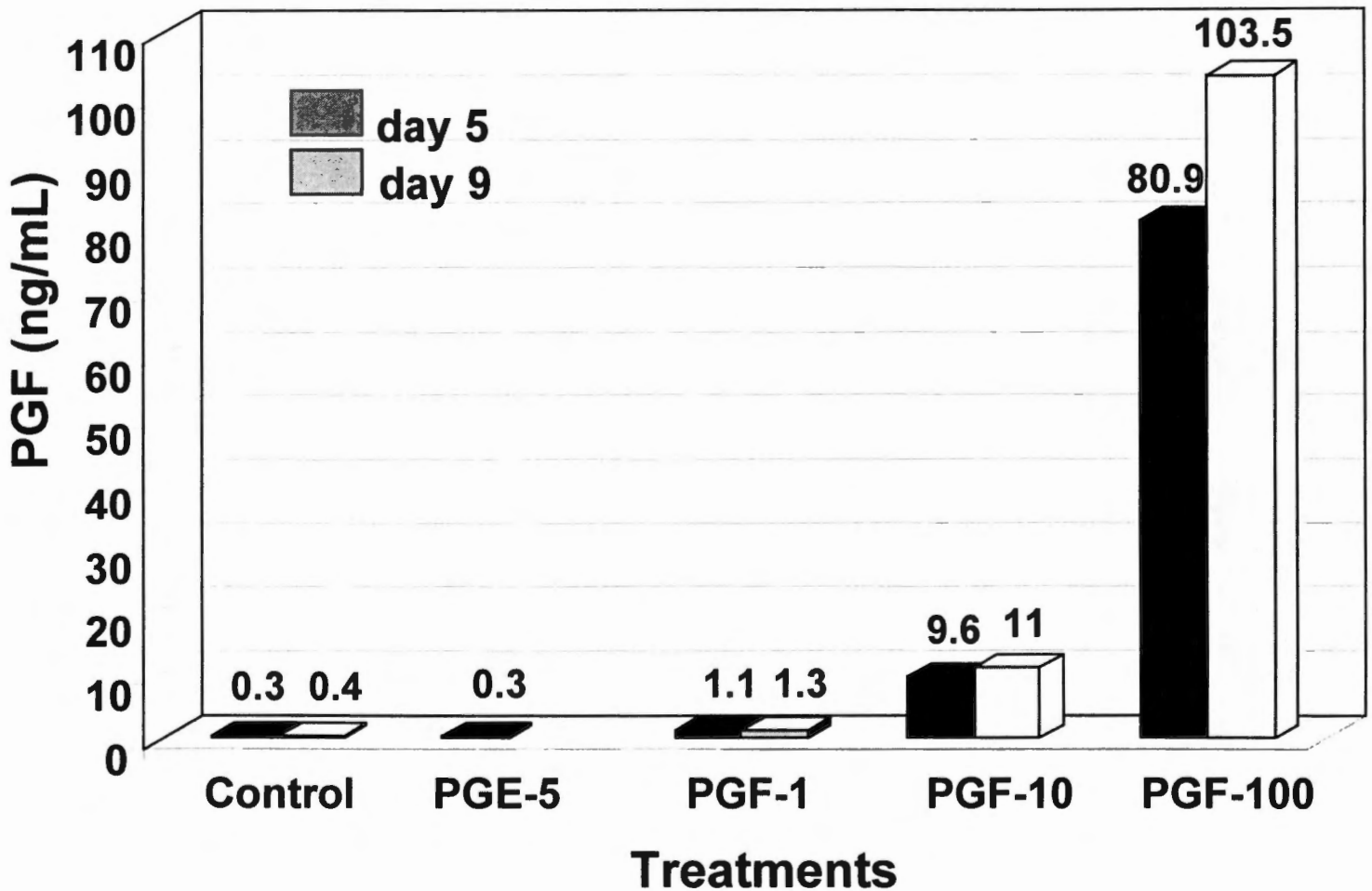

Figure 3-3. Concentrations of $PGF_{2\alpha}$ in the culture medium at the beginning (day 5) and at the end (day 9) of experimental period for Experiment 1. Treatments were control, 5 ng/mL of prostaglandin E_2 (PGE-5), 1 ng/mL of prostaglandin $F_{2\alpha}$ (PGF-1), 10 ng/mL of prostaglandin $F_{2\alpha}$ (PGF-10), and 100 ng/mL of prostaglandin $F_{2\alpha}$(PGF-100).

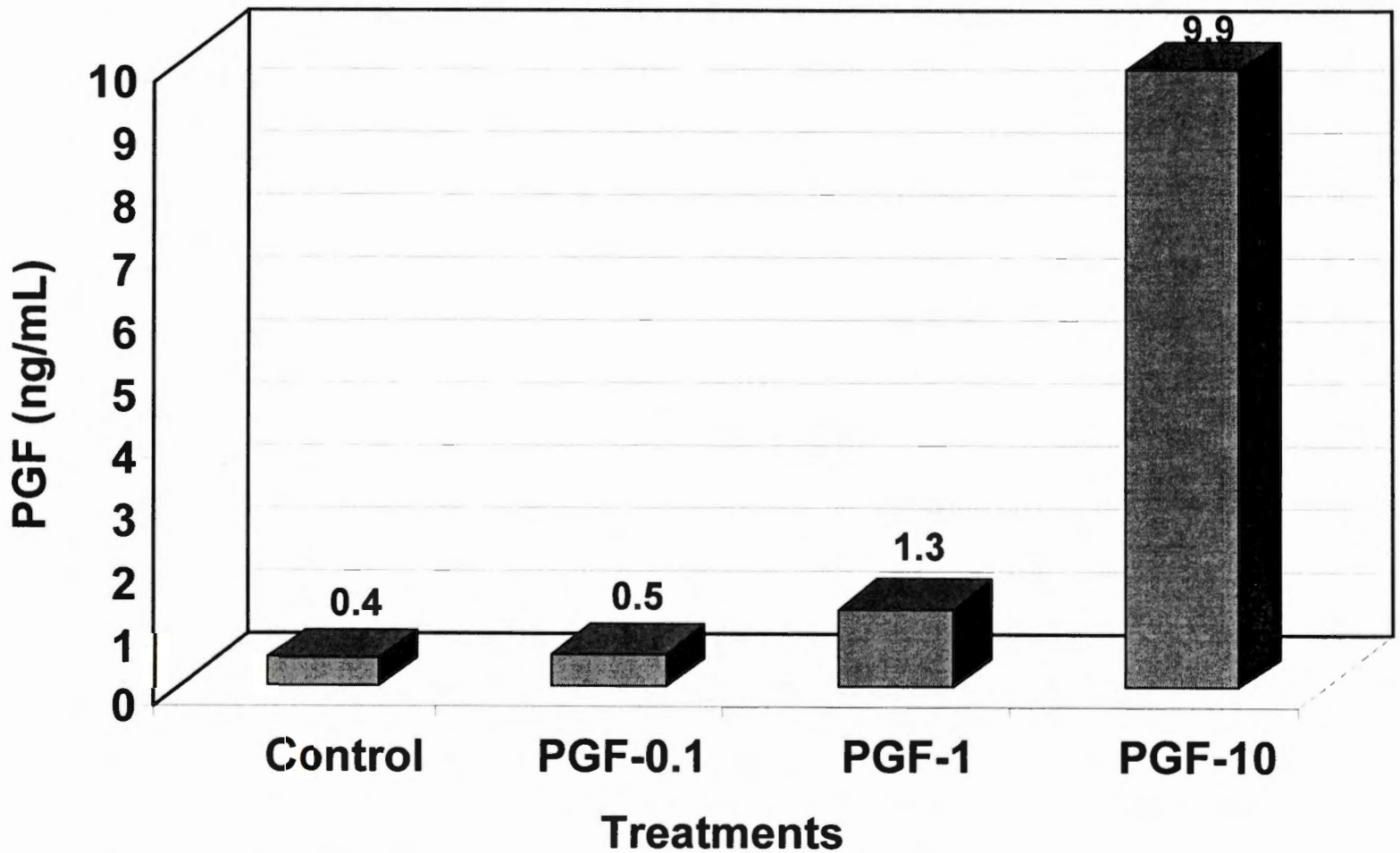

Figure 3-4. Concentrations of $PGF_{2\alpha}$ in the culture medium for Experiment 2. Treatments were control, 0.1 ng/mL (PGF-1), 10 ng/mL (PGF-10), and 100 ng/mL of prostaglandin $F_{2\alpha}$(PGF-100).

No.	Stage
1	Unfertilized
2	2- to 12-cell
3	Early Morula
4	Morula
5	Early Blastocyst
6	Blastocyst
7	Expanded Blastocyst
8	Hatched Blastocyst
9	Expanded Hatched Blastocyst

Statistical analysis

Data for Experiments 1 and 2 were analyzed to test for blastocyst development and hatching rates using an incomplete block design and a randomized block design, respectively. Analysis of variance was performed using mixed models (SAS 8.02, SAS Institute Inc., Cary, NC) and contrasts were tested to identify differences across levels of prostaglandin $F_{2\alpha}$ on embryonic development. Analyses were then validated with Chi-square analysis (SAS 8.02, SAS Institute Inc., Cary, NC).

CHAPTER 4

RESULTS AND DISCUSSION

The aim of this thesis was to investigate effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on development of *in vitro*-produced and *in vivo*-derived bovine embryos. Utilization of these types of bovine embryos provided a model to ascertain effects of $PGF_{2\alpha}$ in culture on pre-compacted (*in vitro*-produced) and compacted (*in vivo*-derived) embryos.

Results from Experiment 1 indicated that culture of 16- to 32-cell pre-compacted (*in vitro*-produced) embryos with 1, 10 or 100 ng/mL of $PGF_{2\alpha}$ in the culture medium reduced blastocyst development when compared to control (30.4 ± 5.3, 41.4 ± 5.1, 33.3 ± 5.3, and 51.8 ± 5.1 respectively, $P=0.002$, Figure 4-1). In addition, culture of embryos in media containing 1 ng/mL of $PGF_{2\alpha}$ had a more detrimental effect than addition of 10 ng/mL of $PGF_{2\alpha}$. However, hatching rates between treatments did not differ and neither blastocyst development nor hatching rates differed between control and PGE_2 treatments ($P>0.10$). Thus, addition of $PGF_{2\alpha}$ to culture medium had a direct negative effect on development of 16- to 32-cell *in vitro*-produced embryos to blastocyst without altering hatching rates.

These results agree with Breuel et al. (1993a) and Buuck (1997) in which addition of $PGF_{2\alpha}$ during *in vitro* culture decreased development of 8-cell rat embryos to blastocyst. Furthermore, administration of $PGF_{2\alpha}$ to progestogen-supplemented cows on days 5 through 8 after mating decreased pregnancy rates (Seals et al., 1998) and embryo quality and development in cattle (Hockett et al., 1998). Lemaster et al. (1999) reported that administration of oxytocin on days 5 to 8 after mating to progestogen-supplemented

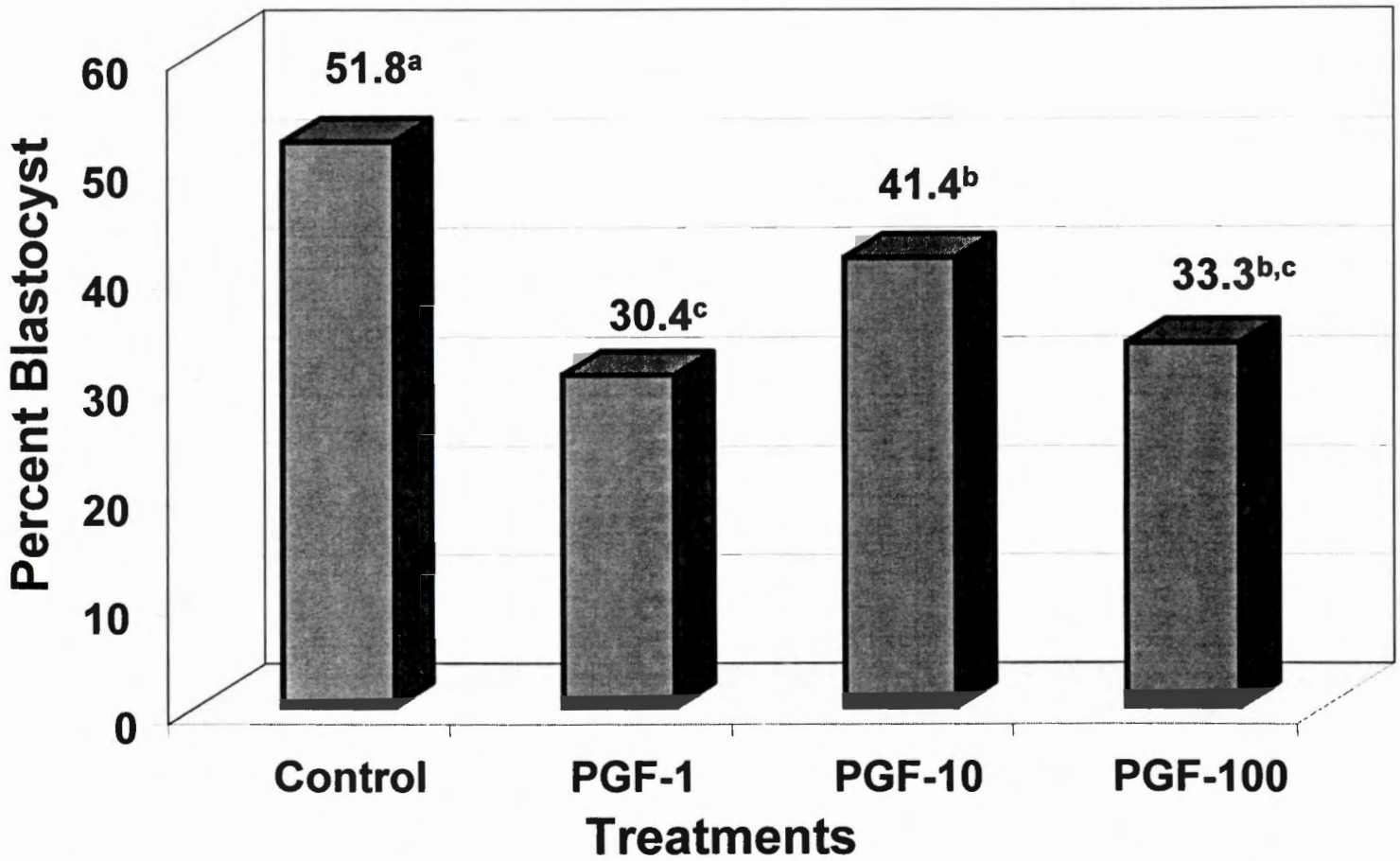

Figure 4-1. Percentage of pre-compacted embryos that continued development to blastocyst in Experiment 1. Treatments were control, 1 ng/mL (PGF-1), 10 ng/mL (PGF-10), and 100 ng/mL of prostaglandin $F_{2\alpha}$ (PGF-100). [a,b,c]Values with different superscripts differ; *P=0.002*). Pooled standard errors were ± 5.1, 5.3, 5.1 and 5.3 for control, PGF-1, PGF-10 and PGF-100, respectively.

cows reduced pregnancy rates by stimulation of uterine release of $PGF_{2\alpha}$. Therefore, reduction in development to blastocyst stage could be related to detrimental effects of $PGF_{2\alpha}$ on embryonic development prior to and/or during the compaction process.

Compaction, which in bovine embryos occurs at the 16- to 32-cell stage, is an essential step of embryonic development in which formation of cell-to-cell adhesion molecules allows blastomeres to differentiate into two distinctive cell populations (the trophectoderm and the inner cell mass) and cavitation to occur (Watson et al., 1992). Utilization of 16 to 32-cell embryos in Experiment 1 served as a model to investigate $PGF_{2\alpha}$ action(s) prior to or during compaction. Formation of gap junctions and cadherins during compaction is considered a key event for continued development and blastocyst morphogenesis (Fleming et al., 2001). In fact, injection of gap junction antisense RNA decreased compaction and blastocyst formation in the mouse embryo (Bevilacqua et al., 1989); whereas, mouse embryos lacking E-cadherin failed to generate a normal trophectoderm and died before implantation (Laure et al., 1994; Riethmacher et al., 1995).

The most understood mechanism by which $PGF_{2\alpha}$ exerts its biological activity is through receptor-mediated events. However, since detrimental effects of $PGF_{2\alpha}$ on embryonic development did not show a "dose response" change in development to blastocyst, actions of $PGF_{2\alpha}$ on bovine embryos may not involve receptor-mediated events. Prostaglandin $F_{2\alpha}$ binds to specific G-protein coupled receptors on targeted cells (Sakamoto et al., 1994). Using the corpus luteum as an example, the ligand-receptor binding activates phospholipase C that produces the hydrolysis of phosphatidylinositol

bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Then, IP_3 induces the release of Ca^{++} from the endoplasmic reticulum increasing intracellular concentrations of calcium, while DAG increases the affinity of PKC for Ca^{++} (Davis et al., 1987). The final outcome is a dramatic increase in intracellular levels of Ca^{++} and activation of PKC. Protein kinase C can then phosphorylate and regulate other proteins. Protein kinase C-dependent excessive phosphorylation of connexin 43 inhibited gap junction communication and impaired ventricular conduction in the heart (Riley and Behrman, 1991; Carlson et al., 1993). Increased intracellular levels of calcium by $PGF_{2\alpha}$ induced apoptosis in luteal cells during corpus luteum regression (Juengel et al., 1993). Calcium stimulates Ca^{++}-dependent endonucleases to cut DNA into internucleosomal fragments, characteristic of the apoptotic process (Rueda et al., 1995). Furthermore, Criswell (1995) also demonstrated that high intracellular levels of Ca^{++} and addition of Ca^{++} ionophore 4-br-A23187 decreased gap junction communication. Thus, $PGF_{2\alpha}$ may exert negative effects on pre-compacted embryos by altering the formation and/or function of gap junctions or by inducing apoptosis through activation of endonucleases. Decreased intercellular communication and stimulation of apoptosis in early embryos will result in impaired cellular differentiation and growth, ultimately resulting in embryonic death.

Prostaglandins are also assumed to traverse membranes by simple diffusion. However, prostaglandins are charged anions at physiological pH and may transverse biological membranes inefficiently (Bito, 1975). This limited simple diffusion appears to be augmented by a carrier mediated prostaglandin transport (PGT; (Shuster, 1998). Prostaglandin transporter is a member of the 12-membrane-spanning superfamily of

transporters and has been cloned in rats and humans (Kanai et al., 1995; Lu et al., 1996). Prostaglandin transporters from both species bind $PGF_{2\alpha}$, PGE_2, and PGD_2 with high affinity and might mediate transepithelial transport of prostaglandins to cells (Lu et al., 1996) and the efflux of newly synthesized prostaglandins from cells (Chan et al., 1998). Clearance of prostaglandins may also be associated with PGT (Kanai et al., 1995). The gene encoding PGT in humans is regulated by fluid mechanical stimuli in cultured endothelial cells (Topper et al., 1998), suggesting that this is an inducible gene potentially capable of responding to physiological stimuli *in vivo*. In the embryo, stretching of the trophectoderm during cavitation could induce PGT especially for binding of PGE_2. Prostaglandin E_2 has been shown to be involved in fluid accumulation during blastocyst formation and hatching of the embryo from the zona pellucida (Biggers et al., 1978). It may be possible that $PGF_{2\alpha}$ could also bind and be internalized into individual blastomeres through PGT that normally would bind PGE_2.

Prostaglandin $F_{2\alpha}$ might also enter the cells by simple diffusion or by binding to PGT and activating peroxisome proliferator-activated receptors (PPAR) in the nucleus. Peroxisome proliferator-activated receptors are a superfamily of nuclear hormone receptors that function as a ligand-dependent transcription factors (Schoonjans et al., 1996). The transcriptional activity of PPAR is enhanced by numerous chemical compounds including fatty acids and prostaglandins (Schoonjans et al., 1996). After activation, PPARs could then regulate transcription of developmentally important genes involved in embryonic development (connexin 43, E-cadherin, Na^+-K^+-ATPase pump) or cause cytotoxicity via apoptosis. Padilla et al. (2000) reported that PPAR γ agonist and PGJ_2 produced cytotoxicity and antiproliferative effects on normal and malignant B cells.

Another action of $PGF_{2\alpha}$ to decrease embryonic development could be the generation of free oxygen radicals. Exposure of rat luteal cells to oxygen radicals induced apoptosis (Riley and Behrman, 1991; Carlson et al., 1993) and loss of fluidity in cellular membranes (Sawada and Carlson, 1991).

In Experiment 2, development to blastocyst of compacted (*in vivo*-derived) morula stage embryos (at least two stages beyond morula stage according to IETS guidelines) did not differ between treatments (68.1% for control, 58.9% for PGF-0.1, 61% for PGF-1 and 60% for PGF-10, $P>0.10$, Figure 4-2). However, culture of embryos in 0.1, 1, or 10 ng/mL of $PGF_{2\alpha}$ for 24 h decreased hatching rates when compared to control (24%, 29.1%, 24.5%, and 44.5% respectively, $P=0.05$, Figure 4-3).

Thus, results from Experiment 2 indicated that continued development of compacted (*in vivo*-derived) morula embryos to the blastocyst stage was not altered by addition of $PGF_{2\alpha}$ to culture medium. However, $PGF_{2\alpha}$ significantly reduced hatching rates of *in vivo*-derived morula. This is in agreement with Maurer and Beier (1976) who reported that addition of $PGF_{2\alpha}$ to culture medium directly decreased the ability of 8-cell rabbit embryos to form expanded and hatched blastocyst, but had no effect on development to early blastocyst. These findings are also consistent with *in vivo* studies in which progestogen-supplemented cows administered $PGF_{2\alpha}$ (days 5 through 8 after estrous) had decreased pregnancy rates following transfer of morula stage embryos (Hernandez-Fonseca et al., 1997). Whereas, transfer of blastocyst stage embryos to progestogen-supplemented cows receiving $PGF_{2\alpha}$ had pregnancy rates similar to controls (E. Keith Inskeep, personal communication). Moreover, Schrick et al. (2001) reported that administration of flunixin meglumine (a COX-1 and COX-2 inhibitor) at the time of

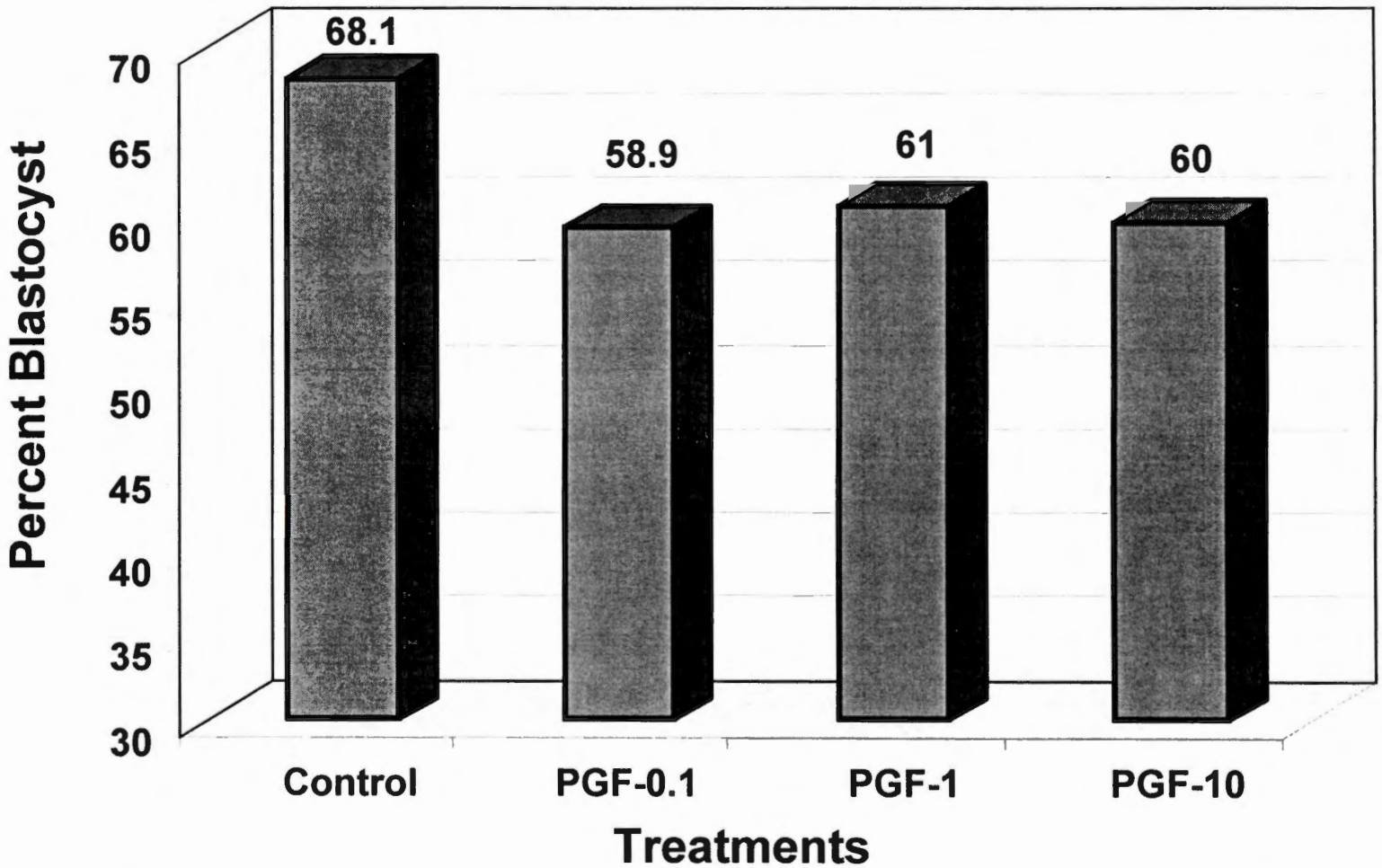

Figure 4-2. Percentage of compacted morula embryos that continued development to blastocyst in Experiment 2. Treatments were control, 0.1 ng/mL (PGF-0.1), 1 ng/mL (PGF-1), and 10 ng/mL of prostaglandin $F_{2\alpha}$ (PGF-10). Pooled standard errors were ± 5.4.

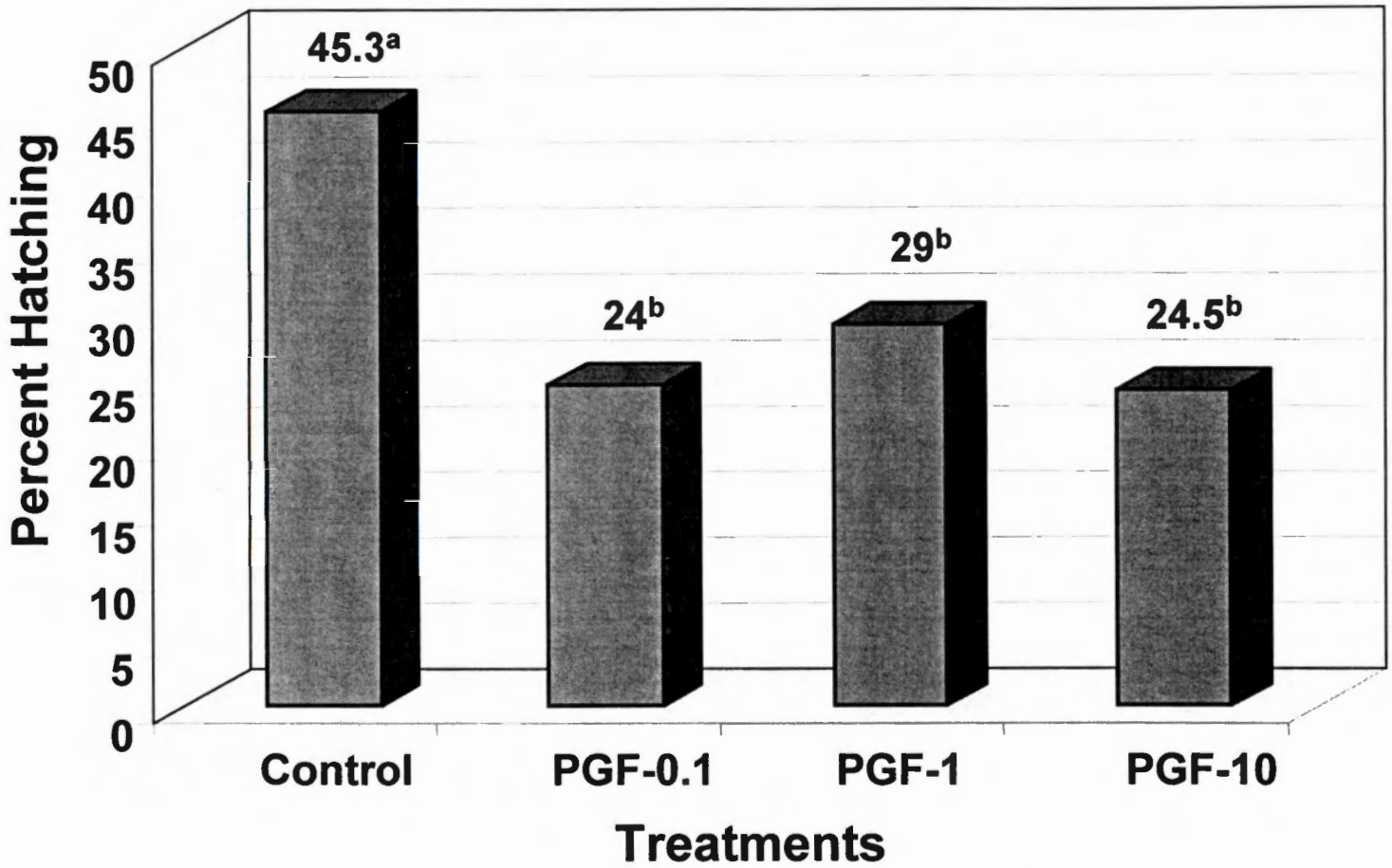

Figure 4-3. Percentage of compacted morula embryos that continued development to blastocyst and hatched from the zona pellucida in Experiment 2. Treatments were control, 0.1 ng/mL (PGF-0.1), 1 ng/mL (PGF-1), and 10 ng/mL of prostaglandin $F_{2\alpha}$ (PGF-10). [a,b]Values differ between treatments; *P=0.05*. Pooled standard errors were ±6.0.

embryo transfer improved pregnancy rates of morula and blastocyst stage embryos in cattle. Thus, results from past and current studies indicate a negative effect of $PGF_{2\alpha}$ on embryonic development after compaction in cattle.

Although the exact mechanisms by which $PGF_{2\alpha}$ is affecting embryonic development at the morula stage are unknown and evidence of $PGF_{2\alpha}$ receptors in preimplantation bovine embryos have not been located in the literature, some modes of action of $PGF_{2\alpha}$ on embryo development can be hypothesized. The reduction in hatching rates of *in vivo*-derived embryos may have resulted from an interruption of Na^+ transport across the trophectoderm epithelium by $PGF_{2\alpha}$. Kim and Yeoun (1983) observed a reduction of Na^+-K^+-ATPase activity after treating luteal membranes with $PGF_{2\alpha}$ (a receptor mediated event). After compaction, accumulation of blastocoelic fluid within the embryo causes expansion and hatching from the zona pellucida (Latham et al., 1999). Localization of an active Na^+-K^+-ATPase at the basolateral area of trophectoderm cell assures movement of Na^+ against a concentration gradient from the outside to the interior of the embryo (Watson and Kidder, 1988); whereas, tight junction formation between trophectoderm cells regulates paracellular transport of Cl^- and controls leakage of the nascent blastocoelic fluid (Fleming et al., 1989).

Possible explanation of deleterious effects of $PGF_{2\alpha}$ on hatching rates would involve disruption and/or dysfunction of tight junction or adherent junctions (E-cadherin) between trophectoderm cells. Tsukita et al. (1991) observed an increase in paracellular permeability of tight junctions due to elevated intracellular levels of Ca^{++}. Furthermore, Morgado-Diaz and de Souza (2000) reported that tyrosine phosphorylation causes disassembly of adherent junctions in cell culture. Both, high intracellular levels of Ca^{++}

and protein phosphorylation are common features associated with the action of $PGF_{2\alpha}$ on target cells.

In conclusion, these studies suggest a direct negative effect of $PGF_{2\alpha}$ on embryonic development of pre-compacted (*in vitro*-produced) and compacted (*in vivo*-derived) bovine embryos. Prostaglandin $F_{2\alpha}$ reduced the ability of 16- to 32-cell pre-compacted embryos to develop to blastocyst and may be related to deleterious actions of $PGF_{2\alpha}$ during or before the time of compaction (during the transition from 32-cell embryo to compacted morula). Furthermore, $PGF_{2\alpha}$ decreased hatching rates of compacted morula embryos possibly through disruption/dysfunction of tight junctions or by alterations in active transport of Na^+ during blastocoel formation. It was interesting to note that different concentrations of $PGF_{2\alpha}$ did not show a "dose response" effect on development of embryos and hatching rates. Thus, detrimental effects of $PGF_{2\alpha}$ on continued embryonic development does not appear to be a receptor-mediated event in either pre-compacted or compacted bovine embryos. Possible mechanisms of action may involve alterations in gap junction and cell adhesion molecules functionality and/or formation, induction of apoptotic mechanisms, or alterations in gene transcription during embryonic development. Future research should be aimed at determining exact mechanisms by which $PGF_{2\alpha}$ alters embryonic development.

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