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Effects of prostaglandin F₂α on in vitro and in vivo development of preimplantation rat embryos

Michael Joseph Buuck

To the Graduate Council:

I am submitting herewith a thesis written by Michael Joseph Buuck entitled "Effects of prostaglandin $F_2\alpha$ on in vitro and in vivo development of preimplantation rat embryos." I have examined the final electronic 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:

Jim Godkin, Alan Mathew

Accepted for the Council:
<u>Carolyn R. Hodges</u>

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Michael Joseph Buuck entitled "Effects of Prostaglandin $F_{2\alpha}$ on *In Vitro and In Vivo* Development of Preimplantation Rat Embryos". 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.

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and recommend its acceptance:

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Accepted for the Council:

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Associate Vice Chancellor and
Dean of The Graduate School

EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ ON *IN VITRO* AND *IN VIVO*

DEVELOPMENT OF PREIMPLANTATION

RAT EMBRYOS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Michael Joseph Buuck

May 1997

ACKNOWLEDGMENTS

ABSTRACT

Two experiments were conducted to determine the effect of
prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on *in vitro* and *in vivo* development
of preimplantation rat embryos. In Trial 1 of the first
experiment, seven-hundred-twenty three 8-cell embryos from
superovulated female Sprague-Dawley rats were collected four
days after natural mating. Embryos were cultured in alpha-
Minimum Essential Medium (αMEM) supplemented with 0.1%
polyvinyl alcohol (control;n=179) or control medium
supplemented with one of the following levels of
prostaglandin $F_{2\alpha}$ 1) 0.1 ng/ml(n=176), 2) 1.0 ng/ml(n=176)
or 3) 10 ng/ml(n=162). After 24 hours of incubation in
their respective treatments, stages of embryonic development
were recorded and morphologically normal embryos were
transferred to control media for an additional 24 h.
Following 24 h of culture, 63% of control embryos had
continued development to the morula or blastocyst stage.
However, only 20, 15, and 16% of the embryos treated with
0.1, 1.0, and 10 ng/ml $PGF_{2\alpha}$, respectively, continued
development to the morula and blastocyst stage. All levels
of prostaglandin $F_{2\alpha}$ decreased the number of embryos

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developing to the expanded blastocyst stage after 48 h of culture (p<0.01). Furthermore, a second trial was performed in which embryos were placed with corpora lutea in 2 of 4 treatments, consisting of the following: 1)control (αMEM;n=30), 2)control-luteal (corpora lutea + αMEM;n=30), 3)PGF-luteal (corpora lutea + 10 ng/ml $PGF_{2\alpha}$;n=30) and 4)PGF-control (10ng/ml $PGF_{2\alpha}$ +αMEM;n=41). The percentage of embryos in the CL + αMEM treatment that reached the blastocyst stage(43.3%) was not statistically different from the controls (37.8%;p>.10). However, the co-culture of corpora lutea and $PGF_{2\alpha}$ tended (p<0.07) to increase the negative effects of $PGF_{2\alpha}$ on blastocyst development(19%) when compared to the $PGF_{2\alpha}$ group.

To investigate the effects of $PGF_{2\alpha}$ on *in vivo* development of preimplantation embryos, prostaglandin $F_{2\alpha}$ (0.5 and 1 mg) was administered to rats twice daily during Days 4-7 of pregnancy. All animals were also injected with 4 mg/day of progesterone from Days 4-11 of treatment. On Day 11, animals were euthanized by CO_2 overdose and the number of implantation sites determined. Prostaglandin $F_{2\alpha}$ was not effective in terminating pregnancy or reducing litter size.

These data suggest that increased levels of prostaglandin $F_{2\alpha}$ have a direct negative effect on *in vitro* embryonic development. However, exogenous $PGF_{2\alpha}$ had no effect on embryonic survival, suggesting that $PGF_{2\alpha}$ may not attain embryotoxic concentrations within the uterus, possibly because of conversion to PGE_2.

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1. INTRODUCTION

In several mammalian species (rat, cattle, and sheep), the uterus causes regression of corpora lutea (CL) via release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Goding, 1974; Cao and Chan, 1993). Administration of $PGF_{2\alpha}$ causes termination of pregnancy in many mammalian species by destruction of the CL, thus reducing circulating levels of progesterone.

However, increases in $PGF_{2\alpha}$ may also have a direct negative effect on embryonic development. In rabbits, addition of prostaglandin $F_{2\alpha}$ during *in vitro* culture decreases the ability of 2-, 4- and 8-cell embryos to develop to hatched blastocysts (Maurer and Beier, 1976). Contrasting results have been reported during *in vitro* development of preimplantation mouse embryos cultured with $PGF_{2\alpha}$ (Kirkpatrick, 1974). Harper and Skarnes (1972) suggested that increasing concentrations of $PGF_{2\alpha}$ increased the number of fetal deaths apart from abortion and that treatment with progesterone prevented abortion, but not fetal death. However, administration of prostaglandin $F_{2\alpha}$, without progesterone supplementation, was found to be effective in terminating pregnancy in the rat when injected

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subcutaneously from Day 4 through 13 of pregnancy (Gutknecht et al., 1969; Labhsetwar,1972).

Prostaglandins have been implicated in both growth of the embryo and the implantation process in the rabbit (El-Banna et al., 1976; Hoffman et al., 1978). Both PGE_2 and $PGF_{2\alpha}$ have been found to be equally as effective in causing decidualization and implantation of the rat embryo (Kennedy, 1985). Furthermore, prostaglandin $F_{2\alpha}$ is converted within the uterus to PGE_2 via 9-ketoreductase (Kennedy, 1985).

Indomethacin, an inhibitor of prostaglandin biosynthesis, delays or inhibits the localized increase in endometrial vascular permeability (Kennedy, 1977; Evans and Kennedy, 1978; Hoffman et al.,1978) and implantation (Saksena et al., 1976; El Banna, 1980). Hoffman et al. (1978) observed an inhibition of embryonic growth with indomethacin treatment between Days 4 and 7 of pregnancy, indicating an importance of prostaglandins. In the hamster, mouse and rat, treatment with indomethacin also disrupted implantation (Saksena et al., 1976; Kennedy, 1977; Evans and Kennedy, 1978; Oettel et al., 1979).

In cows, prostaglandins are often increased during uterine manipulation and irritation, typically encountered

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with artificial insemination and embryo transfer techniques
(Schallenberger et al., 1989). Furthermore, concentrations
of $PGF_{2\alpha}$ in uterine flushings were negatively correlated
with embryonic quality (Schrick et al., 1993). The most
susceptible period of embryonic growth in cattle to the
negative effects of $PGF_{2\alpha}$ appears to be during morula to
blastocyst development (Seals et al., 1996).

Prostaglandin $F_{2\alpha}$ stimulates oxytocin secretion from
the corpus luteum (Fuchs, 1987). Oxytocin then feedbacks on
the uterus and stimulates the secretion of $PGF_{2\alpha}$ which is
responsible for luteolysis (Flint and Sheldrick, 1983).
Removal of the luteal source of oxytocin diminishes the
negative effects of $PGF_{2\alpha}$ on the early developing bovine
embryo, possibly thru interruption of the luteal oxytocin-
uterine $PGF_{2\alpha}$ feedback loop (Lemaster et al., 1996). It is
hypothesized that the decrease in embryo viability
associated with $PGF_{2\alpha}$ is due not only to lowered
concentrations of progesterone as a result of luteolysis,
but also to a direct negative effect $PGF_{2\alpha}$ on the developing
embryo.

2. REVIEW OF THE LITERATURE

Endocrine Events of the Rat Estrous Cycle

The rat has a four day estrous cycle consisting of four distinct phases:estrus, diestrus-1 , diestrus-2, and proestrus. Estrus is the result of increased estradiol (E_2) levels on the afternoon of diestrus-2(Smith et al., 1975). A surge in E_2 occurs during proestrus and initiates the following events: luteinizing hormone (LH) surge, the prolactin surge and the depletion of FSH from the pituitary (Smith et al., 1975). A proestrus rise in progesterone secretion from the corpus luteum occurs simultaneously with the increase in LH. However, the role of the proestrus surge of progesterone has yet to be established.

A divergence in progesterone secretion on the morning of diestrus-2 of the cycle and Day 2 of pregnancy signifies the time when either regression or maintenance of corpora lutea occurs. Prolactin is the major luteotrophic stimulus which converts corpora lutea of the cycle into corpora lutea of pregnancy by initiating and maintaining progesterone secretion (Smith et al., 1975). If pregnancy does not occur, luteolysis takes place followed by a period of

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decreased progesterone production. Prostaglandin $F_{2\alpha}$ is recognized as having a decisive role in the functional regression of the corpus luteum (Rothchild, 1981; Niswender and Nett, 1988).

Biosynthesis and Secretion of Prostaglandins

Several pathways (Figure 1) for the production of prostaglandins from arachidonic acid exist as reviewed by Milvae (1986). The metabolic precursor of prostaglandins is arachidonic acid, a fatty acid obtained from the diet or membrane lipids, which is converted from linoleic acid by desaturase enzymes (Moore, 1985). Following release of arachidonic acid, two enzymes are involved in its subsequent metabolism. The first pathway, involves a lipooxygenase enzyme which produces hydroxyacids and leukotrienes.

The second and more efficient pathway for the production of $PGF_{2\alpha}$ is a cyclooxgenase enzyme which synthesizes prostaglandins and thromboxanes. Aspirin and indomethacin are effective in preventing prostaglandin production through the inhibition of cyclooxygenase (Vane, 1971). Both PGE_2 and $PGF_{2\alpha}$ are metabolized in a single passage through the lung by 15-PG dehydrogenase to the inactive metabolites 15-keto PGE's or PGF.
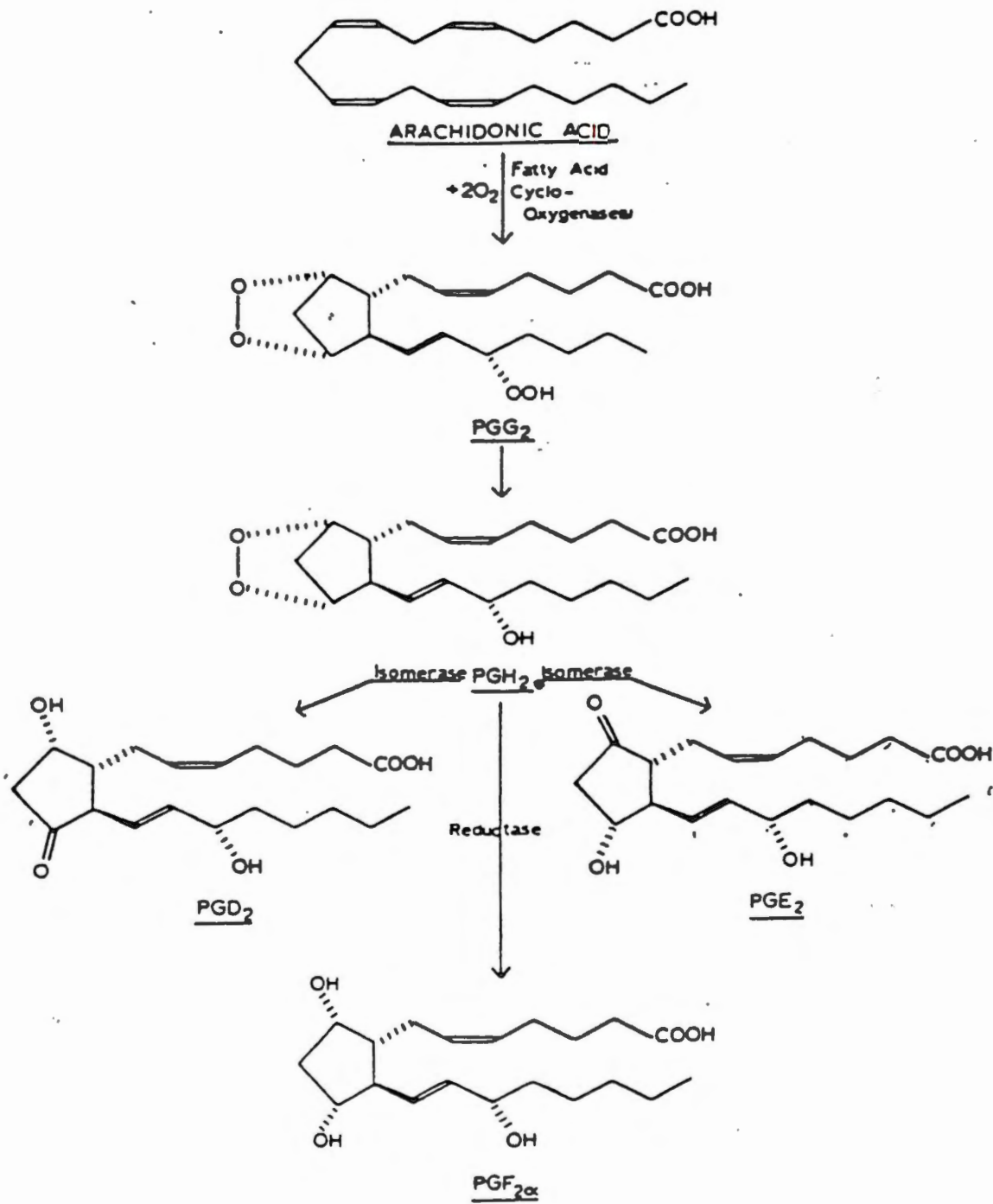
Figure 1: Biosynthesis of Prostaglandins (Hamburg et al., 1973)

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Mechanism of Action of PGF$_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is recognized as having a decisive role in the functional regression of the corpus luteum (Niswender and Nett, 1988). Prostaglandin must be released in a series of five to eight pulses in order to initiate luteolysis in cattle (Kindahl et al., 1976). The administration of $PGF_{2\alpha}$ induces luteolysis in most species, including the rat(Pharriss, 1970). Furthermore, Olofsson et al.(1990) demonstrated that the luteal content of $PGF_{2\alpha}$ in the adult pseudopregnant rat increased during the luteal phase and the luteolytic period, suggesting $PGF_{2\alpha}$ is involved in luteolysis.

Injections of a luteolytic dose of $PGF_{2\alpha}$ into rats does not appear to decrease ovarian blood flow (Behrman et al., 1979. However, it does dramatically decrease the binding of LH to luteal cells. An eventual loss of LH receptors also occurs after treatment with $PGF_{2\alpha}$, caused by inhibition of the actions of prolactin.

An important step in understanding the mechanism of $PGF_{2\alpha}$-induced luteolysis is the antigonadotropic effect of $PGF_{2\alpha}$ on the cyclic adenosine 3',5'-monophosphate (cAMP) system and steroidogenesis (Lahav et al.,1976; Thomas et

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al.,1978). Prostaglandin $F_{2\alpha}$ inhibits LH-induced stimulation of adenylate cyclase, thus reducing cyclic AMP concentrations in luteal tissues (Lahav et al., 1976). This reduction in cAMP concentrations causes the cholesterol esterase enzyme to be converted from its active phosphorylated form to an inactive dephosphorylated form. The net effect of this step is a reduction of free cholesterol for progesterone (P_4) biosynthesis by luteal cell mitochondria. Furthermore, mRNA for the LH receptor decreases late in the estrous cycle, thereby causing functional luteolysis to be irreversible (Bjurulf and Selstam, 1996).

Prostaglandin $F_{2\alpha}$ also increases phosphotidylinositol hydrolysis, specifically hydrolysis of phosphoinositol biphosphate to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol(DAG) within the rat luteal cell (Sender Baum and Rosenberg, 1987). Diacylglycerol can act as a second messenger and activate protein kinase C (PKC), which activates enzymes involved in releasing arachidonic acid to serve as a substrate for prostaglandin synthesis (Nishizuka, 1984).

Prostaglandins have been shown to increase calcium

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concentrations in the large luteal cells (Wegner et al.,
1990) and elevated levels of intracellular calcium has been
shown to be cytotoxic (Rasmussen and Barrett, 1984).
Dorflinger et al. (1983) reported that administration of a
calcium ionophore inhibits LH-stimulated cAMP accumulation
and progesterone secretion in the rat luteal cell,
suggesting that the luteolytic effects of $PGF_{2\alpha}$ are mediated
in a calcium dependent manner.

Biosynthesis of Oxytocin and Luteal Regression

It is considered that ovarian oxytocin secreted by the
corpus luteum is the endogenous oxytocin involved in
luteolysis in several ruminant animals (Flint and Sheldrick
1983; Flint et al., 1990). A positive feedback loop where
luteal oxytocin stimulates the secretion of the luteolytic
agent, $PGF_{2\alpha}$, from the uterus has been reviewed by Fuchs
(1987) and Flint et al. (1990). The positive feedback loop
or the uterine prostaglandin secretory response to oxytocin
becomes functional only late in the estrous cycle, leading
to luteolysis (Silvia and Taylor, 1988).

In the rat, regulation of oxytocin receptors is closely
correlated with the estrogen/progesterone ratio (Soloff et
al., 1979). It has been confirmed that estrogen up-

regulates and progesterone down-regulates uterine oxytocin binding sites (Soloff et al., 1983; Larcher et al., 1995). Cao and Chan (1993) reported that oxytocin treatment in rats increased $PGF_{2\alpha}$ synthesis during luteolysis and that the action of oxytocin was mediated by the oxytocin receptor, as it was blocked by a specific oxytocin receptor antagonist. Furthermore, oxytocin treatment had no significant effect on luteal $PGF_{2\alpha}$ concentrations. Thus, oxytocin induces luteolysis in rats indirectly via uterine $PGF_{2\alpha}$ release, as in ruminants (Melampy et al., 1964). In rats, an indirect transfer of $PGF_{2\alpha}$ is proposed to occur by a countercurrent mechanism between the uteroovarian vein and ovarian artery as reviewed in ruminants by McCracken et al. (1972).

Early Embryonic Development in the Rat

Ovulation and Oocyte Maturation

In the rat, a preovulatory surge of LH causes ovulation of medium and large follicles (reviewed by Berrill and Karp, 1976). The oocytes resume meiosis and continue maturation so that fertilization can occur. During the first day of gestation, the oocytes enter the oviduct where sperm capacitation, oocyte maturation, transport of gametes, fertilization and early cleavage of the embryo takes place.

The oviduct consists of four distinct regions: infundibulum, ampulla, isthmus and the ampullary-isthmic junction.

The oocyte undergoes the final stages of maturation in the ampulla or body of the oviduct. Sperm are capacitated in the isthmus of the oviduct, the portion joining the uterus, prior to fertilization at the ampullary-isthmic junction. The embryonic genome is activated at the 2-cell stage. The fertilized embryo travels down the oviduct by means of ciliary action and muscular contraction of cells lining the walls. Cleavage begins while the embryo is moving down the isthmus and produces an aggregate of cells called the morula, at which time the embryo is transported to the uterus.

The proper uterine environment is necessary for correct development of the embryo. Increased levels of $PGF_{2\alpha}$ have been associated with accelerated rate of movement of mouse embryos through the oviduct (Mercier-Parot, 1976). Therefore, accurate synchrony between the embryo and uterus is necessary for survival and development of the early embryo.

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Morula to Blastocyst Development

It has been estimated that the 8- to 16-cell rat embryo enters the uterus approximately 96 hours after ovulation. During morula development, the daughter cells (blastomeres) flatten on each other to form a rounded embryo and internal cellular components. The combined flattening and polarization are referred to as "compaction". Tight junctions form within the trophoblast when the blastomeres are in close contact during compaction. This formation provides a permeability seal to allow fluids to move from the outside to the inside of the embryo as the result of solute gradient established by active ion transport (i.e. Na^+/K^+ ATPase pump). The accumulation of fluid induces the formation of the blastocoele and allows the blastocyst to form the inner cell mass (ICM) and the trophectoderm (reviewed by Butler, 1987).

Hatching of the Embryo

The blastula, characterized by an internal, fluid filled cavity shortly becomes the free blastocyst by escaping or "hatching" from the enclosing zona pellucida (ZP). This process includes both enlargement and an undulating movement of the blastocyst. The developing

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blastocyst will be released (hatched) from the ZP in the uterus at about 4.5 to 5 days. Expansion of the blastocyst involves hyperplasia and fluid accumulation in the blastocoele. The ZP becomes distended by the blastocyst and breaks in an equitorial plane; however, enzymes may also play an important role in ZP hatching.

After the ZP ruptures, the blastocyst will squeeze between the two edges of the opening in the ZP (reviewed by Bazer et al., 1993). Biggers et al. (1978) suggested that prostaglandins (in particular, the E series) are involved in the hatching process in mice. Prostaglandins appear to increase Na movement into the blastocoele which causes an increase in intracelluar fluid. This accumulation of fluid is a critical component of the hatching process (Borland et al., 1977).

Decidualization and Implantation

The rat endometrium undergoes decidualization, a tissue remodeling process, during embryo implantation. This process involves a transformation of endometrial stromal cells to decidual cells. In the rat, implantation begins shortly after hatching (Day 5-5.5), during which the trophoblastic cells aggressively invade the uterus, and ceases when

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maternal blood vessels are reached and breached (Berrill and Karpp, 1976). To obtain decidualization in ovariectomized rats, the uterus must be sensitized by an appropriate hormonal regimen of estrogen and progesterone (Psychoyos, 1973).

A local inflammatory reaction and an increase in stromal vascular permeability are prerequisites to decidualization and blastocyst implantation. Prostaglandins are vasoactive agents and are important mediators in this process. Indomethacin, an inhibitor of prostaglandin biosynthesis, delays or inhibits the localized increase in endometrial vascular permeability (Kennedy, 1977; Evans and Kennedy, 1978; Hoffman et al.,1978) and implantation (Saksena et al., 1976; El Banna, 1980). Both PGE_2 and $PGF_{2\alpha}$ have been found to be equally as effective in causing decidualization (Kennedy, 1985).

Endometrial binding sites have been reported for PGE_2 (Kennedy, 1983), but the ability of $PGF_{2\alpha}$ to produce decidualization is perplexing because no endometrial receptors have been found (Martel et al., 1985). One possibility is that $PGF_{2\alpha}$ is converted within the uterus to PGE_2, which then acts upon its PGE_2 receptors. This

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conversion is catalysed by 9-ketoreductase which is present in the rat endometrium (Kennedy, 1985). Prostaglandin $F_{2\alpha}$ in concentrations at least 2 orders of magnitude higher than those of PGE_2 must be present in order to compete with PGE_2 for binding sites (Kennedy and Doktorcik, 1988).

It is proposed that changes in the uterine levels of prostaglandins, causing an imbalance of $PGF_{2\alpha}$ to PGE_2 may be the basis for the interference of the implantation process and termination of pregnancy. In the ewe and cow, caruncular tissue preexists and no rapid modification of the endometrium comparable to that observed in rats exist (Lacroix and Kann, 1982).

Development of the Fetus

Fetal development of the rat follows the same developmental schedule as the mouse, with the major difference being that the rat conceptus implants a day and half later than that of the mouse, resulting in a longer gestation period by one and a half days (reviewed by Butler, 1987). After completion of implantation in the rat, extraembryonic parts begin developing. Formation of the heart, pericardium, amniotic folds, and evidence of a head process occurs around Day 8 with the neural plate and

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foregut developing on Day 9 of gestation. Growth and elongation of the fetus continues throughout the gestation period (23 days).

Stages of Embryonic Mortality

In rats, little is known with regard to the timing of embryonic loss. Furthermore, the factor or combination of factors that are responsible for this loss have yet to be determined. Shalgi (1984) has demonstrated that the proportion of *in vivo* or *in vitro* fertilized oocytes that develop to Day 20 fetuses is only 10% less than the proportion of viable Day 13 fetuses, suggesting that the majority of embryonic losses had occurred before Day 13.

In cattle, embryonic mortality is defined by the Committee on Reproductive Nomenclature as any loss which occurs during the first 42 days of pregnancy. In sheep (Edey, 1979) and cattle (Screenan and Disken, 1983), fertilization rates are around ninety to ninety-five percent but only about 60-70% of ovulations give rise to live births. The majority of embryonic loss in subfertile cows is occurring by Day 8 (Ayalon, 1978), although several studies indicate a large proportion of embryonic loss during the period of maternal recognition of pregnancy (Days

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16-24). This early period of pregnancy also seems to account for most losses in sheep (Wilmut et al.,1980).

In litter-bearing animals such as the pig, between 30-40% of conceptuses are lost and fertilization failure is again rare. The majority of the losses in the pig are embryonic. Pope and First (1985) reported that of 15 to 16 ova fertilized, only 9 to 10 piglets are born. Possible reasons for embryonic loss include abnormal embryonic development, an unsuitable uterine environment or luteal dysfunction. Chromosomal abnormalities, particularly polyploidy, exist in a variety of species and can terminate pregnancy, but occur at a relatively low rate (Wilmut et al., 1980).

Several processes are essential in order to prevent embryonic loss. First, an ovulatory follicle must develop an oocyte capable of being fertilized and of undergoing normal embryonic development. Secondly, the oviductal and uterine environments must be capable of gamete transport, fertilization and subsequent embryonic development. Finally, the corpus luteum must function for a sufficient period of time for embryo-derived signals initiating maternal recognition of pregnancy.

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Effect of $PGF_{2\alpha}$ on Early Embryo Development

Prostaglandins have been implicated in both growth of the embryo and the implantation process in the rat (Kennedy, 1977). Administration of $PGF_{2\alpha}$ to rats during early pregnancy, without progesterone supplementation, has been shown to cause fetal death (Labhsetwar, 1972). Furthermore, the antifertility effects were not mediated by inhibition of implantation, suggesting an embryotoxic effect or a reduction in the secretion of progesterone. In mice, administration of $PGF_{2\alpha}$ from the first to the fourth day of pregnancy increased the proportion of early abortions (Mercier-Parot, 1976). Harper and Skarnes (1972) suggested elevated concentrations of $PGF_{2\alpha}$ increased number of fetal deaths apart from abortion, and treatment with progesterone (P_4) prevented abortion but not fetal death in mice. Hoffman et al. (1978) observed an inhibition of embryonic growth with indomethacin (an inhibitor of prostaglandin endoperoxidase synthase) between Days 4 and 7 of pregnancy in the rabbit. Stormshak and Casida (1965) reported that treatment with progesterone (P_4) prevented abortion, but not fetal death when estradiol (E_2) was used to rescue the CL from LH-induced luteolysis in rabbits.

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Schrick et al. (1993) reported that higher concentrations of $PGF_{2\alpha}$ in uterine flushings of cows were related to lower quality of embryos. The most susceptible period of embryonic growth to the negative effects of $PGF_{2\alpha}$ appears to be during morula to blastocyst development (Seals et al., 1996). Furthermore, the regressing CL releases oxytocin which stimulates even higher concentrations of uterine $PGF_{2\alpha}$ (Silvia and Taylor, 1988) and results in pregnancy loss in cows supplemented with exogenous progestogen (Lemaster et al., 1996).

However, blastocysts of several species (porcine, ovine, bovine and laboratory animals)produce $PGF_{2\alpha}$ when incubated with and without arachidonic acid *in vitro* (Lewis, 1989). Prostaglandin $F_{2\alpha}$ from the conceptus is involved in intrauterine migration, ZP hatching, ion transport across the trophectoderm, fluid accumulation in the blastocoele, increased endometrial permeability and glucose metabolism by the blastocyst.

Supplementation Therapy to Maintain Pregnancy

In ovariectomized rats, pregnancy termination can be prevented with administration of progesterone (Peppe and Rothchild, 1974; MacDonald, 1978). Progesterone levels

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administered to ovariectomized rats to maintain pregnancy in various studies ranged from 3 to 20 mg/day. The blastocyst of the pregnant rat can be maintained unimplanted for extended periods of time with progesterone alone. The blastocyst can be induced to implant if the rat is given estrogen in addition to progesterone. The implantation process was supported and pregnancy maintained through Day 20 when injecting 3 mg progesterone/day plus 100 ng/day estradiol benzoate (MacDonald,1978). Administration of prolactin, which initiates progesterone secretion, can support pregnancy but is not required beyond Day 8 (MacDonald, 1978).

Wiltbank et al.(1956) hypothesized that death of bovine embryos after regression of the CL could be prevented if supplementation with a progestogen was begun before luteal regression. Supplementation of the progestogen, melengestrol acetate (MGA), failed to improve maintainance of pregnancy in postpartum cows that underwent early luteal regression. However, subsequent studies indicated the dosage of MGA was too low and resulted in elevated E_2 concentrations. In addition, injection of progesterone (200 mg/day) also failed to maintain pregnancy in cows with short-lived CL (Breuel et

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al., 1993). These results suggest that low fertility associated with a short-lived CL is not only due to a premature regression of the CL, but may result from a oocyte defect or a hostile uterine environment. Schrick et al.(1993) indicated embryos of postpartum cows were capable of pregnancy if placed into a normal cycling recipient.

3. STATEMENT OF THE PROBLEM

Corpora lutea (CL) secrete progesterone for only 2 to 3 days before regressing during the estrous cycle of the rat(Smith et al., 1975). For pregnancy to be established, corpora lutea must be maintained beyond the time of normal luteolysis. Possible reasons for embryonic loss include failure of normal embryonic development, luteal dysfunction, or embryotoxic factors within the uterine environment which destroy the embryo and/or CL.

Several studies have been conducted to determine the exact time of embryonic loss, but results have been variable. Shalgi (1984) demonstrated that the proportion of *in vivo* or *in vitro* fertilized rat oocytes which continued development to Day 20 fetuses was only 10% less than the proportion of viable Day 13 fetuses, suggesting that the majority of embryonic losses had occurred before Day 13. Ayalon (1978) reported the majority of embryonic loss in cattle occurs during the morula to blastocyst stage of development.

Prostaglandin $F_{2\alpha}$ is the luteolytic agent in many mammalian species, including the rat (Pharriss, 1970).

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Prostaglandin levels in uterine venous plasma have been shown to be elevated in the rat on Day 5 of pregnancy (Shaikh et al., 1977) at which time decidualization and implantation take place. Both PGE_2 and $PGF_{2\alpha}$ have been shown to be equally effective in causing decidualization and implantation (Kennedy, 1985). Prostaglandin $F_{2\alpha}$ is converted via 9-ketoreductase to PGE_2 which then acts on its uterine receptors to produce decidualization (Kennedy, 1985). Persaud (1986) found that administration of PGE_2 on Days 5 thru 8 of pregnancy in the rat had no embryotoxic effects. However, prostaglandin $F_{2\alpha}$ has been shown to prevent or terminate pregnancy when administered subcutaneously without progesterone supplementation (Gutknecht et al.,1969; Labhsetwar,1972). It is proposed that changes in the uterine levels of prostaglandins causing an imbalance of $PGF_{2\alpha}$ to PGE_2 may be the basis for the interference of the implantation process and termination of pregnancy.

Data from mice and rabbits indicated that $PGF_{2\alpha}$ caused fetal death or the inability of the embryo to develop to expanded/hatched blastocyst (Harper and Skarnes, 1972; Maurer and Beier, 1976). Furthermore, the antifertility

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effects associated with $PGF_{2\alpha}$ are not due to accelerated egg transport (Labhsetwar, 1972). In cattle, higher concentrations of $PGF_{2\alpha}$ in uterine flushings of cows were related to lower quality of embryos (Schrick et al., 1993). Therefore, the objective of this study was to determine if the decrease in embryo viability associated with $PGF_{2\alpha}$ is due not only to lowered concentrations of progesterone as a result of luteolysis, but also to a direct negative effect of $PGF_{2\alpha}$ on the embryo during *in vivo* and *in vitro* development of the preimplantation rat embryo.

4. MATERIALS AND METHODS

Experiment 1 (*In Vitro* Study)

Thirty mature female rats (3-4 months of age; ≈300g) of the Sprague-Dawley strain were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed under controlled conditions of temperature, humidity and lights (14h light and 10h dark) and were provided standard lab chow and water ad libitum. Normality of estrous cycles was monitored by vaginal smears taken daily between 0800 and 1000 h to determine stage of the estrous cycle by examining epithelial cell type. Only rats that exhibited two consecutive 4-day estrous cycles were used in the study.

On the day after estrus, animals were superovulated by receiving daily subcutaneous injections of 4 IU of FSH (Metrodin, Serono Laboratories, Inc.; Norwell, MA) for 3 consecutive days. Following the last injection, animals were placed with males of proven fertility for mating. On the following morning, matings were confirmed by the presence of spermatozoa in the vaginal lavage (Day 1 of pregnancy).

Trial 1

On Day 4 of pregnancy, animals were euthanized by CO_2 overdose and the oviducts removed and flushed with ~1ml Dulbecco's phosphate buffered saline (DPBS) into a collection dish. The medium was searched using a dissecting microscope for the presence of embryos, which if recovered, were examined further for 1) fertilization, 2) stage of development, and 3) morphology. Morphology was assesed by evaluating eveness (similarity in size) of the blastomeres and the degree of cellular fragmentation. Morphologically normal embryos of the 8-cell stage were assigned to be cultured in alpha-Minimum Essential Medium (αMEM;pH=7.2; Gibco #1200-022; Grand Island, NY) supplemented with 0.1% polyvinyl alcohol (control;n=179) or the control media supplemented with one of the following levels of prostaglandin $F_{2\alpha}$ (Lutalyse; Upjohn Inc.; Kalamazoo, MI): 1) 0.1 ng/ml(n=176), 2) 1.0 ng/ml(n=176) or 3) 10 ng/ml(n=162). After 24 h, stages of embryonic development were recorded and embryos, which had developed to the morula or beyond, were transferred to control media and cultured for an additional 24 h(Figure 2). Stages of embryonic development were recorded after 48 h.
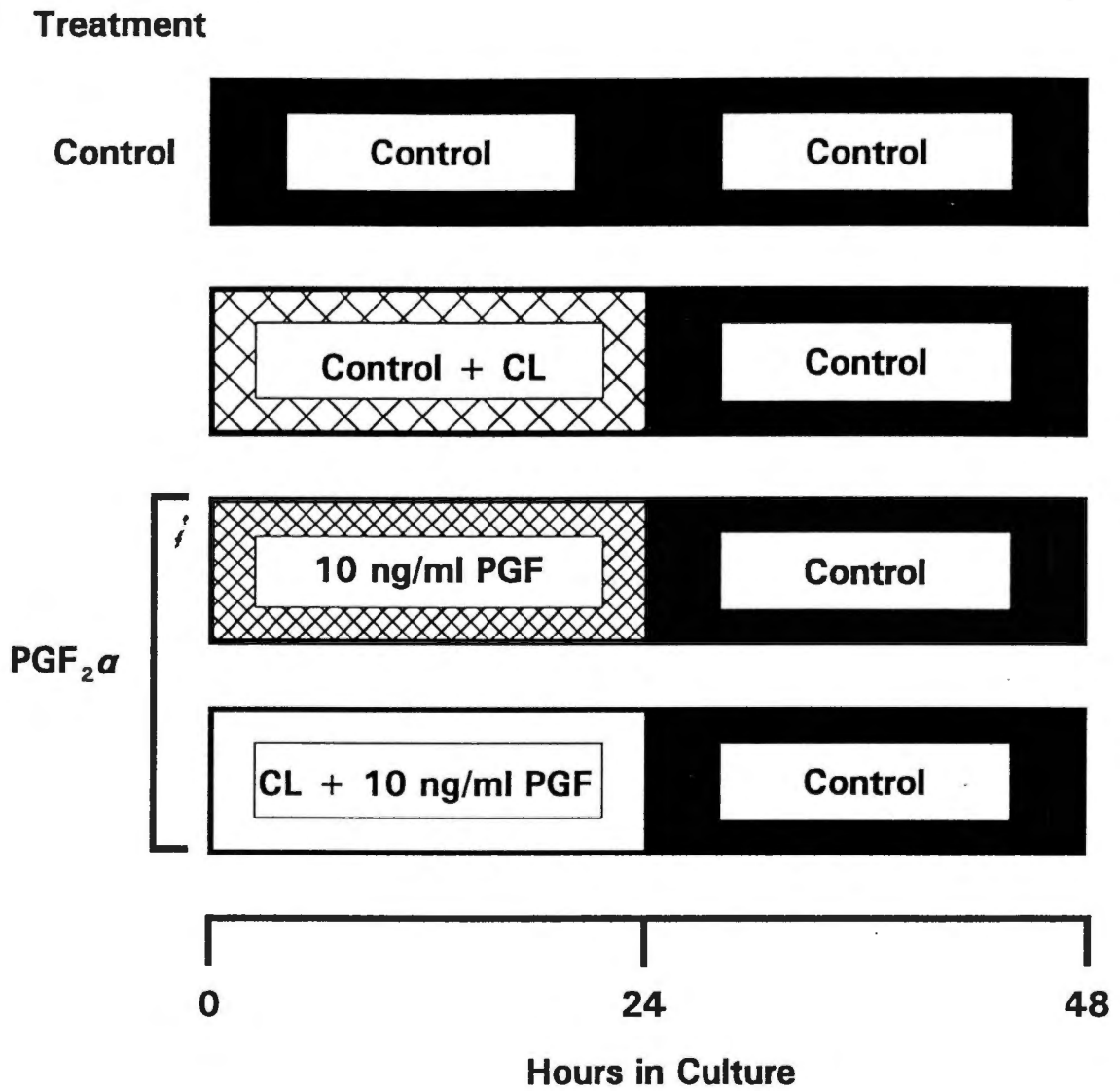
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Treatment

Figure 2. Experimental Procedure for Trial 1.

Trial 2

Ten mature female (3-4 months of age;≈300g) and proven fertile male Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) were utilized in a superovulation scheme as performed in Trial 1. Mating was ascertained by the presence of sperm in the vaginal lavage (Day 1 of pregnancy). Ovaries and oviducts were removed on Day 4 of pregnancy. Oviducts were flushed with Dulbecco's phosphate buffered saline and the number of embryos recorded and scored as in Trial 1. Corpora lutea were recovered from the ovaries using a dissecting microscope. Corpora lutea were washed three times with saline and placed with embryos in 2 of 4 treatments in transwell culture dishes (Falcon; Dickinson Inc.; Lincoln Park, NJ). The number of corpora lutea placed in the treatments corresponded with the number of embryos. Treatments consisted of the following: 1) control (αMEM;n=37), 2) control-luteal (corpora lutea + αMEM;n=30), 3) PGF-luteal (corpora lutea +10 ng/ml $PGF_{2\alpha}$;n=30), 4) PGF-control (10 ng/ml PGF + αMEM;n=41). Embryos were incubated for 48 h under 5% CO_2 in air at 37°C and development was recorded after 48 h of culture (Figure 3).

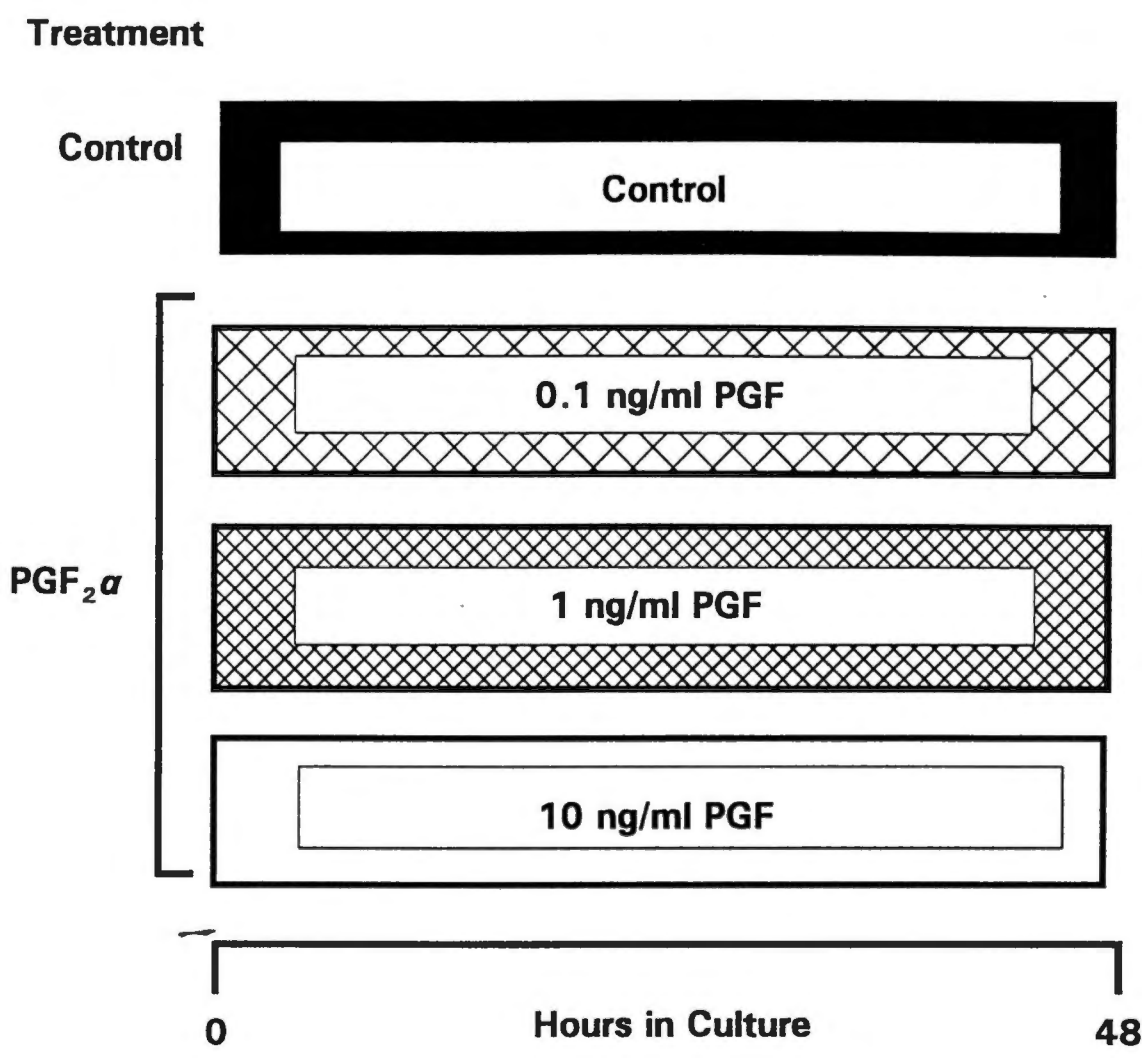Figure 3. Experimental Procedure for Trial 2.

Experiment 2 (*In Vivo* Study)

Twenty five mature female (3-4 months of age; ≈300g) and proven fertile male Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) were utilized to determine the effects of $PGF_{2\alpha}$. Animals were housed under controlled conditions of temperature, humidity and lights (14h light and 10h dark) and were provided standard lab chow and water ad libitum. Normality of estrous cycles was monitored by vaginal smears taken daily between 0800 and 1000 h. Only rats that exhibited two consecutive 4-day estrous cycles were used in the study. Mating was ascertained by the presence of sperm in the vaginal smear (Day 1 of pregnancy).

Animals were separated into three groups on Day 4 of pregnancy: Group 1 (n=11), intact controls; Group 2 (n=7), subcutaneous injections of 500 ug of prostaglandin $F_{2\alpha}$ (Lutalyse; Upjohn Inc.; Kalamazoo, MI) twice daily from Days 4 to 7 of pregnancy; and Group 3 (n=7), subcutaneous injections of 1000 ug of prostaglandin $F_{2\alpha}$ twice daily from Days 4 to 7 of pregnancy(Figure 4). Furthermore, all animals were injected subcutaneously with 4 mg/day of progesterone (Sigma; St. Louis, MO) in corn oil from Days 4 to 11 of treatment(Figure 4). On Day 11, animals were euthanized by
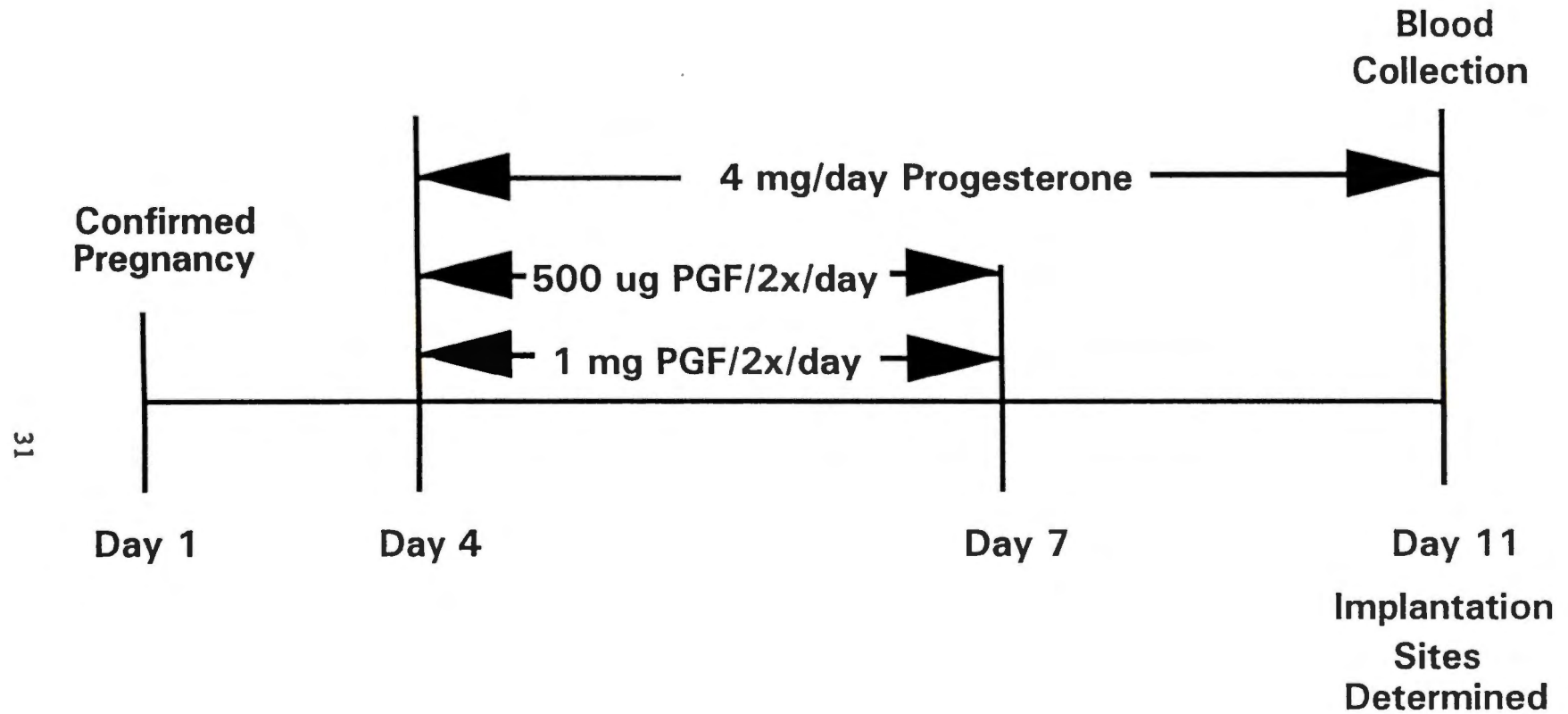
Figure 4. Diagram of Experimental Procedure for Experiment 2

CO_2 overdose and the number of implantation sites determined. Maternal weights, litter size, number of resorptions and number of corpora lutea were also recorded.

Serum Progesterone Levels

Blood was collected by decapitation from control and treated rats on the morning of Day 11 of pregnancy (Figure 4). After allowing the blood to clot at room temperature, serum was separated by centrifugation (2500xg for 20 min) and stored at $-20°C$ until assayed. Concentrations of P_4 in serum were determined as described by Butcher (1977). Sensitivity of the assay was .05 ng/tube (100 μl), with intra- and inter-assay coefficients of variation of 8.6 and 13.6%, respectively.

Statistical Analysis

Effects of prostaglandin $F_{2\alpha}$ on *in vitro* embryonic development were determined using Chi-Square analysis. Both serum P_4 concentrations and implantation data from the *in vivo* study were analyzed statistically with student's *t* test utilizing the General Linear Model (SAS, 1989). Correlations between implantations and corpora lutea were analyzed using Proc Corr of SAS (1989). Effects of treatment were considered significant when P < .05

5. RESULTS

Experiment 1 (*In Vitro Study*)

Trial 1

Prostaglandin $F_{2\alpha}$ was found to have a deleterious effect on embryonic development, regardless of dosage(Table 1). Following 24 h of culture, 63% of control embryos continued development with 35% having reached the morula stage and 28% attaining the blastocyst stage. Only twenty-two percent of the embryos treated with 0.1 ng/ml $PGF_{2\alpha}$ had continued development, with 13 and 7% ($p < .05$) at the morula and blastocyst stage, respectively. Only 15 and 16% of the embryos treated with 1.0 and 10 ng/ml $PGF_{2\alpha}$, respectively, continued development to the morula and blastocyst stage($p < .05$). The percentage of embryos treated with 0.1, 1.0 and 10 ng/ml of prostaglandin $F_{2\alpha}$ that reached the expanded blastocyst stage after 24 hours ($\leq$ 2%) was significantly lower than control embryos (20%; $p < .01$).

Following 48 h of culture, 33% of the total control embryos continued development. However, 52% of control embryos, which were considered alive after 24 h in culture,

continued development following 48 h *in vitro*.

Table 1. Summary of Embryonic Development for Trial 1

Treatment	n	Development[a] 24 hours	48 hours
Control[e]	179	113 (63)[b]	59 (33)[c]
0.1[df]	176	44 (25)	17 (10)
1.0[df]	176	27 (15)	6 (4)
10[df]	162	28 (17)	9 (6)

[a] Values within parenthesis are percentages
[b] Blastocyst, expanded blastocyst
[c] Expanded blastocyst, hatched blastocyst
[d] $PGF_{2\alpha}$ concentration in ng/ml
[e,f] Treatments with different superscripts differ at ($p < .01$)

The percentage of total embryos reaching the expanded blastocyst stage after 48 hours of culture with 0.1, 1.0, and 10 ng/ml of $PGF_{2\alpha}$ were 9, 3, and 4%, respectively, compared to 26% in the control group (Table1; $p < .05$). The percentage of live embryos in the 0.1, 1.0, and 10 ng/ml $PGF_{2\alpha}$ group that continued development after 24 h and transfer to control media were 45%, 19%, and 29%, respectively. All concentrations of prostaglandin $F_{2\alpha}$

reduced the number of embryos reaching the hatched

blastocyst stage ($\leq 1\%$), with the 0.1 ng/ml concentration

of $PGF_{2\alpha}$ being the minimum effective dose in retarding

blastocyst development.

Trial 2

Thirty eight percent of control embryos reached the

blastocyst stage after 48 hours of culture (Table 2).

Table 2. Summary of Embryonic Development for Trial 2

Treatment	n	Development[a] 48 hours
Control (MEM)[c]	37	14 (38)[b]
MEM + Cl[c]	30	13 (43)
10 ng/ml PGF[d,e]	30	9 (30)
10 ng/ml PGF + Cl[d,f]	41	8 (20)

[a] Values within parenthesis are percentages
[b] Blastocyst
[c,d] Treatments with different superscripts differ at (p <.05)
[e,f] Treatments with different superscripts tend to differ (p=.07)

The percentage of embryos in the CL + αMEM treatment that

reached the blastocyst stage (43.3%) was not statistically

different from the controls (37.8%; p>.10). The addition of 10

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ng/ml of $PGF_{2\alpha}$ to the culture media significantly reduced the number of embryos reaching the blastocyst stage (30%;p<.05) compared to the controls. Presence of luteal tissue tended (p=.07) to increase the negative effects of prostaglandin with only 19% of the embryos attaining the blastocyst stage when co-cultured with corpora lutea + 10 ng/ml of $PGF_{2\alpha}$.

Experiment 2

Administration of prostaglandin $F_{2\alpha}$ on Days 4-7 of pregnancy had no effect on embryonic development, regardless of the dose administered. All rats were pregnant and litter size was not reduced by prostaglandin treatment (Table 3). The mean concentration of P_4 in rats treated twice daily with 500 ug of $PGF_{2\alpha}$ was not statistically different (p>.10) from the controls (99.6 ± 5 vs 82.7 ± 14.3 ng/ml; Table 3). However, increasing the dose to 1000 ug $PGF_{2\alpha}$ did decrease the level of serum progesterone (27.4 ± 5.1 ng/ml;p<.05)compared to controls (82.7 ± 14.3 ng/ml). No differences were observed between the number of corpora lutea and implantation sites between the treatments.

Table 3. Effects of $PGF_{2\alpha}$ Treatment *In Vivo* on Pregnancy Rate, Implantation Site Number and Progesterone Concentrations in Mature Rats

Treatment	# of rats Pregnant	# implantation sites [a]	Progesterone (ng/ml) [a]
Control + P_4	11/11	14.5 ± 0.5	82.7 ± 14.3[b]
1 mg/day PGF + P_4	7/7	13.8 ± 2.3	99.6 ± 5.0[b]
2 mg/day PGF + P_4	7/7	14.0 ± 3.2	27.4 ± 5.1[c]

[a] mean ± SEM

[b,c] Means with different superscripts within column differ (p<.05).

6. DISCUSSION

The objective of this study was to determine if the decrease in embryo viability associated with $PGF_{2\alpha}$ is due to a direct negative effect of $PGF_{2\alpha}$ on the rat embryo during *in vitro* and *in vivo* development. A secondary objective was to determine if the rat model could substitute for the cow based on the findings that the quality of bovine embryos was negatively correlated to the concentration of $PGF_{2\alpha}$ in uterine flushings (Schrick et al.,1993). The results from the *in vitro* study support the contention that $PGF_{2\alpha}$ has a direct negative effect on development of 8-cell rat embryos. The number of embryos continuing development was significantly reduced in all $PGF_{2\alpha}$-treated groups. This is in agreement with Maurer and Beier's (1976) findings that $PGF_{2\alpha}$ prevented development of rabbit embryos to the hatched blastocyst stage *in vitro*. These results are in contrast to what has previously been seen in the mouse (Kirkpatrick, 1974).

A decrease in embryonic development was also observed in embryos co-cultured with corpora lutea and $PGF_{2\alpha}$. However, culture of embryos with corpora lutea alone did not

have an effect on blastocyst development, suggesting $PGF_{2\alpha}$ caused regression of corpora lutea which resulted in release of luteal $PGF_{2\alpha}$ (Rothchild, 1981; Niswender and Nett, 1988).

The reduction in development of embryos cultured in medium containing $PGF_{2\alpha}$ may have resulted from an interruption in a number of critical events associated with embryonic development. These events include compaction, water and electrolyte transport and shedding of the zona pellucida. The formation of tight junctions during compaction allows fluids to move from the outside to the inside of the embryo as a result of the solute gradient formed. The accumulation of fluid is an essential component in blastocyst formation and shedding of the zona pellucida. Biggers et al.(1978) reported that prostaglandins (particularly the E series) are involved in the movement of fluid into the embryo and the hatching process in mice. However, the mechanisms through which prostaglandin $F_{2\alpha}$ is detrimental to embryos remains unclear.

Prostaglandins have been shown to be increased in the rat at the time decidualization and implantation occur (Shaikh et al., 1977). Prostaglandin $F_{2\alpha}$ is converted within the uterus via 9-ketoreductase to PGE_2 (Kennedy, 1985).

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Persaud (1986) reported that administration of PGE_2 on Days 5 thru 8 of pregnancy in the rat had no embryotoxic effects. Our findings support the hypothesis that elevated levels of $PGF_{2\alpha}$ resulting from an imbalance in the $PGF_{2\alpha}/PGE_2$ ratio negatively effects blastocyst development.

However, the results of the *in vivo* study indicate that administration of $PGF_{2\alpha}$, along with progesterone treatment, does not have a deleterious effect on embryonic development during early gestation(Days 4-7). These *in vivo* results appear to indicate that the reduction in pregnancy rate in animals treated with $PGF_{2\alpha}$ may be due only to a decrease in progesterone which is required to maintain the proper uterine environment.

Similar results obtained by Fuchs and Mok (1973) showed pregnancy was not interupted during Days 1 thru 7 using intravenous infusions of $PGF_{2\alpha}$ without progesterone supplementation. Prostaglandin $F_{2\alpha}$ was shown to prevent or terminate pregnancy when administered subcutaneously during Days 4 thru 7 of pregnancy without P_4 treatment(Gutknecht et. al., 1969; Labhsetwar, 1972). However, the negative effects of $PGF_{2\alpha}$ were reversed when animals were supplemented with progesterone.

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The elevated levels of progesterone in the $PGF_{2\alpha}$-treated rats (1 mg/day) indicate the corpora lutea did not regress. This suggests that the exogenous dose of prostaglandin $F_{2\alpha}$ was not stimulating the utero-ovarian production of $PGF_{2\alpha}$, which is an important component of the luteolytic mechanism of exogenous $PGF_{2\alpha}$ (Wade and Lewis, 1996). The resistance to the luteolytic effect of $PGF_{2\alpha}$ could be related to changes in $PGF_{2\alpha}$ receptor number, receptor affinity or both. Behrman et al. (1976) reported that functional corpora lutea of the rat are refractory to $PGF_{2\alpha}$-induced luteolysis during early pregnancy. Wright et al.(1980) reported that the resistance to exogenous prostaglandin $F_{2\alpha}$ is mediated by factors other than affinity and capacity of $PGF_{2\alpha}$ receptors in pseudopregnant rats.

Our results show that the increased dose of 2 mg/day of $PGF_{2\alpha}$, along with progesterone supplementation, did regress the corpora lutea but did not effect pregnancy rates or litter size. A possible reason for the sustained pregnancy in $PGF_{2\alpha}$- treated rats may involve its conversion to PGE_2. A "hostile uterine" environment would be avoided if $PGF_{2\alpha}$ levels within the uterus were lowered via conversion to PGE_2.

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In cattle, administration of $PGF_{2\alpha}$ on Days 5-8, along with progesterone supplementation, was found to reduce pregnancy rates(Seals et al.,1996). The dose of $PGF_{2\alpha}$ (0.8 mg/kg) administered to the cow for luteal regression and pregnancy loss was substantially lower than the dose needed in order to regress the CL in the rat(6-8 mg/kg). This indicates that the rats CL is either not as sensitive to the luteolytic effects of $PGF_{2\alpha}$ or the prostaglandin is metabolized more efficiently in the rat. Therefore, the rat is not an efficacious model for evaluating the embryotoxic effects of $PGF_{2\alpha}$ in the cow.

In conclusion, elevated levels of prostaglandin $F_{2\alpha}$ do have a direct negative effect on the developing rat embryo *in vitro*. However, exogenous administration of $PGF_{2\alpha}$, along with progesterone supplementation, did not effect embryonic development. This could be due to a balance in the $PGF_{2\alpha}/PGE_2$ ratio associated with 9-ketoreductase, resulting in lowered concentrations of $PGF_{2\alpha}$ within the uterus.

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