To the Graduate Council:

I am submitting herewith a thesis written by Mitchell E. Hockett entitled "Embryonic Development in Progestogen-Supplemented Cows Administered Prostaglandin $F_{2\alpha}$". I have examined the final 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
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

EMBRYONIC DEVELOPMENT IN PROGESTOGEN-SUPPLEMENTED COWS ADMINISTERED PROSTAGLANDIN $F_{2\alpha}$

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ABSTRACT

An experiment was performed to examine the effects of elevated concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on embryonic development in cows supplemented with exogenous progestogen. Cows were inseminated artificially at estrus (day 0) and 12 h later. A synthetic progestogen (melengestrol acetate; 4 mg/cow·day^{-1}) was supplemented in feed from days 3 through 8. Cows were allotted randomly to receive either 15 mg $PGF_{2\alpha}$ (TRT; n=14) or saline (CON; n=10) at 0600, 1400 and 2200 h from days 5 through 8. Blood samples were collected at 0600 and 2200 h from days 5 day 8 for determination of progesterone and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM). Single embryos were recovered on day 8 by flushing the uterine horn ipsilateral to the corpus luteum (CL) as determined by ultrasonography on day 5. Each embryo was assigned a quality score of 1 (excellent) to 4 (poor), and stage of embryo development was recorded. Concentrations of progesterone were lower from days 5 to 8 in TRT versus CON cows (0.3 ± 0.14 vs 2.01 ± 0.16 ng/mL, respectively; P=0.0001). Overall, PGFM concentrations from days 5 to 8 were elevated in TRT cows (553.2 ± 42.1 pg/mL) compared to CON cows (13.2 ± 50.1 pg/mL; P=0.0001). Embryo quality scores were elevated in TRT cows compared to CON cows (2.9 vs 1.6, respectively; P=0.059). Percentage of

embryos considered transferrable (quality scores 1, 2, and 3) was decreased by administration of $PGF_{2\alpha}$ (Fisher's exact test, P=0.003). Nine of fourteen (64%) TRT embryos were retarded in development at day 8, whereas four of fourteen (29%) TRT embryos developed to expanded blastocyst. All CON embryos developed beyond the morula stage; eight of ten (80%) developed to the expanded blastocyst and two of ten (20%) reached the blastocyst stage upon recovery (Fisher's exact test, P=0.003).

In conclusion, treatment of progestogen-supplemented cows with $PGF_{2\alpha}$ reduced quality and retarded development of embryos prior to the blastocyst. Decreased fertility in conditions elevating PGFM concentrations may be a result of retarded development or reduced quality by $PGF_{2\alpha}$.

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1. INTRODUCTION

Reproductive efficiency of beef and dairy cattle is a primary concern of producers and managers. Beef cattle must wean a calf annually to be profitable. Dairy cows must have a calving interval that allows a maximum amount of time be spent in peak milk production.

Embryonic death may occur at any time during development; however, studies indicated that the greatest percentage of embryonic mortality occurred prior to day 8 of gestation (Ayalon, 1978; Maurer and Chenault, 1983). Causes of embryonic loss include luteal dysfunction and embryonic chromosomal abnormalities. Studies have shown deleterious effects of $PGF_{2\alpha}$ on early embryonic survival when administered on days 5 through 8 after mating in progestogen-supplemented cows (Buford et al., 1996; Hernandez-Fonseca et al., 1997; Seals et al., 1998). These same studies suggested that the regressing corpus luteum (CL) may be producing an embryotoxic factor(s) since lutectomy immediately prior to $PGF_{2\alpha}$ treatment prevented embryonic loss. A subsequent study by Lemaster et al. (1998) indicated that the embryotoxin from the regressing CL was oxytocin (OT) which acted indirectly through increasing uterine concentrations of $PGF_{2\alpha}$.

Prostaglandin $F_{2\alpha}$ may be released during uterine manipulation (Sequin et al., 1974), heat stress (Malayer et al., 1990), and following exogenous oxytocin administration to increase milk letdown and production (Newcomb et al., 1977; Farin and Estill, 1993). Furthermore, concentrations of $PGF_{2\alpha}$ are increased in cattle grazing endophyte-infected tall fescue (Seals et al., 1998). Elevated uterine concentrations of $PGF_{2\alpha}$ have been associated with reduced embryo quality and survival (Schrick et al., 1993). *In vitro* studies have indicated a direct, negative effect of $PGF_{2\alpha}$ on development of rabbit and rat 8-cell embryos (Maurer and Beier, 1976; Breuel et al., 1993a). Day 7 frozen-thawed bovine embryos cultured in varying concentrations of $PGF_{2\alpha}$ exhibited no decrease in development to the blastocyst stage (Fazio and Schrick, 1997). However, extremely high concentrations of $PGF_{2\alpha}$ (10 ng/ml) did inhibit development to expanded/hatched blastocyst. Subsequent *in vivo* studies narrowed the "window" of $PGF_{2\alpha}$ susceptibility prior to day 7 after mating (Hernandez-Fonseca et al., 1997; E. K. Inskeep, personal communication).

These results suggest that the time when embryos are sensitive to the detrimental effects of $PGF_{2\alpha}$ is prior to blastocyst development. Therefore, the objective of the current study was to administer $PGF_{2\alpha}$ to progestogen-

supplemented cows from days 5-8 postmating and evaluate developmental stage and quality of embryos flushed from the uterus on day 8 compared to control cows receiving saline only.

2. LITERATURE REVIEW

Embryonic development

During a normal estrous cycle, the luteinizing hormone (LH) surge initiates resumption of meiosis, maturation and release of the oocyte from the dominant follicle(s) (as reviewed by Espey, 1994). Following fertilization, embryonic development begins in the oviduct. The oviduct consists of four distinct regions: infundibulum, ampulla, ampullary-isthmic junction and the isthmus (Ellington, 1991). Upon release from the ovulatory follicle, the oocyte is captured by the infundibulum and transported to the ampulla. Later stages of oocyte maturation occur in the ampulla, while sperm capacitation occurs in the uterus and isthmus of the oviduct as gametes travel toward one another. At the ampullary-isthmic junction, a capacitated sperm binds and subsequently penetrates the zona pellucida of the matured oocyte. Fertilization of a mature ovum results in the formation of a zygote. Cleavage and subsequent development of the embryo continue in the isthmus until the 8- to 16-cell stage (Figure 1) when the embryo enters the uterus (Ellington, 1991; for review see Bazer et al., 1993).

Ninety-six hours after fertilization (approximately 5 days following onset of estrus), the embryo is referred to as a morula. Prior to the 8-16 cell stage, the early embryo

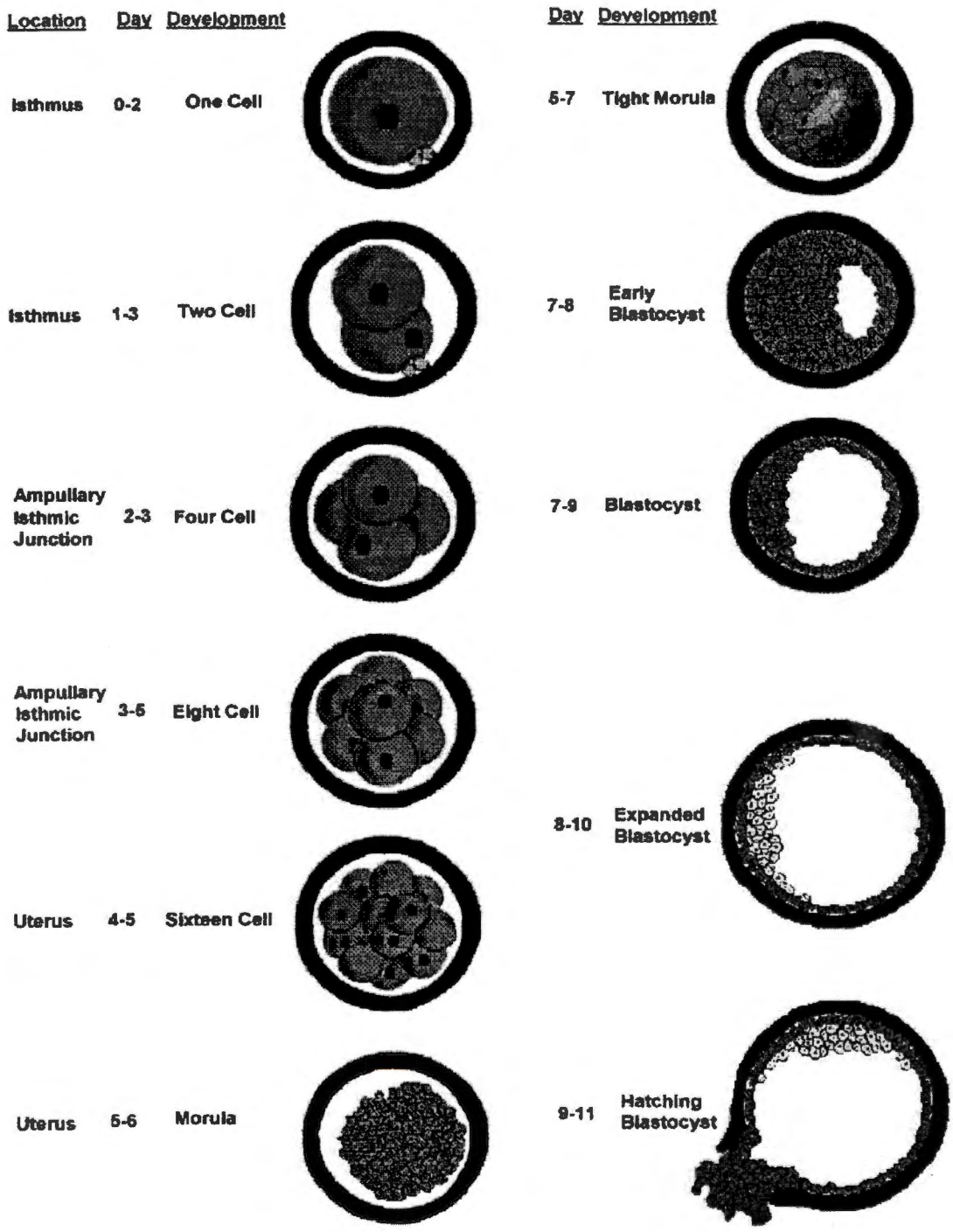


Figure 1. Location and time of embryonic development in the cow. Reprint permission granted by Dr. Rod Geisert, Oklahoma State University.

relies on messenger ribonucleic acids (mRNAs) and proteins of maternal origin for development. However, in order for the embryo to proceed in development, the embryonic genome must "switch on" to produce proteins needed for further development (Barnes and First, 1991).

Cleavage and development continue in the uterine horn ipsilateral to the CL where the embryo must advance through the compaction process (for review see Betteridge, 1988). During compaction, the morula forms a tight ball of cells in which the blastomeres (cells comprising the early embryo) adhere to and flatten against one another so that individual cell outlines are no longer visible (Pratt et al., 1982). The outermost layer of cells polarize and subsequently form the trophectoderm (Koyama et al., 1994) whereas the inner cells of the morula remain apolar and form the inner cell mass (ICM; Kimber et al., 1982). Outer cells form tight junctions with one another to form a barrier (Fleming et al., 1989). These junctions allow fluid to be moved into the embryo by active ion transport via the Na+/K+ ATPase pump to form the blastocoele (for review see Wiley et al., 1990). Inner cells form gap junctions to allow cell to cell communication. This arrangement of cells allows for development of the 3 germ layers: endoderm, mesoderm, ectoderm (for review see Bazer et al., 1993).

The ICM forms the embryo proper while the trophectoderm forms the extra embryonic membranes (for review see Bazer et al., 1993). Eight to 10 days after ovulation, the embryo "hatches" from the zona pellucida (Figure 1) due in part to the expansion of the blastocoele. During hatching, the zona layer becomes torn on the equatorial plane and the embryo exits through the opening.

Between days 15 and 19 of development, the bovine embryo must produce a signal to prevent $PGF_{2\alpha}$-mediated regression of the CL (Northey and French, 1980). This phenomenon has been classically referred to as maternal recognition of pregnancy (for review see Thatcher et al., 1995). Cattle and sheep embryos produce a protein known as bovine trophoblast protein (bTP-1) and ovine trophoblast protein (oTP-1), respectively (Helmer et al., 1987). Ovine trophoblast protein is synthesized by bovine trophectoderm from days 16-24 of pregnancy (Thatcher et al., 1989; Roberts et al., 1992). Godkin et al. (1982) reported pulses of $PGF_{2\alpha}$ necessary for luteolysis were blocked due to binding of oTP-1 to surface glandular epithelial cells of the endometrium. These proteins are also referred to as interferon-tau (IFN-τ; Roberts et al., 1992). Interferon-τ disrupts the cyclooxygenase enzyme complex by inducing an intracellular endometrial inhibitor (Gross et al., 1988). Interferon-τ

also down regulates the number of oxytocin receptors in the endometrium of the uterus; therefore, interrupting the feedback loop of luteal regression (Flint, 1989).

Embryonic loss

As defined by the Committee on Reproductive Nomenclature (1972), embryonic mortality is any loss which occurs during the first 42 days of pregnancy. Failure of normal embryonic development or luteal dysfunction are possible reasons for embryonic loss. Another possible cause includes a hostile uterine environment which may be detrimental to the embryo or CL. Fertilization rates of 90 to 95% are considered normal. Pregnancy rates, however, may be as low as 50-60% (Sreenan and Diskin, 1983).

Embryonic loss has been estimated to be between 20 (Ayalon, 1978) and 42% (Diskin and Sreenan, 1980). The greatest percentage of embryonic mortality in cattle occurs before day 18 after mating (Sreenan and Diskin, 1986). Additional studies have narrowed this time frame to before day 8 (Ayalon, 1978; Maurer and Chenault, 1983). Ayalon (1978) reported that the majority of embryonic loss occurred during the transformation from morula to blastocyst or between days 6 and 7. On the other hand, Diskin and Sreenan

(1980) reported that the majority of embryonic loss occurred during maternal recognition of pregnancy (days 16 to 18).

Synthesis of $PGF_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is a metabolic product of arachidonic acid. Arachidonic acid is a fatty acid which may be obtained from the diet or membrane lipids, which is converted from linoleic acid by desaturase enzymes (Moore, 1985). Several pathways exist for production of prostaglandins through the arachidonic acid cascade (reviewed by Milvae, 1986).

After release of arachidonic acid, two predominate pathways exist (Figure 2). The first, less efficient pathway involves production of hydroxyacids and leukotrienes through a lipooxygenase enzyme (Norman and Litwack, 1987). The second pathway is the most efficient of the two and includes the production of thromboxanes and prostaglandins through the cyclooxygenase enzyme. Through this mechanism, phospholipase A_2 can attach membrane phospholipids to release arachidonate which in turn may be acted upon by either prostaglandin endoperoxide synthase or cyclooxygenase (Norman and Litwack, 1987).

Aspirin and indomethacin are effective in preventing prostaglandin production through inhibition of

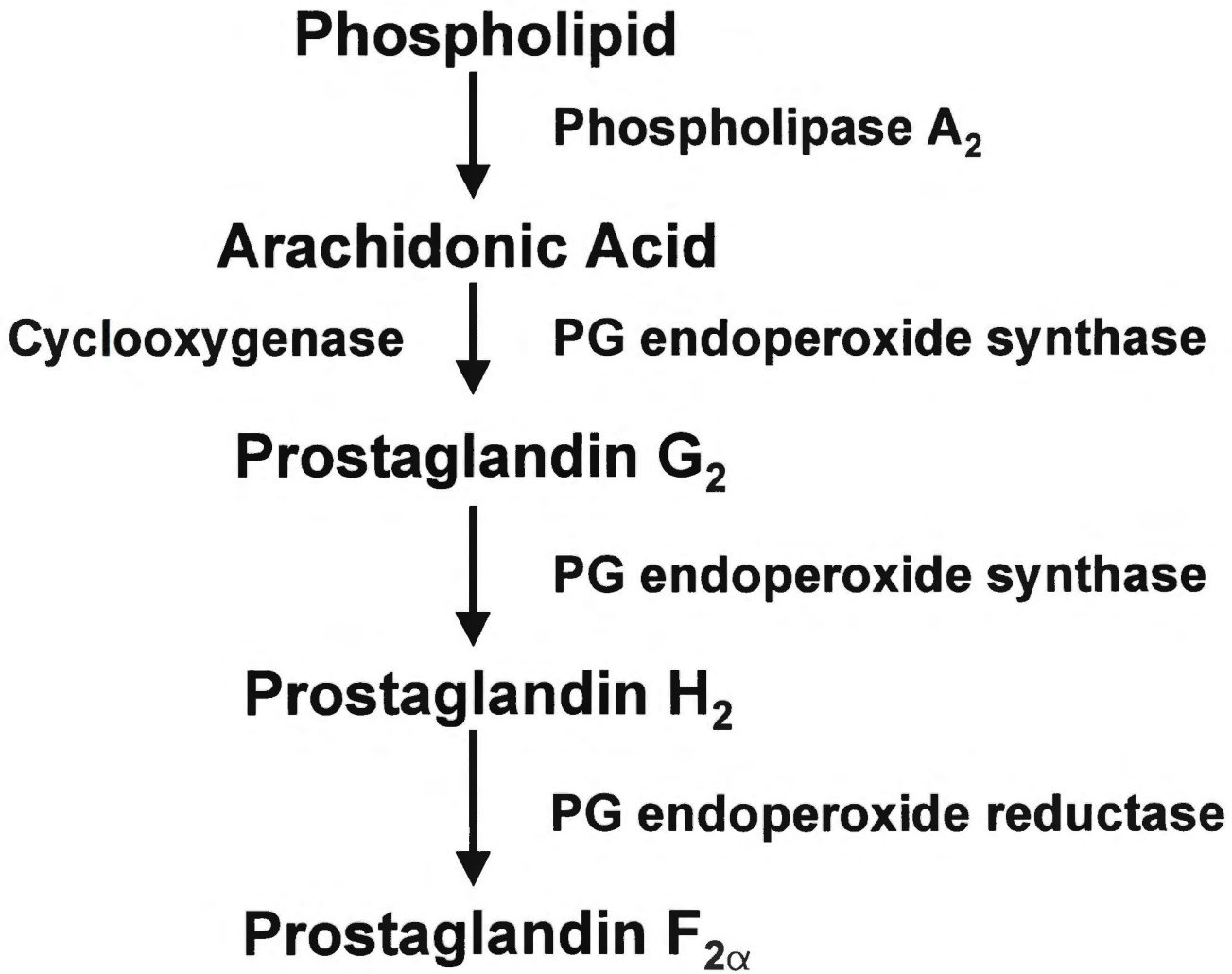


Figure 2. Schematic representation of $PGF_{2\alpha}$ synthesis. (Norman and Litwack, 1987)

cyclooxygenase (Vane, 1971). In addition, aspirin, indomethacin, and flunixin meglumine are all potent inhibitors of endoperoxide synthase (Vane, 1971). Prostaglandin $F_{2\alpha}$ is metabolized very quickly through a single passage of the lung by 15-prostaglandin dehydrogenase to the inactive metabolite, 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM; Kindahl et al. 1976). Kindahl et al. (1976) reported a direct relationship between $PGF_{2\alpha}$ and PGFM in cattle. However, Carver (1989) reported that concentrations of PGFM in blood from the jugular or posterior vena cava did not accurately reflect the production of $PGF_{2\alpha}$ by the uterus in cattle. Nonetheless, PGFM concentrations are used routinely as an indicator of peripheral $PGF_{2\alpha}$ concentrations.

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

The corpus luteum is an extremely vascular endocrine gland responsible for the maintenance of pregnancy through production of progesterone. The CL is comprised of two cell types varying in size. Size differences have resulted in cells of the CL being referred to as "small" (≤20 mm) and "large" (>20 mm) luteal cells. Large luteal cells are responsible for the production of the majority of progesterone found in blood. However, small cells appear to

facilitate production by large cells. Regression of the CL has been reviewed in the literature at great length (Milvae et al., 1986; Knickerbocker et al., 1988; Silvia et al., 1991; Niswender et al., 1994; Pate, 1996).

The CL receives up to 80% of the blood flow to the ovary in ewes (Nett et al., 1976). Prostaglandin $F_{2\alpha}$, a potent vasoconstrictor, decreased blood flow to the ovary during luteolysis in ewes (Nett et al, 1976). It is not known whether this is a symptom or a cause of luteal regression. Furthermore, $PGF_{2\alpha}$ plays both an antisteroidogenic and a cytotoxic role in luteal regression.

Receptors for $PGF_{2\alpha}$ have been isolated on large luteal cells throughout the bovine estrous cycle (Wiltbank et al., 1995). The $PGF_{2\alpha}$ receptor is a seven transmembrane G-protein linked receptor (Sakamoto et al., 1995). Pate (1996) hypothesized that large luteal cells are the target of uterine $PGF_{2\alpha}$. However, administration of $PGF_{2\alpha}$ generally is not successful to induce luteal regression in the cow prior to day 5 post-estrus. The presence of $PGF_{2\alpha}$ receptors implies that other factors are not present to complete the pathway of luteal regression during the early estrous cycle.

During luteal regression, $PGF_{2\alpha}$ is secreted in a pulsatile fashion by uterine endometrial tissue (reviewed by Milvae, 1996). Prostaglandin $F_{2\alpha}$ reaches the iplsilateral CL

by a local veno-arterial countercurrent transfer system (Hixon and Hansel, 1974). Prostaglandin $F_{2\alpha}$ stimulation causes release of oxytocin (OT) from large luteal cells (Flint and Sheldrick, 1982). Oxytocin binds to endometrial OT receptors to stimulate release of $PGF_{2\alpha}$ at approximately 6 hour intervals (as reviewed by Silvia et al., 1991) initiating the OT-$PGF_{2\alpha}$ feedback loop and pulsatility of $PGF_{2\alpha}$ release.

When luteal receptors bind $PGF_{2\alpha}$, receptors are translocated across the membrane and cause activation of phospholipase C (PLC; Davis et al., 1987). Phospholipase C causes hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG; Berridge, 1987). Diacylglycerol increases the affinity of protein kinase C (PKC) for calcium while IP_3 causes release of calcium from intracellular stores. The end result is a dramatic increase in free intracellular calcium concentrations and activation of PKC (reviewed by Nett et al., 1976).

This increase in intracellular calcium concentrations has been implicated in the cytotoxic effect of $PGF_{2\alpha}$ by stimulating apoptosis to occur. Apoptosis is characterized by condensation of chromatin, compacting of cytoplasmic organelles, formation of vesicles on the cell membrane

surface, and specific DNA degradation into regularly sized fragments (Rueda et al., 1995). The cleavage of DNA, a characteristic of apoptosis, may be determined by end labeling with ^{32}P-labeled dideoxy-adenosine triphosphate and the presence of a DNA ladder. This DNA ladder has been identified in the CL at luteolysis and is similar to that observed in other cases of apoptosis (McGuire et al., 1994). Furthermore, DNA laddering was observed in luteal tissue at days 8, 10, and 12 of the estrous cycle in ewes treated with $PGF_{2\alpha}$ but not in controls. Both control and $PGF_{2\alpha}$-treated ewes had DNA laddering at day 14 of the estrous cycle (period of normal luteolysis; Rueda et al., 1995). Therefore, $PGF_{2\alpha}$ appears to exert a cytotoxic effect on luteal cells by increasing intracellular calcium concentrations and stimulating apoptosis.

The antisteroidogenic effect of $PGF_{2\alpha}$ is primarily exerted by activation of protein kinase C (PKC). Protein kinase C inhibits cholesterol transport to the mitochondria where the P450 side chain cleavage enzyme awaits to convert cholesterol to pregnenolone (Norman and Litwack, 1987). In rats, treatment with $PGF_{2\alpha}$ resulted in a decrease in plasma membrane fluidity and an increase in superoxide radical formation (Sawada and Carlson, 1991). Synthesis of the superoxide radical is controlled by the PKC-second messenger

system (Sugino et al., 1996). Wu et al. (1992) reported that the superoxide radical inhibited LH stimulation of cAMP and decreased activity of protein kinase A (PKA); therefore, decreasing the presence of cholesterol in the mitochondria and ultimately decreasing the production of pregnenolone. Prostaglandin $F_{2\alpha}$ has been reported to reduce steady-state levels of mRNA for 3beta-hydroxysteroid dehydrogenase, thus decreasing the conversion of pregnenolone to progesterone. Thus, $PGF_{2\alpha}$ may exert its antisteroidogenic effect of decreasing progesterone production through multiple pathways.

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

The negative effect of prostaglandin $F_{2\alpha}$ on embryonic development was first reported in the rabbit. Stormshak and Casida (1965) reported progesterone could prevent abortion, but not fetal death in rabbits, when estrogen was used to rescue the corpus luteum from LH-induced luteolysis. Increasing concentrations of $PGF_{2\alpha}$ increased the number of fetal deaths and treatment with progesterone prevented abortion but not fetal death in mice (Harper and Skarnes, 1972).

Premature release of $PGF_{2\alpha}$ has been associated with first ovulation in the postpartum cow and leads to a

decrease in length of the luteal phase (Ramirez-Godinez et al., 1981; 1982; Peter et al., 1989; Cooper et al., 1991). Cows bred at their first postpartum ovulation failed to maintain pregnancy even when exogenous progestogen was provided to replace the regressing CL (Breuel et al., 1993b). Schrick et al. (1993) transferred day-6 embryos from cows with short luteal phases into normally cycling recipients and reported that pregnancy could be maintained, indicating that changing the uterine environment allowed embryonic development beyond that of the postpartum cow. However, when "good" quality embryos were transferred into cows with short luteal phases and supplemented with exogenous progesterone, pregnancy rates were 50% lower than in cows with normal luteal phases (Butcher et al., 1992). This study implies a hostile uterine environment may have toxic effects on embryonic development. Schrick et al. (1993) reported elevated concentrations of $PGF_{2\alpha}$ in the uterine lumen of postpartum cows compared to normal cycling cows. Cows with higher $PGF_{2\alpha}$ concentrations had lower quality embryos ($r= -0.42$, $P=0.07$). Therefore, $PGF_{2\alpha}$ was implicated in playing a direct role in decreasing embryonic survival in postpartum cows as demonstrated in mice (Harper and Skarnes, 1972).

Prostaglandin $F_{2\alpha}$ has been shown to have a direct effect on embryos from other species also. Maurer and Beier (1976) reported that culture of rabbit embryos in $PGF_{2\alpha}$ (0.1-10 ng/mL) had no effect on development to blastocyst, but decreased continued development to expanded or hatched blastocyst. Breuel et al. (1993a) found that 0.1-10 ng/mL of $PGF_{2\alpha}$ in culture media prevented development of 8-cell rat embryos to morula and blastocyst. However, *in vivo* administration of $PGF_{2\alpha}$ in progestogen-supplemented rats from days 4 to 9 did not result in a decreased number of implantation sites (Buuck, 1997). The author hypothesized that the rat may not be a good model for the cow due to the presence of 9-keto-reductase which catalyzes conversion of $PGF_{2\alpha}$ to PGE_2. Furthermore, rat embryos compact as early as the 8-cell stage as opposed to the cow which undergoes compaction around the 32-cell stage. Breuel et al. (1993a) reported blastocyst stage embryos were recovered in control media after 24 h in culture (day 5 of pregnancy), however, bovine embryos do not reach blastocyst stage until approximately day 7 of pregnancy. Therefore, the window of time during which embryos are susceptible to the deleterious effects of $PGF_{2\alpha}$ may be smaller in the rat than in the cow.

In early postpartum cows supplemented with exogenous progesterone and injected with either saline, flunixin

meglumine, or flunixin meglumine plus lutectomy, pregnancy rates were higher in cows receiving flunixin meglumine plus lutectomy. Normal cycling cows supplemented with progesterone and administered $PGF_{2\alpha}$ during days 4-7 or days 5-8 after mating had lower pregnancy rates than saline or flunixin meglumine treated cows (Buford et al., 1996). Furthermore, lutectomy immediately prior to $PGF_{2\alpha}$ treatment prevented embryonic loss. Similarly, Seals et al. (1998) reported that $PGF_{2\alpha}$ administration to progestogen-supplemented cows between days 5-8 decreased pregnancy rates compared to saline. The same treatment had no effect on pregnancy rates if administered between days 10-13 or 15-18. Pregnancy rates were increased in $PGF_{2\alpha}$-treated cows receiving exogenous progestogen if lutectomy was performed on day 5 before treatment was initiated.

To further delineate the timing of $PGF_{2\alpha}$ toxicity, day-7 frozen-thawed embryos of good or excellent quality were cultured in media containing 0, 0.1, 1.0 or 10 ng/mL $PGF_{2\alpha}$ (Fazio and Schrick, 1997). Percent development to blastocyst did not differ between control media (0), 0.1, 1, or 10 ng/mL treatments after 72 h of culture. Transfer of day-7 embryos to cows administered $PGF_{2\alpha}$ and exogenous progestogen resulted in pregnancy rates similar to those of control animals (E. K. Inskeep, personal communication).

However, Hernandez-Fonseca et al. (1997) reported that transfer of day 6 embryos to cows administered $PGF_{2\alpha}$ and exogenous progestogen resulted in decreased pregnancy rates compared to cows receiving saline. Thus, these experiments narrowed the window of $PGF_{2\alpha}$ toxicity on the bovine embryo to prior to day 7 following mating.

These trials offer several suggestions regarding the embryotoxicity of $PGF_{2\alpha}$. Effects of $PGF_{2\alpha}$ in cycling cows may be seen early in embryonic development (between days 4-7 or days 5-8 post mating) but not after day 10 post mating or after day 7 *in vitro* and *in vivo*. Lutectomy increased pregnancy rates in progestogen-supplemented cows receiving $PGF_{2\alpha}$ suggesting that the regressing CL may be deleterious to embryonic survival. This observation is further supported by work in the rat (Buuck, 1997) where culture of embryos with both $PGF_{2\alpha}$ and the luteal tissue further retarded embryonic development below that of $PGF_{2\alpha}$ alone. This study implies that some factor from the regressing CL could have toxic effects on embryonic development.

Oxytocin is known to be released from the CL during normal luteolysis caused by $PGF_{2\alpha}$ administration (Flint and Sheldrick, 1982; Schallenberger et al., 1984; Stormshak et al., 1994). Injection of oxytocin on days 5-8 in cycling beef cows supplemented with exogenous progestogen with or

without the presence of a CL decreased pregnancy rates (33% and 30%, respectively) compared to saline (80%) (Lemaster et al., 1998). However, administration of oxytocin and flunixin meglumine restored pregnancy rates equal to that of control animals. Therefore, oxytocin does not appear to directly affect embryonic development and any indirect effects may be restored by preventing $PGF_{2\alpha}$ production. Also, the fact that oxytocin can decrease pregnancy rates in luctectomized animals rules out other "toxic factors" being produced by the regressing CL and promotes $PGF_{2\alpha}$ as the embryotoxic factor.

Events initiating $PGF_{2\alpha}$ secretion

Many situations exist where concentrations of $PGF_{2\alpha}$ may be elevated and cause detrimental effects on embryonic development. For example, dairy cows in today's high production scenario may be extremely susceptible to elevation of $PGF_{2\alpha}$. Butler (1998) noted that cows in negative energy balance had higher levels of uterine $PGF_{2\alpha}$ and may have an increased sensitivity for release of $PGF_{2\alpha}$. Also, many producers have adopted the practice of administering oxytocin to improve milk letdown and production. Farin and Estill (1993) noted that oxytocin administration resulted in elevated levels of $PGF_{2\alpha}$ and an

increase in the number of short cycles. Therefore, administering oxytocin on a regular basis may result in CL regression and/or elevated concentrations of uterine $PGF_{2\alpha}$ which may be directly detrimental to the early embryo.

Prostaglandin $F_{2\alpha}$ may also be elevated during mastitis. Elevated concentrations of $PGF_{2\alpha}$ have been reported in milk from cows with Gram-negative mastitis (Giri et al., 1984). Furthermore, intrauterine infusion of endotoxin (Gilbert et al., 1990) resulted in shortened estrous cycles. Therefore, dairy cows in negative energy balance and administered oxytocin, or experiencing clinical mastitis caused by a Gram-negative pathogen may have elevated concentrations of $PGF_{2\alpha}$ which may negatively affect embryonic development.

Beef cows are also at risk for elevated concentrations of $PGF_{2\alpha}$ and decreased reproductive efficiency. In addition to those scenarios discussed with dairy cows, beef cows may be subject to elevated $PGF_{2\alpha}$ due to grazing endophyte-infected tall fescue. Cows consuming endophyte-infected tall fescue have elevated levels of PGFM in plasma (Seals et al., 1998).

Additionally, $PGF_{2\alpha}$ may be released in either dairy or beef cows due to uterine manipulation associated with artificial insemination or embryo transfer (Schallenberger et al., 1989). Both dairy and beef cows experiencing heat

stress are characterized as having elevated $PGF_{2\alpha}$ concentrations of $PGF_{2\alpha}$ (Malayer et al., 1990). Therefore, the interaction of endophyte-infected fescue consumption and mastitis or heat stress may compound the elevation of $PGF_{2\alpha}$ concentrations.

Thus, $PGF_{2\alpha}$ is commonly elevated in both beef and dairy cows. Elevated concentrations of $PGF_{2\alpha}$ are often associated with decreased reproductive efficiency, namely decreased pregnancy rates. Research has indicated that $PGF_{2\alpha}$ may have a direct negative effect on pregnancy rates.

3. STATEMENT OF THE PROBLEM

Embryonic mortality results in direct economic losses to beef and dairy producers. The estimated cost of wintering open beef cows in the United States is $1.1 billion annually (Inskeep and Peters, 1982). Dairy producers experience a loss of $1.50 to $2.00/head for each day a cow is open past 90 days of production (Graves, 1987).

Increased embryonic mortality may in part be due to elevated $PGF_{2\alpha}$ resulting from manipulation of the reproductive tract during embryo transfer or that which occurs during disease states such as mastitis (Giri et al., 1984) or fescue toxicosis (Seals et al., 1998). Schrick et al. (1993) reported that elevated uterine concentrations of $PGF_{2\alpha}$ in postpartum cows were correlated inversely to embryo quality.

Moreover, $PGF_{2\alpha}$ administration from days 4-7 or 5-8 following insemination decreased pregnancy rates in cows that were supplemented with progesterone (Buford et al., 1996; Seals et al., 1998). Pregnancy rates were similar for cows supplemented with progesterone and treated with $PGF_{2\alpha}$ during days 7-9 and receiving a day-7 embryo (E. K. Inskeep, personal communication). However, recipient cows treated with $PGF_{2\alpha}$ on days 6-8 and receiving exogenous progestogen exhibited decreased pregnancy rates when day-6 embryos were

transferred into the uterine horn ipsilateral to the CL (Hernandez-Fonseca et al., 1997).

Negative effects of $PGF_{2\alpha}$ may be due to direct effects on the early embryo or as a result of alterations in the uterine environment. To test the hypothesis that $PGF_{2\alpha}$ negatively affects early embryonic development, an experiment was designed to evaluate embryo quality and development in cycling cows receiving either $PGF_{2\alpha}$ or saline during days 5-8 postbreeding while being supplemented with progestogen.

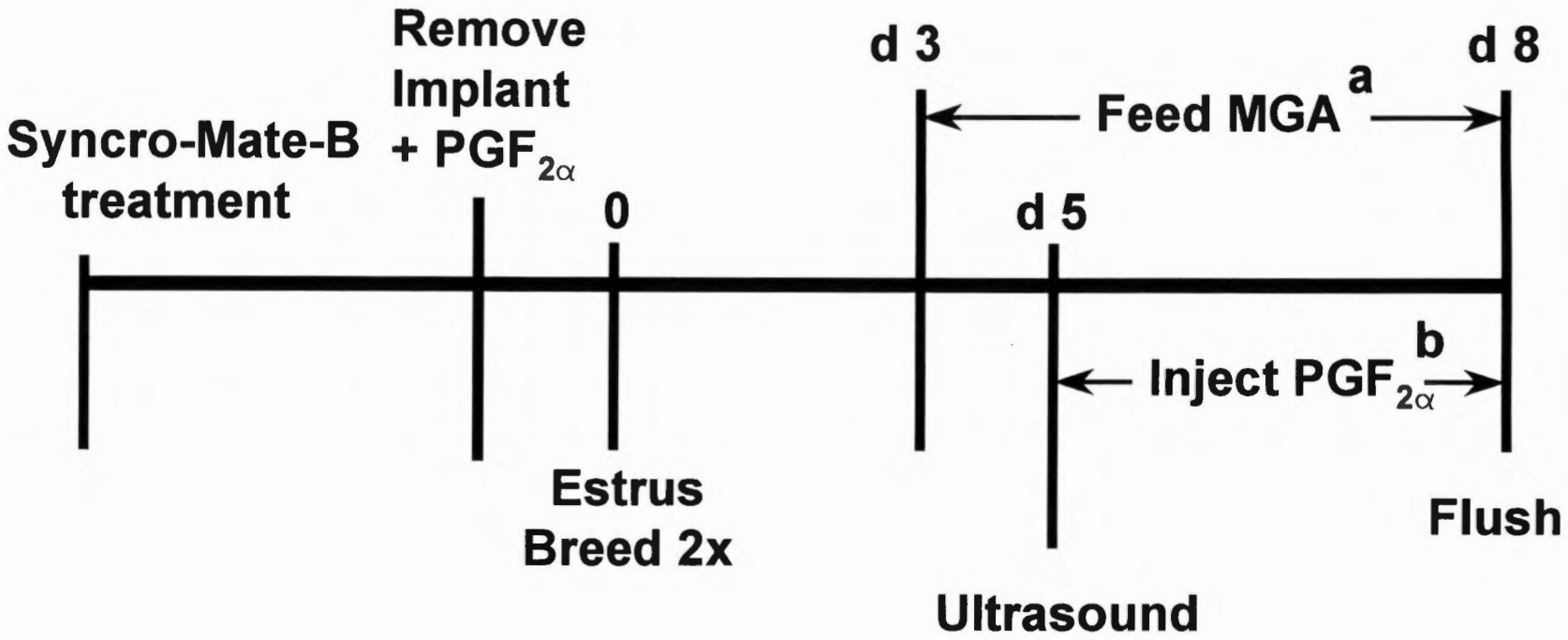
4. MATERIALS AND METHODS

General methods

Estrus was synchronized in cycling beef cows (n=70) using a Syncro-Mate B (Rhone Merieux, Athens, GA)/Lutalyse (Pharmacia and UpJohn, Kalamazoo, MI) protocol (Figure 3). Cows were administered a 6 mg norgestomet implant and injected intramuscularly with 3 mg norgestomet and 5 mg estradiol valerate. Implants were removed nine days later and cows were administered 25 mg $PGF_{2\alpha}$ intramuscularly (Lutalyse). Cows were fitted with Heatwatch ® heatmount detectors (DDX, Boulder, CO) for estrous detection in addition to visual surveillance twice daily. Cows were bred artificially at estrus (d 0) and again 12 h later using one Holstein bull of known fertility to remove any "male" effect. All cows were fed 4 mg of melengestrol acetate (MGA; 1 mg MGA per .45 kg pelleted feed, Appendix A; Tennessee Farmers Cooperative, Knoxville, TN) beginning on d 3 (Seals et al., 1998). Cows were allowed ad libitum access to bermuda grass pasture, orchard grass hay and water. Additional feed was provided for supplemental energy.

Experimental procedures

Cows were assigned randomly to receive either 3 ml saline (CON) or 15 mg $PGF_{2\alpha}$ (TRT) at 0600,1400 and 2200 h



[a]4 mg/cow*d

[b]15 mg i.m. at 0600, 1400, 2200

Figure 3. Schematic design of experiment

beginning on d 5 through 0600 h on d 8 (Figure 3). Ultrasonography was performed immediately prior to treatment on d 5 with an Aloka 500 ultrasound unit equipped with a 7.5 MHz linear transducer (Model UST-556I-7.5, Corometrics Medical Systems, Wallingford, CT). Ultrasonography was performed to verify the presence and location of the CL before initiation of $PGF_{2\alpha}$ treatment and regression of CL.

Embryo recovery and evaluation

A single embryo was recovered by flushing the uterine horn ipsilateral to the CL with 1 L of Dulbecco's phosphate buffered saline (PBS; Gibco, Inc., Grand Island, NY) containing 1% fetal calf serum (FCS; Gibco, Inc.). Uterine flushes were scored (range 1-3) on each cow with regards to the difficulty and amount of uterine manipulation required. A flush score of 1 was indicative of an easy flush with very little manipulation and 3 was an extremely difficult flush with excessive manipulation.

Flush media (3 mL) were collected from the second passage of medium through the uterine horn of each cow. Flush samples from cows with a flush score of 1 or 2 were used for analysis of $PGF_{2\alpha}$ and 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM). The remainder of the collected flush medium was filtered through a 0.22 micron filter

(Veterinary Concepts Incorporated, Spring Valley, WI) and searched for the presence of an embryo. Embryos were removed and placed into a holding medium (PBS with 15% FCS), scored for quality following the scheme proposed by Elsden and Seidel (1982), and stage of development was recorded. Quality scores were assigned by two experienced technicians unaware of treatment as follows:

Quality score 1: Perfectly symmetrical inner cell mass, evenly granulated, evenly colored with no serious deformities in the zona pellucida and at least 85% of the cell mass intact. Stage of maturity corresponded to the stage expected for the day of collection.

Quality score 2: Slight variation of all the above in quality score 1, may have several extruded blastomeres but one definite healthy large cell mass existed that contained 50% or more of the entire mass. Maturity was near stage of development expected on the day of collection.

Quality score 3: May have irregular zona pellucida, discolored or retarded development, but at least one heathy cell mass that contained 25% or more of the entire cell mass.

Quality score 4: Degenerated, retarded, or dead embryo.

Blood preparation and radioimmunoassays

Jugular blood (12 ml) was collected prior to administration of treatments at 0600 and 2200 and placed in chilled tubes containing 200 μl sodium citrate (25 mg/ml of H_2O; Baxter Scientific Products). Blood and flush samples were placed on ice until processing at the laboratory. Blood samples were centrifuged (2500 x g) for 20 min and plasma was collected. Plasma and uterine flush samples were stored at -20°C until radioimmunoassays were performed. Plasma samples were assayed to determine concentrations of progesterone (P_4) and PGFM. Uterine flush samples were assayed for $PGF_{2\alpha}$ and PGFM.

Progesterone concentrations in plasma were determined using a solid phase radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) as described by Seals et al. (1998). Sensitivity of the assay was 0.02 ng/mL. Intra- and inter-assay coefficients of variation were < 5%.

Concentrations of PGFM in plasma and flush media were determined as described by Silvia and Taylor (1989) with modifications described by Homanics and Silvia (1988). Sensitivity of the assay was 15 pg/tube (200 μl) and the intra-assay coefficient of variation was < 5%. Antibody for the PGFM assay was provided by William Silvia, University of Kentucky, Lexington.

Prostaglandin $F_{2\alpha}$ concentrations in uterine flush samples were determined by the procedure of Cooper et al. (1991). Sensitivity of the assay was 25 pg/tube, with an intra-assay coefficient of variation (CV) of <10%.

Statistical analysis

Changes in concentrations of P_4 and PGFM were compared by analysis of variance for a split-plot design with treatment as the main plot and time as the subplot using the MIXED procedure of SAS (1997). Relationships between uterine PGFM, uterine $PGF_{2\alpha}$ and embryo score were determined using Proc Corr of SAS (1997). Embryo scores and embryonic development were analyzed by Fisher's Exact Test (SAS Institute, 1997).

5. RESULTS AND DISCUSSION

Hormone analysis

Overall progesterone concentrations were lower in TRT versus CON cows (0.3 ± 0.14 vs 2.01 ± 0.16 ng/mL, respectively; P=0.0001). Furthermore, treatment affected progesterone concentrations over time. Progesterone concentrations increased in CON animals over time, characteristic of normal luteal function. Animals administered $PGF_{2\alpha}$ exhibited decreased progesterone over time as would be observed during luteal regression (Figure 4; P=0.0001). Furthermore, cows in the TRT group had elevated overall PGFM concentrations (553.17 ± 42.05 pg/mL) compared to CON (13.17 ± 50.09 pg/mL; P=0.0001). The interaction of time and treatment affected significantly PGFM concentrations with PGFM concentrations remaining steady in CON cows but increasing over time in TRT cows (Figure 5; P=0.0001).

These results are complimentary to those of Seals et al. (1998) who performed a similar experiment except looking at pregnancy rates instead of actual embryo development and quality. The administration of $PGF_{2\alpha}$ resulted in elevation of PGFM, the metabolized form of $PGF_{2\alpha}$. Additionally, $PGF_{2\alpha}$ administration caused subsequent luteolysis and resulted in

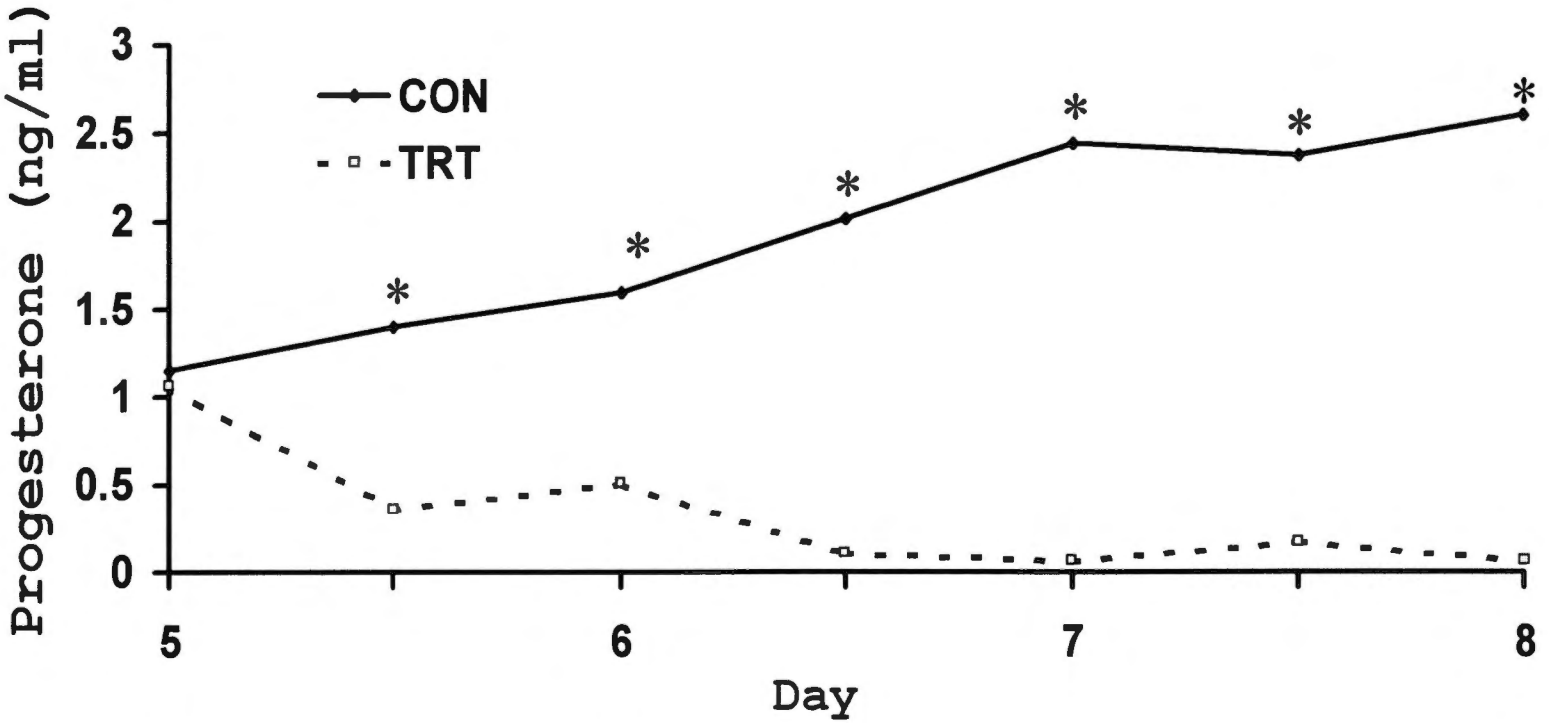


Figure 4. Concentrations of progesterone during the experimental period. Times identified with an asterisk (*) differ (P=0.0001) in progesterone concentrations between treatments.

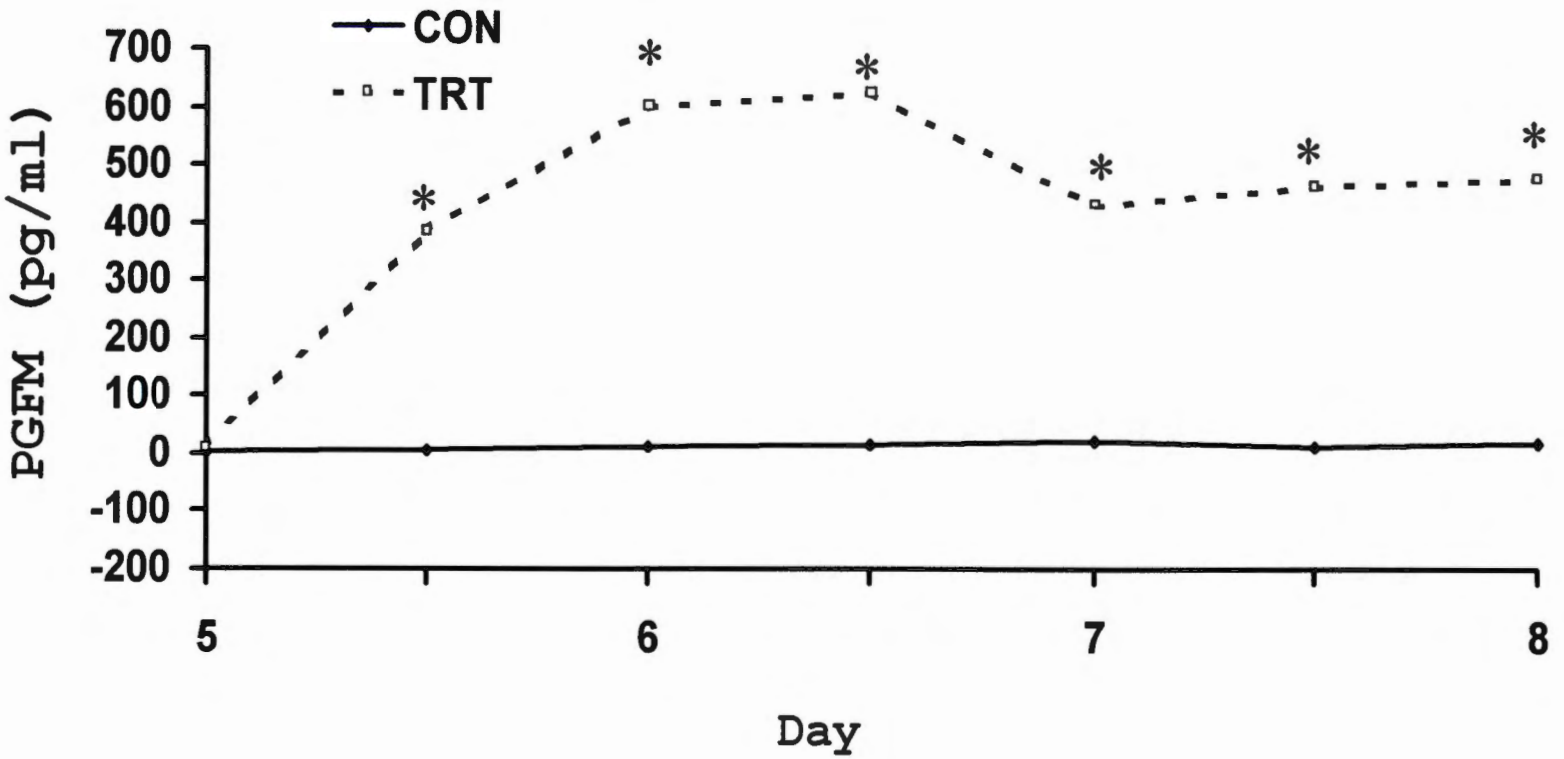


Figure 5. Concentrations of PGFM during experimental period. Times identified with an asterisk (*) differ (*P*=0.0001) in PGFM concentrations between treatments.

decreased progesterone concentrations in TRT cows.

Uterine luminal concentrations of $PGF_{2\alpha}$ in flush medium did not differ between CON and TRT cows (818.98 ± 81.57 vs 780.87 ± 103.98, respectively; *P*=0.78). Uterine luminal PGFM concentrations also did not differ between CON and TRT cows (79.81 ± 37.97 vs 136.39 ± 32.09, respectively; P=0.26). These data are not surprising. The technique for collection of uterine lumen samples to determine concentrations of $PGF_{2\alpha}$ is not the most optimum technique. Manipulation of the reproductive tract cannot be avoided during passage of the catheter through the cervix and placement in the uterine horn. Therefore, release of $PGF_{2\alpha}$ is unavoidable by this technique. However, samples from cows with excessively difficult flushes were not analyzed for $PGF_{2\alpha}$ or PGFM concentrations to decrease some effects uterine manipulation might impose. The preferred method of sampling would include death of the animal, removal of the reproductive tract from the animal, and flushing the tract with saline.

Embryo recovery

Following synchronization, 58 of 70 cows exhibited estrus. Uterine flushes were attempted on 58 cows and 52 attempts were successful to completion. Six flushes were not

completed due to inability to pass the cervix or inhalation of air into the uterus.

Embryo recovery was successful in 28 of 52 flushes (54%). Of this total, 3 unfertilized ova and 1 zona pellucida were found. Samples from these animals were not included in data analysis. Out of the 52 cows which were successfully flushed, 24 were CON yielding 13 successful recoveries (54.2%) and 28 were TRT yielding 15 successful recoveries (53.6%).

One hypothesis raised in the past was that $PGF_{2\alpha}$ may speed passage of the embryo through the uterine horn and thereby have a negative effect on development and pregnancy rates. Fuchs (1987) suggested that uterine motility and gamete transport are accelerated by either $PGF_{2\alpha}$ or OT. Our recovery percentages suggest that embryos did not travel down the uterine horn at differing rates in TRT and CON cows to the point of altering recovery.

Development and quality scores of embryos

One objective of this experiment was to determine if administration of $PGF_{2\alpha}$ had a negative effect on development and quality of embryos between days 5-8 of pregnancy in cows supplemented with exogenous progestogen. Evaluation of quality scores and development of collected embryos

indicated that elevated concentrations of $PGF_{2\alpha}$ decreased development and quality of embryos. Embryo quality scores revealed an increase in mean quality scores in TRT compared to CON cows (2.9 ± 0.30 vs 1.6 ± 0.35, respectively; P=0.059) indicating a reduction in the quality of TRT embryos. Percentage of embryos at each stage of development differed between treatment groups (Figure 6, Fisher's Exact Test P=0.003). Although 29% (4/14) developed to expanded blastocyst, 64% (9/14) had not developed past the morula stage. Furthermore, 80% (8/10) of the CON embryos progressed to expanded blastocyst and 20% (2/10) of the embryos were at the blastocyst stage upon recovery. All CON embryos had developed to maturity corresponding to day of development.

Percentage of embryos at each quality score differed between treatments (Figure 7, Fisher's Exact Test P<0.003). Quality score 1 embryos comprised 60% (6/10) of CON embryos compared to 29% (4/14) of TRT embryos. Furthermore, 64% (9/14) of TRT embryos were quality score 4 while none of the CON embryos were quality score 4. The appearance of embryos in the TRT group with a quality score of 4 were noted as having dispersed cells with a fragmented (dark salt and pepper) appearance. Therefore, treatment with $PGF_{2\alpha}$ resulted in a lower percentage of embryos being excellent in quality and a greater percentage of embryos being poor in quality.

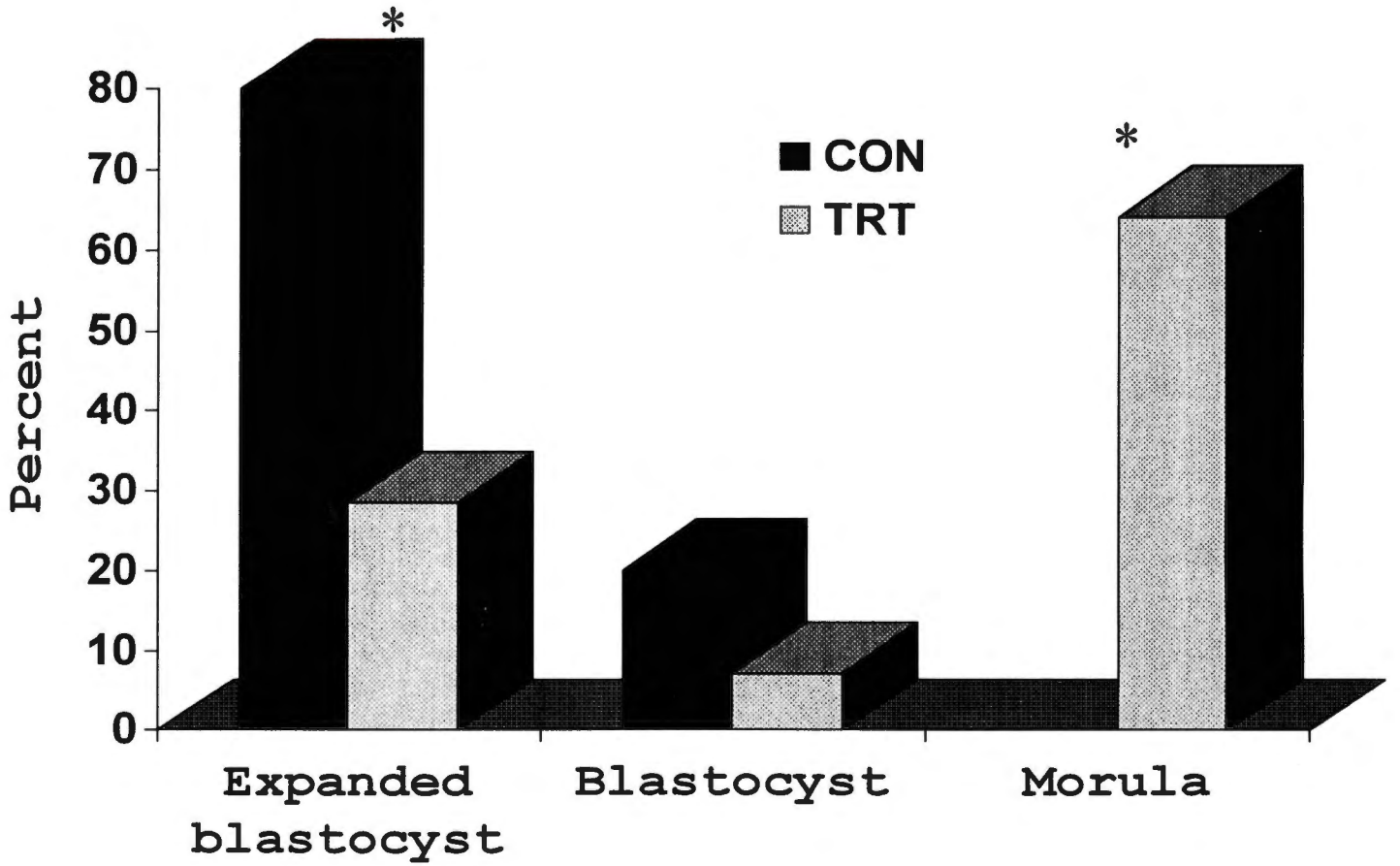


Figure 6. Development of embryos during the experimental period. Stages identified with an asterisk (*) differ in development between treatment (P=0.003).

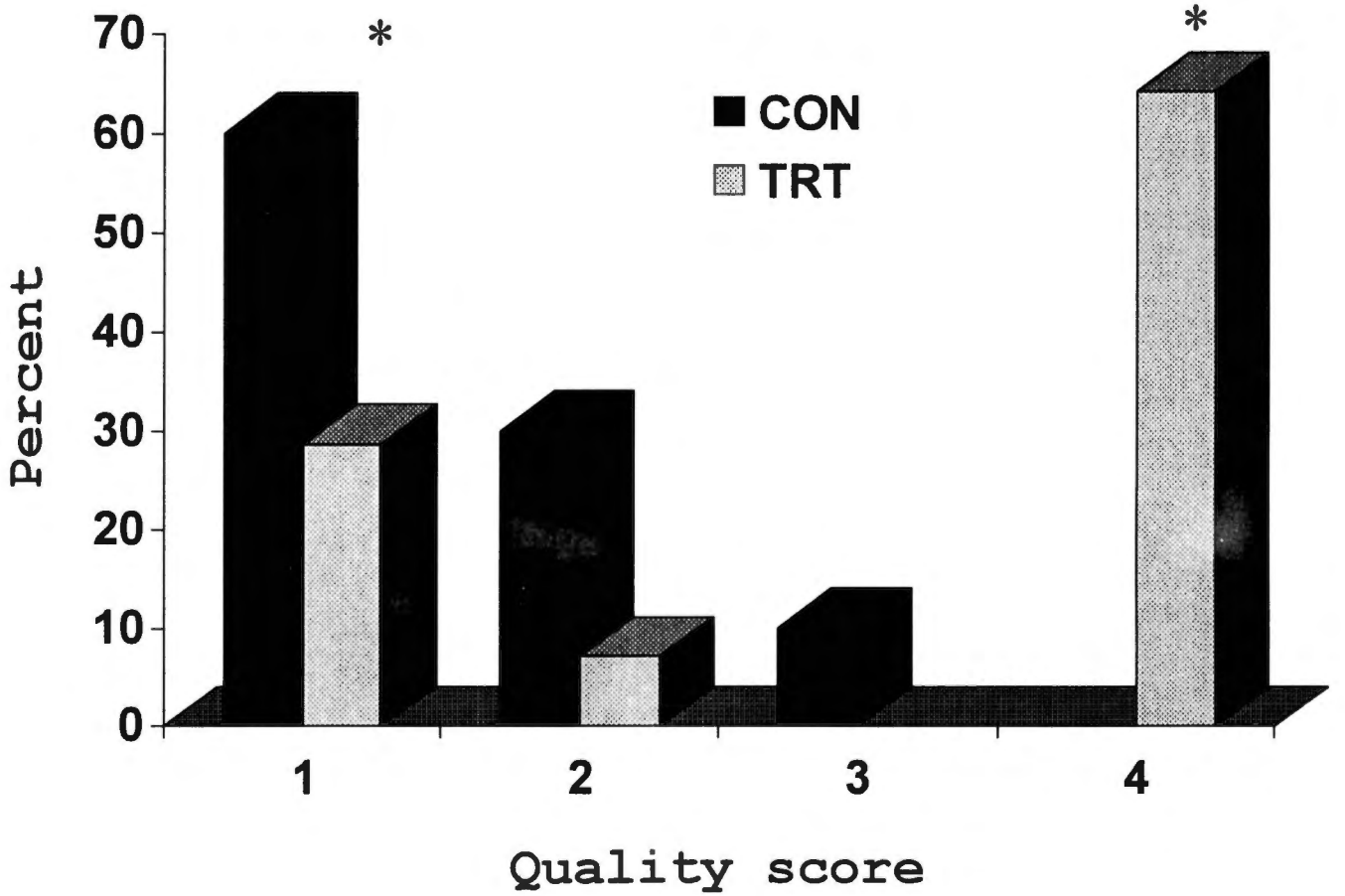


Figure 7. Quality score of embryos during the experimental period. Stages identified with an asterisk (*) differ in development between treatments (P=0.003).

These results complimented those of Seals et al, (1998) which reported a decrease in pregnancy rates in progestogen supplemented cows administered $PGF_{2\alpha}$. The pregnancy rates (71% CON, 23% $PGF_{2\alpha}$) reported by Seals et al. (1998) are similar to the percentage of embryos found to be of good or excellent quality.

Embryos with a quality score of 1, 2 or 3 are considered viable and worthy of transfer to recipient cows. Comparison of CON and TRT embryos on this basis resulted in 100% (10/10) of CON embryos considered transferrable (viable, quality score 1, 2, or 3) while only 36% (5/14) of TRT embryos were considered transferrable (P=0.002). However, 64% (9/14) of TRT embryos lacked development and/or quality to be considered viable for embryo transfer.

A positive correlation between embryo quality score and PGFM concentrations on day 8 at 0600 h (r=0.47, P<0.05) and on day 8 at time of flush (r=0.74, P=0.0001) was observed. Additionally, uterine luminal concentrations of PGFM tended to be related to embryo quality score (r=0.38, P=0.07). In contrast, no relationship was observed between uterine luminal concentrations of $PGF_{2\alpha}$ and embryo score (r=0.18, P=0.41). Uterine concentrations of $PGF_{2\alpha}$ and PGFM were highly correlated to one another (r=0.75, P=0.0001). The significance of the correlation of uterine luminal PGFM

concentrations with embryo score is unknown. At present, no reports have been published to evaluate effects of PGFM on embryonic development, although the possibility does exist that high concentrations of PGFM could have a detrimental effect on embryos.

Although very little research has been performed in the area of $PGF_{2\alpha}$ and embryonic development, several mechanisms may account for negative effects of $PGF_{2\alpha}$ on embryonic development. Prostaglandin $F_{2\alpha}$ stimulates calcium influx into cells (for review see Niswender et al., 1994). This is the mechanism which is responsible for apoptosis of luteal cells during regression. During luteal regression, $PGF_{2\alpha}$ binds to its receptors and stimulates PLC and subsequent release of IP-3 (Davis et al., 1987). Inositol tri-phosphate causes mobilization of intracellular stores of calcium into the cell. Calcium then stimulates Ca^{2+}/Mg^{2+} dependent endonucleases to cut DNA into internucleosomal fragments (Rueda et al., 1995). These fragments form the characteristic DNA ladder used as a landmark for apoptosis. Prostaglandin $F_{2\alpha}$ administration to ewes induced DNA fragmentation in luteal tissue (Rueda et al., 1995). Presence of $PGF_{2\alpha}$ receptors on the surface of the embryo or individual blastomeres has not been reported. The presence

of $PGF_{2\alpha}$ receptors may be a requirement for $PGF_{2\alpha}$ to negatively affect embryos through this proposed mechanism.

Also observed during CL regression is the production of reactive oxygen species (for review see Niswender et al., 1994). Free radicals may work much like elevated levels of intra-cellular calcium to degenerate DNA into fragments or by attacking the integrity of the cell membrane (Wagner et al., 1996; Thomas et al., 1997). Furthermore, free radicals may modify low density lipoprotein (LDL) by oxidation to produce a toxin. The molecule 7 beta-hydroperoxycholest-5-en-3 beta ol (7 beta-OOH-Chol) has been purified and is responsible for 90% of the cytotoxicity of the lipids of oxidized LDL (Chisolm et al., 1994). This compound has been found in human atherosclerotic lesions and the plasma of diabetic animals, and it may play a role in tissue damage that occurs in certain diseases (Chisolm et al., 1994). The period of luteal regression may be vulnerable to this mechanism as LDL is abundant on the surface of luteal cells and free radical production occurs during this time frame as well. The initiation of luteal regression by injection of $PGF_{2\alpha}$ may stimulate this pathway to produce a cytotoxic component.

Two other possible mechanisms by which $PGF_{2\alpha}$ may interrupt embryonic development include disruption of gap

junction formation and function. Cadherins and connexins are two proteins vital to cell communication and gap junction formation. Cadherins are a member of a family of cell adhesion molecules (CAM). Epithelial cadherin (E-cadherin) is seen on the cell surface of epithelial cells. E-cadherin allows cells of like type to adhere to one another. This is most important during the process of compaction. Embryonic cells adhere to one another and gap junctions are opened which allow cells to communicate with one another. Blastomeres then organize according to polarity and function. Furthermore, outer cells form tight junctions which are necessary for cell support, a function most important when uterine fluid is pumped into the embryo to form the blastocoel.

Culture of myoctes from neonatal rat hearts in arachidonic acid, a precursor to prostaglandin synthesis, has produced reversible uncoupling of gap junctions (Schmilinsky et al., 1997). Crow et al. (1994) reported that culture of bovine embryonic lens cells with two different ionophores caused an increase in intracellular calcium concentrations and decreased intracellular dye transfer.

Connexins are proteins which make up the channels of the gap junction. At present, 13 subgroups exist which have been identified (DeSousa et al., 1997). Connexin 43 has been

identified and studied in the mouse embryo. Koo et al. (1997) reported that phosphorylation of connexin 43 on serine residues correlated with a decrease in gap junctional communication in clone 9 rat liver cells. Connexin 56, the lens gap junction protein, has been shown to be modified through phosphorylation by protein kinase C or protein kinase A *in vitro* (Berthoud et al., 1997). Both protein kinase C and protein kinase A are products of $PGF_{2\alpha}$ binding to its receptor. Phosphorylation of connexins decreases opening of gap junctional channels and therefore decreasing cell communication. Protein kinase C, which is elevated during times of luteal regression, is capable of phosphorylating connexins. Gap junctional intercellular communication between luteal cells is decreased by culture with a PKC agonist (TPA, Grazul-Bilska et al., 1996).

Conclusion

In summary, results of the current experiment indicate that $PGF_{2\alpha}$ has a detrimental effect on embryonic development by decreasing the rate or ability of an embryo to develop past the morula stage. Additionally, $PGF_{2\alpha}$ decreased the quality score of embryos recovered on day 8 and quality scores were highly correlated to blood PGFM values and tended to be related to PGFM in uterine flush samples.

Prostaglandin $F_{2\alpha}$ may interfere with the process of compaction which occurs at the morula (16-32 cell) stage in the bovine. Future studies should be aimed at delineating mechanisms by which $PGF_{2\alpha}$ exerts a negative influence on quality and development of the early bovine embryo.

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APPENDIX

APPENDIX

Ingredients

Processed grain by-products, plant protein products, grain products, forage products, cane molasses, calcium carbonate, salt, lignin sulfonate, potassium sulfate, magnesium sulfate, zinc oxide, manganese oxide, ferrous sulfate, magnesium oxide, copper sulfate, cobalt carbonate, ethylene diamine dihydroiodide, calcium oxidate, sodium selenite, vitamin A acetate, vitamin D-3, vitamin E supplement and mineral oil.

Guaranteed Analysis

Crude protein (min.)	25.00%
Crude fat (min.)	2.50%
Crude fiber (max.)	9.75%
Calcium (min.)	1.25%
Phosphorus (min.)	0.55%
Salt (min.)	1.30%
Potassium (min.)	1.00%
Vitamin A (min.)	5,000 IU/lb
Vitamin D (min.)	1,500 IU/lb

Active Ingredient

Melengestrol Acetate	2,000 mg/ton

Manufactured By
Tennessee Farmers Cooperative
200 Waldron Road
Lavergne, TN 37086

VITA

Mitchell Earl Hockett was born April 22, 1974 in Murfreesboro, Tennessee. He attended primary and middle school in Coffee County, Tennessee, where his family owned a Holstein dairy farm. Mitch graduated from Coffee County Central High School in 1992. In the summer of 1996, he graduated with a B.S. degree in Animal Science from the University of Tennessee. In the fall of 1998, he graduated with a M.S. degree in Animal Science from the University of Tennessee.

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