The use of altrenogest to control reproductive function in beef cattle

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THE USE OF ALTRENOGEST TO CONTROL REPRODUCTIVE FUNCTION IN BEEF CATTLE

A Dissertation

Submitted to the Graduate Faculty of
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

in

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by
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ABSTRACT

There has been great controversy involving progesterone (P₄) levels during early pregnancy in cattle. The objectives of these experiments were to determine the effect of an early low dose administration of P₄ or altrenogest (ALT) on pregnancy rates in repeat breeder (RPB) females, if an increase in pregnancy rates could result from a direct effect of P₄ on the embryo and if ALT could support pregnancy in the absence of a functional CL. Firstly, ALT was evaluated for use as a progestin in cattle by synchronizing estrus in beef heifers. There were no differences in the number of females displaying behavioral estrus or in pregnancy rates when synchronized with ALT or MGA. A second experiment was designed to determine the effect of P₄ or ALT supplementation during days 3 to 5 on pregnancy rate in RPB cattle. It was determined that 15 mg of P₄ or ALT during days 3 to 5 increased pregnancy rates compared with nontreated breeding periods. A third experiment was designed to determine if P₄ exerted a direct effect on the embryo. In vitro produced (IVP) embryos were cultured in the absence of a co-culture system. At day 3, post-insemination, embryos were cultured in the presence of P₄ and evaluated on days 6 to 9. On day 7 post-insemination, there were significantly more grade 1 blastocysts from the P₄ group compared with other treatment groups. Also, embryo developmental rates were increased when cultured in the presence of P₄ and more of these embryos developed to the hatched blastocyst stage compared with other treatment groups. After a direct effect of P₄ on developing IVP bovine embryos existed, it was determined that these embryos did not possess P₄ receptors. Finally, it was demonstrated that ALT could support pregnancy in the absence of a functional CL. These experiments demonstrated that ALT could serve as a progestin in cattle and when administered in low doses during early pregnancy could improve pregnancy rates in RPB cows. These results are likely due to a direct effect of P₄ on the embryo; however, this mechanism is by means other than binding the PR.
CHAPTER 1
INTRODUCTION

It is well known that progesterone (P₄) has been shown to be necessary for the establishment and maintenance of pregnancy in mammals. In addition, P₄ has been proven essential for the initiation of reproductive function in the female. In cattle, P₄ treatment of prepubertal females has been demonstrated to hasten the initiation of cyclicity in heifers (Short et al., 1976; Heitzman et al., 1979). Also, P₄ treatment of post-partum cows has decreased the number of days to mating (Brown et al., 1972; Yelich et al., 1988; Yelich et al., 1995a; Patterson et al., 1995). Wilmut et al. (1980) reported that a normal period of P₄ exposure during the luteal phase was necessary to achieve normal pregnancy rates during the subsequent estrual period. However, the effects of elevated P₄ during early pregnancy on the developing bovine embryo are still under investigation.

Elevated P₄ levels during corpus luteum (CL) formation (metestrus) may have effects on the developing embryo. Increased circulating P₄ levels at the time the embryo is in the oviduct has been reported to hasten the rate of embryo transport into the uterus in mice (Kendle and Lee, 1980) and cattle (EL-Banna and Hafez, 1970; Crisman et al., 1980). This treatment hastened embryo transport along with stimulation of uterine cell proliferation and differentiation, as well as increased blood flow to the bovine uterus (Johnson et al., 1997; Rider et al., 1998; Gray et al., 2001). An early rise in P₄ has been reported to alter the pattern of protein synthesis, secretion and overall increase uterine protein secretions in cattle (Garret et al., 1988). Furthermore, it has been noted that when early circulating P₄ increased there were increased embryo developmental rates (Lawson et al., 1983; Lawson and Cahill, 1983; Garrett et al., 1987; Garrett et al., 1988). Thus, with earlier embryo entry into the uterus, there will be a stimulated, receptive uterus to support further embryo growth and differentiation.

An early increase in P₄ levels in pregnant or nonpregnant cows, however, has not been consistently demonstrated (Shemesh et al., 1968; Pope et al., 1969; Henricks et al., 1970; Henricks et al., 1971; Sreenan and Diskin, 1983). Ashworth et al. (1989) and Mann and Lamming (1996) reported that hastened CL development and function would likely increase pregnancy rates in cattle. There is indirect evidence of faster CL development positively impacting pregnancy rates, when comparisons were made of the reproductive performance of pubertal and mature females (Davies and Beck, 1993; Silva
et al., 2002). In sheep, ewe lambs had lower levels of P₄ during metestrus and diestrus and lowered reproductive performance compared with mature ewes (Davies and Beck, 1993). However, research has failed to demonstrate differences between fertile beef and dairy cows regarding early P₄ levels during metestrus or diestrus (Shemesh et al., 1968; Pope et al., 1969; Robertson and Sarda, 1971; Bulman and Lamming, 1978; Geisert et al., 1988). The question of whether or not there is an early increase in P₄ levels during metestrus (presumably from earlier developed luteal tissues) in pregnant females compared with nonpregnant females remains to be answered. Lukaszewska and Hansel (1980) reported that P₄ levels were significantly higher in pregnant females compared with nonpregnant females by day 8 (estrus = day 0). Later, Hwang et al. (1988) demonstrated that the bovine embryo produces luteotrophic prostaglandins. These luteotrophins had been previously reported to cause increased P₄ levels in the ewe (Pratt et al., 1979). These reports demonstrate that viable bovine or ovine embryos have the ability to increase P₄ levels during early pregnancy.

The next logical step was to administer P₄ to mated females in an attempt to increase pregnancy rates. Unfortunately, this approach has resulted in varied success rates in cattle. Even with the lack of success, there have been reports of increased pregnancy rates over controls with P₄ supplementation from day 1 to day 7 following onset of estrus in dairy cattle (Henrick, 1953; Dawson, 1954; Wiltbank et al., 1956; Johnson et al., 1958). However, these studies used various levels of P₄ supplementation and are primarily veterinary case reports. The majority of these reports, however, indicated that P₄ supplementation from day 6 onward (estrus = day 0) failed to increase pregnancy rates (Sreenan and Diskin, 1983; Munro and Bertram, 1990; Van Cleef et al., 1991; Stevenson and Mee, 1991; Mann et al., 1998).

Attempts to increase P₄ levels by administration of luteotrophins have also failed to significantly increase pregnancy rates over that of control females. Administration of luteotrophins (human chorionic gonadotrophin, hCG or gonadotrophin releasing hormone, GnRH) during mid-cycle (day 7 or later) to artificially inseminated dairy and beef cattle have significantly increased circulating P₄ levels (Wiltbank et al., 1961; Rajamahendran and Sianangama, 1992; Lulia et al., 1994). However, the majority of these studies report no difference in pregnancy rates between treated and nontreated females (Holness et al., 1982; Sreenan and Diskin, 1983; Walton et al., 1990; Sheldon
and Dobson, 1993). These studies, taken collectively, indicate that increasing endogenous P₄ levels during mid to late luteal phase does not increase embryo survival rates. These findings are noteworthy because it is at this time that the embryo is releasing luteotrophic substances (Lewis et al., 1982; Hwang et al., 1988; Lewis, 1989).

The previous findings led to two hypotheses for the role of P₄ in the successful development during pregnancy: (1) P₄ increase must occur during early metestrus for a successful pregnancy to be established and (2) elevated P₄ during the luteal phase exerts a beneficial effect directly on embryonic growth and survival. The second hypothesis has recently gained some support. Mann et al. (1996) demonstrated that elevated P₄ levels during the early luteal phase (day 4 and later) resulted in a reduced luteolytic signal and thereby, allowed the embryo to have a greater chance of sending an optimal signal for maternal recognition of pregnancy. Furthermore, it was demonstrated that elevated P₄ levels result in conversion of uterine prostaglandin synthesis from PGF₂α (luteolytic) to PGE₂ (luteotrophic), elevated interferon tau (a maternal recognition signal) synthesis by the conceptus and a need for lower amounts of interferon tau to prevent luteal regression (Mann et al., 1998).

At this time it appears that these two hypotheses are not mutually exclusive and therefore, the further development of the first hypothesis was part of the objective of this dissertation. This objective was based on the findings of Gustaffason and Larson (1983) who demonstrated that when embryos were collected from cows of lowered fertility on day 7 (estrus = day 0) and transferred to fertile cows, this resulted in reduced embryo survival. Conversely, when embryos were collected on day 7 (day 0 = estrus) from fertile cows and transferred to cows with lowered fertility, embryo survival was normal (Gustaffason and Larson, 1985). These findings suggested that early embryonic developmental (day 3 until sustained luteal function) under the influence of elevated P₄ could possibly exert an effect on increasing embryonic survival. Also, there has been a need to determine if P₄ can exert a direct effect on the embryo.

Another objective of this dissertation was to determine if, the progestin, altrenogest could serve as a biologically active progestin in ruminant species, such as the cow. By determining whether altrenogest could serve a bovine progestin, it could be used to further evaluate the effect of progestins on early embryo development, as well as, maintaining pregnancy in beef cattle.
CHAPTER 2
LITERATURE REVIEW
Role of Oral Progestins in Control of Reproduction

MGA in Control of Reproduction

Melengestrol Acetate (MGA) is an orally active progestin commonly used for
estrus suppression and as a growth promotant in beef cattle. For MGA to be orally
active, the progestin has been chemically modified at carbons 6 (methyl and double
bond additions), 16 (methylene addition) and 17 (acetoxy addition) (Lauderdale, 1983).
These modifications allow MGA to remain chemically active as it transverses the rumen
into the circulation.

MGA was originally developed for use as a growth promotant in feedlot heifers
and has been shown to consistently improve weight gain and feed efficiency (Bloss et
al., 1966). Hafez (1987) subsequently reported that MGA improved weight gain and feed
efficiency by preventing behavioral estrus and blocking ovulation, while allowing follicular
development to continue on the ovaries. This resulted in sustained estrogen (E\textsubscript{2})
levels, which stimulated a synergistic effect on growth hormone (GH) release that resulted in
increased nitrogen retention and increased protein synthesis.

In cattle, MGA is ~30 times more potent than progesterone (P\textsubscript{4}) in pregnancy
maintenance and 125 times more potent than P\textsubscript{4} for suppressing estrus (Lauderdale,
1983). When fed at a rate of 0.25 mg per head per day, estrus was suppressed in all
heifers and at a rate of 0.50 mg per head per day follicular growth and development
would still occur in the absence of a corpus luteum (CL) (Bloss et al., 1966). However,
Young et al. (1966) reported that when MGA was fed (for 154 days) at a rate of 0.20 mg
per head per day behavioral estrus was only suppressed in 12 of 20 females, while at a
rate of 0.40 mg per head per day, only 3 of 20 females exhibited behavioral estrus and
at a rate of 0.60 mg per head per day no females displayed behavioral estrus.
Furthermore, Zimbelman and Smith (1966a) indicated that 0.42 mg per female per day
of MGA was the minimum requirement to prevent ovulation and behavioral estrus in beef
cattle. An important finding indicated that MGA was equally effective when administered
orally or intramuscularly and when administered orally, MGA was absorbed rather than
degraded in the rumen and/or intestines (Zimbelman and Smith, 1966b). Prior to its use
for estrous cycle synchronization, MGA was reported to maintain pregnancy in bilaterally
ovariectomized cows (Zimbelman and Smith, 1966c). A dose of 4 mg per female per day of MGA was found to be the minimum dose required to maintain pregnancy in bilaterally ovariectomized cows. This was 20 times greater than the dose required to prevent ovulation in beef cattle (Zimbelman, 1963). More recently, MGA has been used to support pregnancy in females where detection of endogenous P₄ levels were important (Wright et al., 1994; Bridges et al., 2000).

Use of MGA to control estrus in cycling cows utilizes long term (14 to 18 days) daily administration of MGA at doses ranging from 0.5 to 1.0 mg of MGA per head per day (Zimbelman and Smith 1966b; Zimbelman and Smith, 1968a; Zimbelman and Smith, 1968b; Roche and Crowley, 1973; Patterson et al., 1989). In these studies, the interval from removal of MGA from the ration to the onset of estrus ranged from 3 to 7 days (Zimbelman and Smith, 1968a; Zimbelman and Smith, 1968b; Roche and Crowley, 1973; Patterson et al., 1989). It should be noted that decades before, Zimbelman and Smith (1966b) reported low pregnancy rates among MGA-treated females following artificial insemination (AI). Subsequent reports verified a decrease in pregnancy rates that occurred in long-term MGA-treated females when compared with those of nontreated control females (Hill et al., 1971; Lamond et al., 1971). There have been two reports of no decrease in pregnancy rates following AI in dairy cows administered long-term MGA treatment (Roussel et al., 1969; Boyd, 1970), although decreases in pregnancy rates among AI dairy cows have been reported with long-term administration of P₄ (≥14 days) and other progestins (Trimberger and Hansel, 1955; Ray et al., 1961; Hansel, 1967; Wiltbank et al., 1967; Lamond et al., 1971; Wettemann and Hafs, 1973; Roche, 1974a; Roche, 1974b).

With long-term MGA treatment failing to produce respectable pregnancy rates among cattle following AI, alternative protocols were developed for the use of MGA. An attempt to improve pregnancy rates, following long-term treatment with MGA, by administration of hCG or LH at the time of AI failed to improve pregnancy rates in MGA-treated females (Zimbelman and Smith, 1968a; Zimbelman and Smith, 1968b; Roche and Crowley, 1973). Researchers aware that long-term progestin treatments decreased pregnancy rates following AI, continued searching alternative approaches using MGA. Soon after, a short-term treatment (≤14 days) with MGA was developed, which produced pregnancy rates similar to control treated females although the degree of synchrony was
greatly reduced (Wiltbank and Kasson, 1968; Roche, 1974a; Sreenan and Mulvehill, 1975). It was later demonstrated that long-term administration of MGA or other progestins induced persistent dominant follicles in cattle (Yelich et al., 1997; Cavalieri et al., 1998; McDowell et al., 1998). This was caused by P₄ down-regulating LH receptors, which are required for ovulation and final maturation of the oocyte (Batra and Miller, 1985; Thiery and Martin, 1991). In addition to the decreased viability of the oocyte, a persistent dominant follicle altered the steroid hormone secretion pattern and thus, secretory patterns of the oviduct thereby adversely affect fertilization in vivo (Binelli et al., 1999).

To circumvent the decreased fertility associated with long-term MGA administration and to prevent the loss of synchrony with short-term MGA administration, a short-term MGA protocol was combined with administration of PGF₂α. Although Fike et al. (1997) demonstrated that infertility due to persistent dominant follicles could be overcome by preventing ovulation of the persistent follicle and inducing ovulation of a second follicle this procedure was subsequently not practical for on-farm use. Using the combination of short-term MGA feeding and PGF₂α females were administered MGA for 5 days and administered PGF₂α following the end of MGA treatment. However, Moody et al. (1978) reported that when using this approach pregnancy rates remained low. When the MGA treatment was extended to 7 days and combined with PGF₂α, pregnancy rates among beef cattle still remained low (Patterson et al., 1986; Beal et al., 1988). A protocol was then developed where MGA was administered for 14 to 16 days and PGF₂α was administered 16 to 17 days following the last feeding of MGA (Brown et al., 1988). This protocol resulted in increased pregnancy rates which were attributed to a 17 day delay from MGA removal and the administration of PGF₂α. This delay would allow for the majority of the females to be in diestrus at the time of PGF₂α administration (King et al., 1982). This amendment of the delay from last feeding of MGA to PGF₂α administration was combined with a more abbreviated period of MGA administration (~14 days) to further improve pregnancy rates following estrous cycle synchronization and AI in cattle.

Mauck et al. (1988) compared the 14-day MGA feeding period with a 7-day feeding period and determined that the 14-day protocol was superior in pregnancy rates and degree of synchrony. Later, Coleman et al. (1990) evaluated the effect of a 21-day MGA treatment period with or without subsequent PGF₂α administration (14 days later)
and found that pregnancy rates were markedly reduced without PGF$_{2\alpha}$ administration. Today, the optimal MGA protocol for beef cows and heifers is a 14-day MGA treatment period followed by PGF$_{2\alpha}$ administration 15-days later for cows and 17-days later for heifers (Kesler et al., 1996).

MGA has also been utilized for estrous cycle synchronization of postpartum cows. Using the current MGA protocol (14-day MGA administration followed 17 days later by administration of PGF$_{2\alpha}$) and AI in postpartum cows acceptable estrual response, pregnancy rates and increased weight gain were achieved (Yelich et al., 1988). Yelich et al. (1995a) concluded that MGA was effective in postpartum females provided that synchronization was initiated when cows had a good body condition score. In addition, it was reported that 48-hour calf removal increased synchronized pregnancy rates in postpartum cows synchronized with MGA (Yelich et al., 1995b). The pregnancy rates achieved following synchronization with MGA and AI in postpartum cattle were in agreement with the pregnancy rates reported for postpartum cows by Patterson et al. (1995).

In prepubertal and pubertal heifers, synchronization rates and pregnancy rates were consistently higher following MGA-estrous cycle synchronization and AI compared with nontreated control heifers and heifers undergoing estrous cycle synchronization with other progestins (Deutscher et al., 1989; Goehring, 1989; Jaeger et al., 1992; Patterson and Corah, 1992).

There have been attempts to test the effectiveness of MGA in non-ruminant species. Loy and Swan (1966) demonstrated that 10 to 20 mg per day per head of MGA or 100 mg injections of MAP (6α-methyl-17α-acetoxyprogesterone) were not effective in controlling estrus or ovulation in the mare. It was concluded that P$_4$ was catabolized to the urinary metabolite 5α allopregnane rather than its primary urinary metabolite 5β pregnane. This may have altered the progesterone compound rendering it inert in the mare. Additional studies demonstrated that although MGA or norgestomet would support pregnancy in bilaterally ovariectomized cows, however, they were ineffective in maintaining pregnancy in bilaterally ovariectomized mares (McKinnon et al., 2000).
Altrenogest for Control of Reproduction in the Mare

Altrenogest (allyltrenbolone, ALT) is an orally active progestin developed for use in the horse. Altrenogest has been chemically modified by addition of a hydroxyl group and 3 carbon chain placed on carbon 17 of the progestin (Peters, 1992). Research has demonstrated that ALT has low anabolic activity and is 20 times less potent than testosterone or similar progestins when compared for effects on muscle growth in castrated rats (Peters, 1992). It was further noted that 50% of altrenogest residue becomes bound to binding proteins and becomes biologically inactive. Based on these findings (Peters, 1992), the withdrawal period for ALT (known commercially as Regumate) for equids is 15 days.

There have been no deleterious effects reported of ALT administration to healthy cycling mares when fed for 86 days at a rate of 0.044 mg per kg of body weight, 0.132 mg per kg of body weight or 0.220 mg per kg of body weight (Shideler et al., 1983). This observation was based on evaluations for differences in white blood cell counts, platelet number, creatinine levels, cholesterol, signs of inflammation and overall organ appearance following necropsy.

The initial development of ALT was for controlling estrus in mares. Squires et al. (1979) demonstrated that when ALT was orally administered to estrual mares (early in the breeding season) at a rate of 0.044 mg per head per day for 12 days, behavioral estrus ceased within 3 days following initiation of ALT treatment and estrus remained inhibited for the duration of treatment. However, ovulation was not inhibited in all ALT-treated mares during this time. Following the cessation of ALT treatment, ALT-treated mares had a mean interval to estrus of 4.5 days and had a significantly shorter time interval from the end of treatment to ovulation and the onset of estrus to ovulation compared with nontreated mares.

Squires et al. (1979) also reported that initially transitional mares treated with ALT had a high incidence of continuous estrus compared with a high incidence of discontinuous estrus seen in control-nontreated transitional mares. In this initial testing of ALT, Squires et al. (1979) tested for the most efficient stage of estrous cycle to begin administration of ALT for estrous cycle synchronization and it was reported that the interval from ALT-treatment to estrus was shorter when ALT-treatment was initiated during diestrus compared with that of estrus. Although ALT was effective in the control of
estrual behavior and in estrous synchronization, the inability of ALT to block ovulation in all mares made this 12-day treatment period unlikely to be the most effective regime, since it did not allow for sufficient time for CL regression to occur before the end of treatment (Squires et al., 1979).

Further testing of ALT in transitional mares revealed that it was effective in abbreviating the transition period in these females (Turner et al., 1981; Webel and Squires, 1982). When transitional mares were administered ALT (0.044 mg/head/day) for 15 days there was a significant decrease in the length of estrus and the number of inseminations required without a decrease in pregnancy rate (Turner et al., 1981). Webel and Squires (1982) reported similar effects of ALT-treatment in transitional mares and demonstrated that ALT-treatment during late transition significantly decreased the interval from treatment to pregnancy.

In the mare, the effect of ALT-treatment on follicular development appears to be dependant on the size of follicles present on the ovary at time of ALT administration. If ALT was administered when follicles ≥ 20 mm were present, follicular growth was suppressed and the number of large follicles (>30 mm) and follicle diameter was reduced (Turner et al., 1981; Webel and Squires, 1982; Squires et al., 1983). In these reports of a reduction in development of large follicles (>30 mm) and follicle diameter usually occurred during a reported increased FSH levels towards end of treatment period (Turner et al., 1981 and Squires et al., 1983). Subsequently, Wiepz et al. (1988) demonstrated that ALT and not norgestomet (another progestin) suppressed follicular activity in late transitional mares and the rise in serum LH following ALT removal was greater than detected in nontreated control females.

Although ALT had been shown to improve breeding efficiency in transitional mares, the failure to block ovulation in the mare remained a problem. When ALT was administered to estrual mares, ovulation occurred in 75% (9 of 12) of treated females and CL were formed in all of these females (Squires et al., 1983). However, when PGF_{2α} was administered to ALT-treated cycling, ovulation in diestrous mares was blocked in 60% (3 of 5) of the mares and behavioral estrus was suppressed in all mares (Lofsedt and Patel, 1989). It was also noted in the cycling mares that large follicles could develop during ALT treatment and that ALT had only 60% of the binding affinity of P_4 in the hypothalamus and that amount may not be sufficient to block gonadotrophin release. In
support of this theory, it was shown that P₄ in doses of 100 mg per head per day or
greater administered mid-cycle were successful in inhibiting behavioral estrus and
ovulation, but 50 mg per head per day of P₄ would inhibit behavioral estrus only (Loy and
Swan, 1966). The discrepancies reported on the efficiency of blocking ovulation and
follicular dynamics are probably due to the dose administered as well as the phase of
reproductive transition at which ALT was administered.

ALT has also been utilized for asynchronous embryo transfer in the mare (Pool et
al., 1987). In females treated with ALT immediately following ovulation, that received a
day-6 or day-7 embryo on day 2 to 5 of ALT-treatment could became pregnant. In the
same experiment it was noted that when ALT-treatment was initiated at day-9 or greater
following ovulation, there was an increased pregnancy rate when recipient females
received day-6 or day-7 embryos. These findings demonstrated that early pregnancy
could be supported with ALT administration. Furthermore, these findings demonstrate
that ALT-treatment could increase the number of females in a group available for use as
embryo transfer recipients. Also, supplementation with ALT has been demonstrated to
prevent embryo loss in 60-day or less pregnant mares that were administered
Salmonella typhimurium (endotoxin) (Daels et al., 1991).

Not only has ALT been demonstrated to support early pregnancy in the absence
of luteal tissue, it has been reported to support pregnancy through gestation in the
ovariectomized mare (Hinrichs et al., 1985; Hinrichs and Kenny, 1987; Hinrichs et al.,
reported that pregnancies could be established in ovariectomized recipient mares with
supplementation of 300 mg of P₄ daily until ~90 days of gestation. However, when 22 mg
of ALT daily was used to support pregnancy, it was ineffective at maintaining pregnancy
to term (Hinrichs et al., 1986). This finding was in contrast to that of McKinnon et al.
(1988), who demonstrated that 22 to 25 mg of ALT administered daily to ovariectomized
pregnant mares was capable of supporting pregnancy to term.

With ALT-treatment prior to and after embryo transfer there was concern
regarding postnatal effects on the developing offspring (Naden et al., 1990a; Naden et
al., 1990b). With a gestation of ~336 days, mares as with cattle must become pregnant
while nursing an offspring. Therefore, it would be of importance to determine detrimental
effects of ALT on lactation and foal growth. However, there have been no reports of
detrimental effects of ALT on the developing offspring. Following a series of GnRH challenges in stud colts produced from ALT-treated dams (ALT administered from day-20 to day-325 of gestation) there were no differences as a result of treatment of the dam with ALT (Naden et al., 1990a). In addition, no differences were found in the time to puberty or in hypothalamic function of fillies produced from dams treated with ALT from day-20 to day-325 of gestation (Naden et al., 1990b). Furthermore, Sigler et al. (1989) demonstrated that treatment with 0.044 kg ALT per head per day to lactating mares had no effect on milk yield, milk composition or foal growth as compared with untreated control mares.

**Altrenogest in Control of Reproduction in Swine**

In an early report on the use of ALT for the synchronization of estrus and ovulation in gilts, 33 of 38 gilts exhibited estrus within 2 to 7 days following the last ALT administration, when 12.5 mg of ALT per head per day was administered for 19 days (Davis et al., 1979). Although an increase in ovulation rate with no decrease in fertilization rates was noted an increased incidence of cystic follicles occurred in ALT-treated gilts. It was proposed that the increase in cystic follicles may have been a response to the failure of these females to consume the entire dose of ALT. Following this initial testing of ALT in gilts, it was demonstrated that the minimum dose required for effective synchronization of estrus in gilts or sows was 10 to 20 mg of ALT per head per day (Kraeling et al., 1981), while doses of 20 to 40 mg of ALT per head per day were the most effective for synchronization of estrus and ovulation, without induction of follicular or luteal cysts. In this study, the interval from the last treatment with ALT to the onset of estrus was significantly longer for gilts (5.4±0.3 days) compared with that of sows (6.0±0.2 days).

In conjunction to determining optimal dosage of ALT, research continued on determining the optimal length of ALT administration. Davis et al. (1979) reported an increased ovulation rate in gilts treated with 12.5 mg of ALT for 19 consecutive days, while Pursel et al. (1981) demonstrated that gilts treated with 15 mg of ALT for 18 consecutive days had a significantly greater live litter size at birth and weaning compared with nontreated control gilts. In a later study, synchronized pregnancy rates were not different for ALT-estrous cycle synchronization (70.7%) compared with nontreated controls (73.5%).
The consistent efficiency of estrous cycle synchronization and ovulation in gilts resulting from ALT treatment, allowed for improved timed insemination opportunities in swine breeding herds. Davis et al. (1985) reported that pregnancy rates, litter size and live pigs at farrowing were not different for gilts treated with 15 mg of ALT for 18 days and time inseminated at 5, 6 and 7 days following ALT-treatment cessation. It should be noted that the timing of insemination after ALT withdrawal is influenced by breed of gilts or sows (Martinat-Botté et al., 1985). It was later reported that ALT-estrous cycle synchronization could increase the number of gilts detected in estrus compared with the nontreated control gilts (Davis et al., 1987). An increased litter size has been reported in ALT-treated gilts mated at their pubertal estrus (Davis et al., 1987), although there have been other reports that ALT treatment during the prepubertal period was ineffective in improving breeding success (O’Reilly et al., 1979; Kraeling et al., 1982; Wood et al., 1992).

To further increase the precision of control of estrus and ovulation in gilts, the ALT-treatment was combined with gonadotropin administration. These efforts resulted in a decrease in the time from end of ALT treatment to onset of estrus without changing ovulation rate or litter size (Varley et al., 1989).

The effects of ALT on ovarian activity in gilts are similar to the effects reported in the mare. ALT treatment at 15 mg per head per day effectively suppressed behavioral estrus, however, the time from ALT withdrawal to the onset of estrus was dose dependant with higher doses of ALT hastening the onset of estrus (Redmer and Day, 1981). Differences in ovulation rate were not detected in this study, although Diehl et al. (1986) demonstrated that ALT-treatment of inbred pigs with genetically reduced ovulation rates, increased the ovulation rate without affecting fertilization. There was a decrease in the number of ovulatory-size follicles (>8 mm) in gilts that received 15 mg of ALT for 18 days but not in gilts that received 2.5 mg for 18 days (Redmer and Day, 1981). In contrast, gilts that received a higher dose of ALT (15 mg) had a reduction in the number of cystic follicles compared with gilts that received a low dose (2.5 mg).

Estradiol levels were elevated in gilts treated with low daily doses of ALT, while the circulating LH and P₄ levels were unaffected by this treatment. Conversely, E₂ levels decreased in gilts treated with high daily doses of ALT and LH and P₄ levels were unaffected (Redmer and Day, 1981). These findings were validated by those of Martinat-
Botté et al. (1985), who reported that the decreased E$_2$ levels during treatment with high doses of ALT ($\geq$15 mg), due to a decrease in large follicles (>6 mm), which are the predominant source of E$_2$. In support of this theory, Guthrie and Bolt (1985) demonstrated that a high dose of ALT (20 mg daily) prevented the estrogen-triggered LH surge and behavioral estrus in eCG-treated gilts.

It has been hypothesized that the major factor contributing to piglet survival at birth was fetal hypoxia occurring during parturition (Randall, 1972). Therefore, the ability to control the time of parturition in the sow could increase the number of live births by allowing management to increase surveillance during this time. In sows, the time of parturition can be controlled with PGF$_{2\alpha}$ but the onset of parturition often ranges from 8 to 48 hours following PGF$_{2\alpha}$ treatment (Guthrie, 1985). P$_4$ has also been used to control the time of farrowing in sows, however, if administration is continued past day 114 of gestation problems with lactation and uterine involution occur (Nellor et al., 1975). Administration of 16 mg of ALT per day from day 111 to 118 in pregnant sows followed by E$_2$ treatment 6-hours following last ALT administration successfully delayed farrowing, but there was an increase in stillborn piglets (Varley and Brooking, 1981). Following this observation Guthrie et al. (1984) demonstrated that 20 mg of ALT (days 109 to 112) followed by PGF$_{2\alpha}$ treatment hastened the onset of parturition after PGF$_{2\alpha}$ administration compared with females treated only with ALT. There was no increase in stillbirths observed in the ALT+PGF$_{2\alpha}$-treated females compared with control-treated females during this experiment. In a subsequent report the lack of increase in stillbirths noted with ALT-PGF$_{2\alpha}$ controlled parturition protocol was validated by Guthrie et al. (1987).

After determining that ALT could function as a progestin in swine researchers began to work in earnest to determine if ALT administration could decrease the interval from farrowing to estrus. Treatment with ALT from 7 days prior to weaning decreased the number of sows that experienced delayed estrus without decreasing the degree of synchrony in the subsequent estrus following group weaning (Martinat-Botté et al., 1985). Stevenson et al. (1985) reported that treatment with ALT for 7 days prior to weaning (4-week lactation period) actually increased the interval to estrus 14.5±0.2 days in ALT-treated females compared with 5.6±0.2 days for the nontreated control sows but increased the farrowing rate of sows inseminated at their first estrus following weaning. In early weaned sows (~2 weeks of lactation), ALT treatment increased the number of
post-weaned sows (97%) detected in estrus following weaning (Koutsotheodoros et al., 1998). Also, ALT-treated sows had an increased ovulation rate and there was a tendency for increased embryo survival rate in these females found at the time of sacrifice.

**Altrenogest in Control of Reproduction in Exotic Species**

There have been few reports on the use of ALT in species other than the horse or the pig. However, Schwarzenberger et al. (1999) reported an okapi, that had experienced five abortions due to placental deficiency, was administered 10 mg of ALT per day from day-50 post-mating until 30 days prior to expected parturition. In this experiment, the dose of ALT was reduced by 1 mg per day over a 10-day period ending at the end of ALT-treatment. A female calf was born from this female and no deleterious health affects were noted in the dam during ALT-treatment. The female calf did die 30 days following parturition, however, this was due to kidney hypoplasia that was not linked to ALT-treatment.

**Effects of P₄ on the Oviducts during Early Pregnancy**

**Disruption of Fertilization**

In domestic farm animals (e.g., cattle and sheep), P₄ levels are relatively low during ovulation and fertilization. At 14 hours after ovulation in the cow, circulating P₄ levels remain below 0.5 ng/ml (Henricks et al., 1970; Wettemann and Hafs, 1973) and by day 3 post-ovulation levels are still below 2 ng/ml (Henricks et al., 1971; Wettemann and Hafs, 1973), however, by 6 days following ovulation P₄ levels in both pregnant or nonpregnant cows are ≥3 ng/ml and peak around day 14 post-ovulation (Henricks, et al., 1970, Wettemann and Hafs, 1973).

Low P₄ levels during ovulation and fertilization have been shown to be beneficial to fertility in many species. Chang (1967) demonstrated that in vivo fertilization could be effectively disrupted by administration of P₄, at the time of fertilization in rabbits. In addition, it was noted that when rabbits were treated during estrus with P₄, unfertilized ova and degenerate embryos were often recovered from the uterus. Chang (1967) suggested that the P₄ treatment hastened embryo transport from the oviduct to the uterus prior to the embryo developing to a stage capable of surviving in the uterus. In another polytoccus species, the pig, increased P₄ levels shortly prior to ovulation was proposed to be the cause of increased polyspermy at fertilization (Day and Polge, 1968). Hunter (1968) demonstrated that P₄ administration to hamsters 3 days prior to the time
of fertilization greatly reduced the number of fertilized oocytes. Decreased fertilization rates, as a result of P₄ administration prior to ovulation, are likely a response of P₄ down-regulating LH release thereby delaying ovulation and lowering fertility (Batra and Miller, 1985; Thiery and Martin, 1991). However, this hypothesis does not explain the increased frequency of disruption of fertilization when P₄ is administered following ovulation.

The disruption of fertilization when P₄ treatment was initiated prior to mating was shown to be caused, in part, by decreased sperm transport in the reproductive tract (Nutting and Mares, 1970). In contrast, in utero deposition of semen in P₄-treated rabbits increased fertilization rates. The theory of P₄ causing disruption of fertilization was strengthened in the pig, when P₄ administration 8 hours prior to ovulation resulted in an increased incidence of polyspermy compared with nontreated control females (Hunter, 1972). It was concluded that P₄ administration caused a relaxation of endometrial tissue of the oviduct allowing more spermatozoa to reach the ampullary-isthmus junction (site of fertilization) contributing to the increase in polyspermy in swine.

Chang (1969) demonstrated in pseudopregnant rabbits induced to ovulate mid-cycle and in P₄-treated rabbits there was a greater decrease in fertilized oocytes, and an increased incidence of oocytes collected from the uterus and increased embryo degeneration compared with that of nontreated control does. It was concluded the longer the exposure period to P₄ the more dramatic the disruption to fertilization and embryo transport. Even though the majority of these oocytes were unfertilized the oocytes that did undergo successful fertilization and rapid transport to the uterus, they were still capable of developing. When these 1-day old fertilized oocytes were recovered from these females and transferred to control treated females, 65% of the embryos continued development.

In contrast, when rabbit does at 23 days of pseudopregnancy were treated with P₄ during estrus and embryos were recovered on day 2 following estrus, 87% of these treated rabbits had viable embryos, but when day-22 pseudopregnant rabbits were treated the same way and embryos recovered on day 6, only 45% of the embryos were viable (Chang, 1969).
**Effect on Oviductal Glycoprotein**

The environment in which oviduct-stage embryos develop is believed to be under the control of circulating E₂ and P₄ (Leese, 1988). It has been demonstrated that in vitro cultures of rabbit oviduct epithelium synthesized a sulphated glycoprotein identical to those demonstrated to exist in oviductal fluid (Barr & Oliphant, 1981; Oliphant and Ross, 1982; Oliphant et al., 1994). A similar glycoprotein was then discovered in sheep and was termed estrogen associated glycoprotein (EAP) (Sutton et al., 1984). It is believed that P₄ stimulates the production, while E₂ stimulates the release, of these glycoproteins (Leese, 1988). In support of this hypothesis, the secretion of these EAPs increases during estrus and decreases by days 3 to 4 following estrus in the cow at the time the CL is becoming mature and increasing P₄ secretion. Gandolfi et al. (1989) demonstrated that the oviduct epithelial cells secrete specific glycoproteins during specific time intervals in which the embryo transverses the oviduct.

Kapur and Johnson (1986) reported that the embryo could sequester oviductal proteins into the perivitelline space possibly to later serve as a source of amino acids for further development. The role oviductal protein plays in embryo development is not completely understood. However, Whittingham (1968a, 1968b) reported that mouse embryos cultured on oviduct tissue collected during metestrus had a higher rate of development than mouse embryos cultured on oviduct tissue collected during diestrus. This finding was further supported by the finding that fertilized sheep oocytes had higher cleavage rates when cultured in the presence of EAPs (Nancarrow and Hill, 1995). However, when sheep zygotes were cultured in the presence of EAPs the cleavage rate was reduced (Nancarrow and Hill, 1995). These observations may indicate a role of P₄ dominance over E₂ to suppress of EAP secretion at different stages of embryo development in the oviduct.

There is still confusion regarding the mechanism of how P₄ is involved in EAP secretion. It was demonstrated that oviduct tissue from the ampulla secreted more sulfated glycoproteins in the presences of E₂ and then P₄ when compared with that of nontreated control rabbit ampulla tissues (Erickson-Lawrence et al., 1989). This demonstrated that the time of maximum glycoprotein secretion in the oviduct is concurrent with increasing P₄ influence over the oviduct, as the addition of E₂ alone failed to increase sulfated glycoprotein secretion over that of nontreated control.
(Erickson-Lawrence et al., 1989). Thus, it may be concluded that P₄ exerts some type of control over oviductal glycoprotein secretion while the embryo is developing within the oviduct.

The effect of P₄ on EAPs is a possible result of P₄ effects on oviductal fluid release and EAP sequestering. Cline et al. (1977) demonstrated in mice that P₄ administration following estrus resulted in a greater accumulation of oviduct fluid in the ampulla region compared with E₂-treated or nontreated control pregnant females. Of the embryos collected from these females, those collected from E₂-treated females had the lowest developmental rates and had significantly more degenerate embryos from the nontreated control and P₄-treated groups.

These results suggest that one of the roles of P₄ during embryo development in the oviduct is to negate the deleterious effects of asynchronous E₂. In cattle, with impaired fertility due to persistent dominant follicles, there is an increase in E₂ (Fike et al., 1997). This increase has been demonstrated to decrease the amount of glycoprotein adversely altering the oviductal microenvironment in which the embryo develops (Binelli et al., 1999). The mechanism by which P₄ can regulate these secretions has not been reported. However, localization of P₄ in the bovine oviduct ipsilateral to the ovary bearing the CL has been reported (Wijayagunawardane et al., 1996). It was demonstrated in cattle that the ipsilateral oviduct contained a significantly greater concentration of P₄ during early pregnancy than during the luteal phase although no differences were detected between the follicular phase and the interval post-ovulation.

**Effects of P₄ on Embryo Transport into the Uterus**

In various mammalian species, P₄ controls the transport of embryos from the oviducts to the uterus. Roblero and Garavagno (1979) demonstrated that when pregnant mice were ovariectomized on day-2 post-coitus, embryo transport into the uterus was inhibited. Addition of E₂ failed to reverse this affect, while the supplementation of P₄ initiated and continued normal transport of embryos into the uterus. Kendle and Lee (1980) reported that the administration of antiprogestin compounds (day 1, 2 or 3 post-mating) to pregnant mice also inhibited normal embryo transport to the uterus. However, P₄ supplementation again initiated normal transportation, although there was a need for a continued presence of P₄ to maintain embryo transportation to the uterus. Similarly, when pregnant rats were ovariectomized on days 1 to 3 following ovulation, embryo
transport was disrupted (accelerated transport of some embryos and retention of others) (Forcelledo et al., 1982). However, when P$_4$ was supplemented following ovariectomy, embryo transport was restored to normal. Furthermore, Fuentealba et al. (1988b) reported that embryo transport in nonpregnant rats (embryo transfer recipients) occurred more slowly than in pregnant rats. This could be due to differences in P$_4$ levels in the oviducts of pregnant and nonpregnant rats, as previously shown in cattle (Wijayagunawardane et al., 1996). These findings supported the hypothesis that P$_4$ controls embryo transport to the uterus.

In sheep, Holst (1974) reported that 8-cell embryos arrive in the uterus ~66 hours following ovulation. However, when day-2 sheep embryos were transferred into untreated ovariectomized ewes on day 2, embryo transport was disrupted as in the rat (Forcelledo et al. 1982). Interestingly, normal sheep embryos were found remaining in the uterus of these untreated ovariectomized ewes when transferred into the uterus on day 2 (Moore et al., 1983). Supplementation of E$_2$ and P$_4$ beginning 5 days prior to transfer and continued until embryo recovery on day 2 in ovariectomized pregnant ewes failed to restore normal embryo transport. It was hypothesized that this abnormal transport response was from an inappropriate in vivo hormone levels. The results are clear, that the disruption of endogenous P$_4$ can disrupt normal embryo transport, which further indicates a controlling mechanism of P$_4$.

In cattle, ovum transport through the ampulla of the oviduct occurs rapidly (8 to 10 hours following ovulation), most likely from cilia action (EL-Banna and Hafez, 1970). Embryo transport through the isthmus into the uterus, however, occurs more slowly and is likely under the control of muscular contractions. EL-Banna and Hafez (1970) further reported that embryos remained in the isthmic region for sometime before they were transported into the uterus, 3.5 to 4 days following ovulation. In superovulated females under the influence of higher P$_4$ levels, embryos were found to be transported more quickly though the oviducts, presumably by decreasing the effect of an isthmus block (EL-Banna and Hafez, 1970). Later, Crisman et al. (1980) further verified that P$_4$ was the controlling factor in embryo transport in the cow. In naturally mated heifers, administration of P$_4$ 24 hours following the onset of estrus stimulated the rate of embryo transport into the uterus. In contrast, the administration of E$_2$ failed to increase the rate of embryo transport over that of embryos from nontreated control females.
It is unknown whether P$_4$ exerts a controlling influence on embryo transport in the mare. Weber et al. (1991) found that when pregnant mares were administered PGE$_2$, significantly more embryos (6 of 11) were recovered in the uterus compared with vehicle and nontreated control pregnant mares. They also demonstrated that the equine embryo was capable of synthesizing and secreting PGE$_2$ at this time and that PGE$_2$ controlled the rate of embryo transport into the uterus of the mare instead of P$_4$. Mann and Lamming (2001) have proposed that early P$_4$ administration in the cow could effectively alter the ratio of PGE$_2$ to PGF$_{2\alpha}$ synthesis in the bovine uterus in favor of PGE$_2$, although it is unknown if this altered synthesis pattern occurs in the mare.

**Effects of P$_4$ on Uterine Growth and Proliferation**

In mammals, the uterus is composed of two primary functional layers; the endometrium and the myometrium, with secretory glands found in the endometrial layer (Perry, 1945). The endometrium is composed of two tissue types, a luminal epithelium and glandular epithelium (Perry, 1945). Generally, the bovine endometrium is populated by a large number of aglandular carnucles, which are stromal protrusions covered by luminal epithelium (Wimsatt, 1950; Atkinson et al., 1984). The area between the carnucles is densely populated with endometrial glands that produce secretions which promote embryo development (Bazer, 1975). These secretions are composed of a various assortment of enzymes, growth factors, lymphokines, hormones and proteins.

In the mouse uterus, it has been demonstrated that epithelial cells proliferate in response to E$_2$ during the early stages of pregnancy (Finn and Martin, 1967). However, on day 3 of pregnancy there is a transition from increased presence of epithelial cells to an increase presence of stromal cells (glandular cells), which are regulated by P$_4$ (Martin and Finn, 1968). Tachi et al. (1972) reported that P$_4$ administration could increase the number of synchrony of stromal cell proliferation in the mouse.

There is evidence that P$_4$ can act on the uterine glands in concert with the growth factors that it regulates. Piva et al. (1996) reported that stromal cells cultured in vitro in a serum-free culture medium were stimulated to synchronously re-enter the cell cycle by addition of basic fibroblast growth factor (bFGF) and P$_4$. Rider et al. (1998) demonstrated that this effect could be duplicated with other growth factors (epidermal growth factor, EGF and transforming growth factor alpha, TGF-α) in the presence of P$_4$. This effect is a result of synergism between the growth factors and P$_4$, as it was
demonstrated that \( P_4 \) or the growth factors alone could not duplicate this effect (Rider et al., 1998).

In addition to cell growth, \( P_4 \) can also increase the vascular growth and blood flow to the uterus (Johnson et al., 1997). Finn and Martin (1967), Martin and Finn (1968) and Johnson et al. (1997) also demonstrated that \( P_4 \) supplementation in ovariectomized sheep stimulated tissue remodeling within the uterus. In addition, Johnson et al. (1997) reported that \( P_4 \) treated ovariectomized sheep had increased uterine weights. This is not unexpected since \( P_4 \) has been demonstrated to increase cell growth and proliferation of uterine endometrium (Rider, 1998).

Uterine \( P_4 \) receptors (PR) have been shown to be stimulated by the pre-ovulatory \( E_2 \) surge (Ing and Tornesi, 1997). In response to \( E_2 \) administration in ovariectomized ewes, PR upregulation was prolonged in the middle and deep endometrial stromal tissues, where the majority of the uterine glands are located (Ing and Tornesi, 1997). However, the greatest expression of PR occurred in the superficial lining of the luminal epithelium, where the embryo interacts with the uterus. These PR were found to be expressed within 24 hours following administration of \( E_2 \).

Zheng et al. (1996) demonstrated that \( E_2 \) receptors (ER) and PR were inversely related during early pregnancy in ovine uterine tissues, with the exception of the luminal stroma. Kimmins and MacLaren (2001) have reported that PR were predominantly, absent in the luminal epithelium in cycling and pregnant cows. There was also a reduction in PR in the glandular epithelium layer and in the carnucular regions compared with intercarnuclar endometrium regions in pregnant cows. In addition, it was reported that there was a reduction in oxytocin receptors (OR) in the luminal epithelium of pregnant cows, which may indicate a role of PR reduction in maternal recognition of pregnancy. The role of \( P_4 \) in maternal recognition of pregnancy will be reviewed later in this chapter.

During pregnancy, \( P_4 \) exerts a calming effect on myometrial contractions of the ovine uterus (Rhodes and Nathanlielsz, 1990) preventing the disturbance of the developing embryos once they have entered the uterus. Bonafos et al. (1995) reported the uterine tone changes morphologically and that contractions increase during the pre-ovulatory phase and then greatly decrease following ovulation in the cow.
Effects of P₄ on the Synthesis and Secretion of Uterine Proteins and Growth Factors

It is believed that oviductal protein secretion can improve embryo development and that uterine protein secretion plays an equal or greater role in embryo survival. Dixon and Gibbons (1979) reported that uterine secretions from P₄-treated nonpregnant cows yielded five major protein fractions. Two of these fractions were composed of serum proteins, while the other three fractions contained a minimum of nine nonserum proteins that contained acid phosphatase and lactoferrin. When bovine uterine endometrial extracts were cultured in vitro, the majority of bovine uterine-specific proteins tended to be acidic polypeptides opposed to the basic polypeptides that predominate in the gilt, ewe and mare (Bartol et al., 1985).

Lactoferrin, a well known histotroph protein, has been reported to have antibacterial properties in women (Masson et al., 1968). Bazer (1975) reported that many of these proteins in the pig have the same enzymatic and P₄ binding properties as those found in allantoic fluid from pregnant sows. These allantoic proteins were produced and secreted from uterine endometrial glands and absorbed via the placenta and sequestered within the allantoic fluid.

Prior to these discoveries, other unique uterine proteins have been described. Krishnan and Daniel (1967) discovered blastokinin, a unique uterine protein believed to stimulate blastocyst formation in the rabbit. It was later demonstrated that blastokinin was more important for the growth of blastocyst-stage rabbit embryos rather than for development to the blastocyst stage (Whitten and Biggers, 1968; Roberts et al., 1976a). It was later realized that blastokinin and another uterine-specific protein termed uteroglobin were the same compound (Bazer, 1975).

In a review of uterine secretions in the pig, it was reported that uterine specific proteins were induced by P₄ (Davis and Blair, 1993). Chen et al. (1975) and Basha et al. (1979) have reported that uteroferrin release from the uterine glands and transfer of iron from uterus to the conceptus was controlled by P₄. Roberts et al. (1976b) reported that lysozyme, β-hexosamindase and cathepsin were also P₄ induced uterine proteins, in swine. Uteroferrin-associated polypeptides (Davis and Blair, 1993), retinol-binding protein (Adams et al., 1981), plasmin and trypsin inhibitor (Fazlezbas et al., 1985) and β-endorphin (Li et al., 1991) have been found in uterine secretions of the pig and appear to
be regulated by $P_4$. Furthermore, the release of a uterine specific protein termed “purple protein” has been reported to be under the controlled by $P_4$ (Squires et al., 1972).

Along with the numerous uterine proteins present in uterine secretions, there are many growth factors present as well. Brigstock et al. (1989) demonstrated that EGF, insulin-like growth factor I (IGF-I), insulin-like growth factor-binding protein (IGFIBP), colony stimulating factor (CSF), acidic-fibroblastic growth factor (aFGF) and bFGF were present in the uterine fluid of a multitude of species. In addition, Simmen et al. (1988) demonstrated that an EGF-like peptide was present in the uterine secretions of the pig. Many of these factors were reported to stimulate uterine cell growth and endometrial glandular development (Brigstock et al., 1989). Also, the synthesis and release of IGF-I, IGFIBP and CSF are apparently regulated by $P_4$ (Brigstock et al., 1989).

Different factors can affect the normal pattern of uterine secretions of the female. In pigs, it has been reported that pubertal females are capable of producing all known uterine specific proteins, however, the quantity was markedly reduced compared with that of mature females (Murray and Grifo, 1976). However, when embryos recovered from adult ewes were transferred to adult ewes and ewe lambs, there were no differences in lambing rates (Quirke and Hanrahan, 1983). It was concluded that uterine function was not a likely cause of decreased lambing rates in younger ewes but their conclusion did not account for the fact that the embryos transferred were all exposed to the uterine environment of a mature female during early embryonic development.

Gustaffson and Larson (1983) have shown that when day-7 embryos were collected from repeat breeder cows (RPB) and transferred to RPB females or fertile control females, pregnancy rates were low in both treatment groups. However, when day-7 embryos were collected from fertile cows and transferred to RPB females and control females, the pregnancy rates were normal for embryo transfer (Gustaffson and Larson, 1985). These studies suggest that exposure to a “healthy” uterine environment from day 1 to day 7 of the estrous cycle may be critical during early embryo development.

Comparisons between the uterine function of “fertile” and “infertile” cows have demonstrated that there are differences between total protein present in uterine flushings as early as day 5 of the estrous cycle in RPB (infertile) females compared with control (fertile) females (Guise and Gwazdauskas, 1986). This difference continued
through day 10 but was no longer apparent by day 15 of the estrous cycle. P_4 levels were also reported to be altered in RPB cows and were suggested to be a cause of the differences in uterine protein concentrations (Guise and Gwazdauska, 1986). In addition to lowered total uterine protein, RPB females have lower endometrial glycogen and nucleic acid content compared with fertile females (Bugalia et al., 1988). The, RPB females were found to have significantly less acid phosphatase and alkaline phosphatase enzyme activity in the uterine endometrium. The lower alkaline phosphatase activity is indicative of a disruption or deficiency in carbohydrate metabolism within the uterine environment.

Deficiencies in uterine metabolism have also been demonstrated in cows carrying abnormal developing embryos, as determined by embryo recovery on day 7 of gestation. Specifically, uterine flushings from these females possessed significantly more glucose and protein compared with uterine flushings from cows carrying normal embryos (Wiebold, 1988). There was a significantly higher protein content (during first 7 days of pregnancy) in the uteri of females carrying an abnormal developing embryo, which is opposite to what had been previously reported. However, 70% of the abnormal embryos were at the cleavage stage on day 7 and therefore, these findings could represent an artificial increase in uterine protein due to the absence of a viable embryo to metabolize the uterine carbohydrates and proteins.

An elaborate comparison was made between naturally mated ewes that had received a day-6 embryo (estrus = day 0) on day 4 (asynchronous transfer), a day-6 embryo on day 6 (synchronous transfer) or a day-6 embryo on day 10 (asynchronous transfer) and naturally mated ewes that received a day-9 embryo on day 6 (asynchronous transfer), a day-9 embryo on day 9 (synchronous transfer), day-9 embryo on day 12 (asynchronous) or a day-9 embryo on day 10 of a naturally mated ewe treated with P_4 on days 4 to 9 post-estrus (Wilmut and Sales, 1981). It was discovered that there were no differences in the total protein recovered from endometrial tissue cultures from these ewes, however, there were differences between treatment groups in the levels of 30 different proteins evaluated from ewes. These findings demonstrated that the presence of an advanced stage embryo could accelerate uterine function to match the stage of the embryo and not the stage of the gestation (Ashworth and Bazer, 1989). These findings also demonstrated neither P_4 nor pregnancy altered total protein
production, however, they altered the levels of individual proteins (Ashworth and Bazer, 1989). Alterations of uterine secretion during P₄ supplemented from days 1 to 5 of early pregnancy have also been reported in the cow (Garrett et al., 1988).

Interestingly, differences in the endometrial morphology and secretory status of pregnant and nonpregnant females have also been found (Bonafos et al., 1995). As determined via ultrasonography, pregnant females had a significantly greater endometrial echo texture score beginning on day 4 of the estrous cycle (estrus = day 0) compared with nonpregnant females. The echo texture of the uterus signifies the thickening for the uterus in preparation of supporting an embryo.

Geisert et al. (1988) reported that as pregnancy continued the complexity of polypeptide synthesis increased. In addition, there is an increase in two low molecular weight basic polypeptides in endometrial tissue from pregnant females compared with nonpregnant cows. However, this increase has also been reportedly to occur with progression through the estrous cycle. On day 4 of the bovine estrous cycle (estrus = day 0) only 13 uterine proteins were recovered compared with 78 uterine proteins recovered on day 8 of the estrous cycle (Bartol et al., 1981). It was also noted that there was a significant increase in prostaglandins present in the ipsilateral uterine horn of pregnant females compared with the ipsilateral uterine horn of nonpregnant females. Correspondingly, it is known that the bovine embryo is capable of producing prostaglandins on days 7 to 18 of the estrous cycle (Shemesh et al., 1979; Lewis et al., 1982; Hwang et al., 1988). Collectively, these findings demonstrate how P₄ during pregnancy can regulate the energy substrates in the uterus available to the developing embryo during early pregnancy.

**P₄ Effects on Immune System Function during Pregnancy**

In the cow, research has been conducted in an effort to determine the effect of P₄ on immunosuppression necessary for the developing embryo to continue pregnancy. In a classical experiment, Rowson et al. (1953) reported that P₄-treated females (regardless of stage of estrous cycle) were found to be more likely to develop intrauterine infections than untreated females. More recently, it was reported that bovine uterine flushings collected at day 3 of the estrous cycle were only marginally immunosuppressive while day-10 flushings were not immunosuppressive and day-18 flushings (regardless of pregnant or nonpregnant) were highly immunosuppressive.
(Fisher et al., 1985). These findings indicate that a window of opportunity exists for an early embryo to continue development within the uterus with minimal immune response.

It has been demonstrated that uterine immunosuppression could occur in two ways: (1) through uterine derived immunosuppressive proteins and (2) through embryonic-derived immunosuppressive proteins (Fisher et al., 1985). Subsequently, Hansen and Newton (1987) demonstrated that P₄ induced bovine uterine protein could bind IgA and IgM but they could not bind IgG. In the ewe, the immunosuppressive proteins were demonstrated to be P₄ inducible and could effectively delay allograft rejection (Stephenson and Hansen, 1987). These findings suggest that elevated P₄ levels could reduce the incidence of embryo mortality, as a response of uterine immune response to foreign cells until maternal recognition occurs.

Low and Hansen (1988) reported that P₄ could inhibit lymphocyte proliferation in the uterus. Also, in vitro culture of uterine fluid from P₄-treated ovariectomized ewes could reduce the lymphocyte proliferation response and could further lessen antibody responses (Stephenson and Hansen, 1990). Later it was reported that there was a decline in the number of intra-epithelial lymphocytes in the uterus, as early pregnancy continued (Hansen, 1995). However, the P₄ levels required to inhibit lymphocytes in the bovine uterus were much higher than P₄ levels in the blood therefore, it has been hypothesized that P₄ may induce uterine proteins that also inhibit lymphocytes during pregnancy (Hansen, 1997).

**Effects of P₄ on Embryo-Uterine Synchrony during Early Pregnancy**

It has been established that uterine-specific proteins are regulated by P₄, which predominates during metestrous and diestrous periods in various mammals (McCarthy et al., 1977; Fischer, 1989; Yang and Foote, 1990). It was not known, however, how manipulation of the release of these proteins (embryo-uterine synchrony) would affect embryo growth. In rabbits, P₄ administration from 3 days before to the day of mating (mating = day 0) was found to disrupt fertilization and the majority of the fertilized oocytes died by day 4 following mating (McCarthy et al., 1977). However, when these embryos were recovered from the P₄-treated does and transferred to nontreated recipient does on day 3 following mating, most survived compared with those recovered from P₄-treated does on day 4 and transferred to day-4 (mating = day 0) nontreated recipient does. When the uterine fluid from these rabbits was evaluated, there was a
greater amount of uterine protein found in P₄-treated donors compared with nontreated recipient females. It was later demonstrated that uterine fluid from day-2 (mating = day 0) P₄-treated does would support the development of day-2 embryos from P₄-treated does and day-3 embryos from nontreated control does. In addition, it was demonstrated that uterine fluid from day-3 (mating = day 0) nontreated control does would support the development of day-3 embryos from nontreated control does and day-2 embryos from P₄-treated does. These findings illustrated that the synchrony between the embryo and the uterus was important to establish pregnancy and more importantly, is likely controlled by circulating P₄ and subsequently P₄-induced uterine proteins.

Asynchrony between embryo and uterus does not always result in early embryo death. For example, it has been demonstrated in rabbits that when 8-cell embryos were transferred into advanced staged uteri (embryo recipient more days post-estrus than embryo donor) embryo growth was markedly stimulated (Fischer, 1989). Specifically, the cell diameters were larger, there was an increase in protein synthesis and cell proliferation was greater for embryos transferred into older uteri than for embryos transferred to synchronous uteri. However, when advanced stage embryos were transferred to younger female uteri, the embryos exhibited an increase in degeneration, a decreased in protein synthesis and retardation of development. This study demonstrated that the day of gestation was not as important as the stage of maturation of the endometrium for embryo development. A synchronous match between the embryo and stage of uterine maturation is critical to the establishment and maintenance of pregnancy, with this synchrony controlled by P₄.

In the absence of P₄ treatment during early pregnancy, embryo-uterine synchrony is more critical to the successful maintenance of pregnancy. When day-3 rabbit demi-embryos and normal embryos were transferred into day-2 or day-2.5 nontreated recipient does, demi-embryo and normal embryo survival rates were low as it was when day-3 normal embryos were transferred day-3.5 to day-4 nontreated recipients (Yang and Foote, 1990). Only asynchrony of up to 12 hours would permit further embryo development in recipients at acceptable rates.

This window of opportunity for embryo transfer has also been reported in sheep. Wilmut and Sales (1981) reported that when day-3 embryos were transferred into day-6 (estrus = day 0) recipients, there was a marked increase in the developmental rates of
the embryos, although the conceptus failed to develop to term. However, when these asynchronous embryos were transferred back into a synchronous recipient, following their 3-day exposure to an advanced stage uterus, these embryos maintained their ability to produce offspring. When this experiment was repeated by transferring day-6 embryos into the uteri of day-9 embryo recipients, no embryos developed to term. However, a few of these embryos developed to term when transferred back to a synchronous recipient following their 3-day exposure to an advanced uterus (Wilmut and Sales, 1981). These experiments demonstrated that the ovine embryo has limited ability to regulate its development in an attempt to establish synchrony with the uterus. There appear to be developmental stage limits, as early staged embryos were more adept at establishing synchrony than more advanced staged embryos.

Lawson and Cahill (1983) reported that \( P_4 \) can be used to alter the embryo-uterine relationship when ewes were administered 25 mg \( P_4 \) on day 0 to day 3 (day 0 = estrus) of the estrous cycle was shortened by 4 days. More importantly, on day 6 of this altered cycle the uterus was capable of supporting embryos recovered on day 10 from nontreated control females. This study further demonstrated \( P_4 \) may have a functional mechanism involving maternal recognition.

When embryos are exposed to advanced stage uteri either via embryo transfer (ET) or \( P_4 \) supplementation (during early pregnancy) of the embryo increases its metabolic rate and the reverse occurred when the embryos are exposed to a less developed uterus (Wilmut and Sales, 1981). Lawson et al. (1983) reported that when embryos collected from ewes 4 days after mating were transferred into day-1 or day-2 (estrus = day 0) ewes, the embryos were unable to develop past the early blastocyst stage. This was markedly different from day-4 embryos that where transferred into the uteri of day-6 or day-7 recipients which experienced accelerated growth and development and resulted in larger diameter at time of embryo recovery. In addition, there were fewer degenerate embryos recovered from advanced stage uteri. However, this was found only with early stage embryos, as day-9 embryos transferred into day-11 embryo recipients failed to develop, while day-9 embryos transferred into day-6 recipients continued to develop in utero to some extent.

In contrast, administration of \( P_4 \) on day 10 to day 24 of pregnancy in ovariectomized pigs failed to significantly improve or alter embryo-conceptus
development (Gentry et al., 1973). Correspondingly, variations in embryo development due to ovulation intervals has been noted in the pig and hypothesized as a cause of increased early embryo mortality (Pope et al., 1986). Day-6 embryos (estras = day 0) transferred to day-7 recipients were morphologically less developed at day 12 and 13 compared with day-7 embryos transferred to day-6 recipients (Pope et al., 1986). Interestingly, Morgan et al. (1986) indicated that estradiol (E\textsubscript{2}) administration could influence uterine secretions in the pig, as does P\textsubscript{4} in other farm animal species. Based on these findings, the pig is one species in which P\textsubscript{4} is critical for the maintenance of pregnancy but may not play a dominant role in embryo-uterine synchrony.

In the cow, the differences between embryo and uterine synchrony can not exceed 36 hours and still result in acceptable pregnancy rates via ET (Donaldson, 1985). However, as shown in the ewe, early stage bovine embryos (early morulae) were capable of withstanding uterine asynchrony better than later stage blastocysts (Donaldson, 1985). In the cow, as in other species, P\textsubscript{4} administration (during early metestrus) could modify the degree of synchrony between the embryo and uterus (Garrett et al., 1987; Garrett et al., 1988; Geisert et al., 1991a). To improve pregnancy rates from ET in cattle, better quality, advanced stage embryos should be transferred to more synchronous recipients while early stage, good quality embryos could be transferred to slightly more asynchronous recipients. Bovine embryos are more likely to withstand the alteration of their uterine environment and thus, establish pregnancy.

P\textsubscript{4} administration (day 1.5 to day 5, estrus = day 0) can be utilized to stimulate synchrony between the bovine embryo and uterus (Geisert et al., 1991a). Garrett et al. (1987) demonstrated that 100 mg of P\textsubscript{4} administered from days 1.5 to 4 (estras = day 0) resulted in more advanced stage embryos (hatching or hatched blastocysts) recovered on day 5 of the estrous cycle compared with embryos (8-cells to morulae) recovered from nontreated control females on day 5. In addition, when embryos were collected on day 14 of the estrous cycle, embryos from P\textsubscript{4}-treated females were significantly greater in length than embryos from nontreated control females. Garrett et al. (1988) reported that P\textsubscript{4} treatment (days 1.5 to 4, estrus = day 0) altered the uterine-embryo relationship by altering of uterine synthesis and release of polypeptides. P\textsubscript{4} supplementation was utilized to induce these alterations for asynchronous ET in the cow. Following 100 mg of P\textsubscript{4} administered on days 1.5 to 4, embryos collected from day-8 cows were transferred
to these day-5 P₄-treated females, and normal embryo development continued (Geisert et al., 1991a).

**Effects of P₄ on Embryo Developmental Rates**

Elevated P₄ levels during fertilization or immediately following (prior to 2 days after ovulation) has been demonstrated to prevent early cleavages in oviduct-stage embryos. Whitten et al. (1957) demonstrated that presumptive mouse zygotes were inhibited from further development when cultured in vitro in the presence of P₄. This effect was apparently dose dependant, with doses ≥8 µg/ml of P₄ completely inhibited further embryo development. High concentrations of P₄ also inhibit first cleavage and further development of presumptive rabbit zygotes in vitro (Whitten et al., 1957). However, when presumptive zygotes were moved to a P₄-free medium they resumed cleavage within 2 to 3 hours following their removal (Daniel and Levy, 1964). Mouse embryos subjected to 8 µg/ml of P₄ at the 2-cell or 4-cell stage failed to continue development in vitro. However, this effect was not evident when 8-cell, morula or early blastocyst stage mouse embryos (uterine-staged embryos) were exposed to the same P₄ treatment (Kirkpatrick, 1971).

There have also been reports of increased degeneration in these in vitro-cultured mice embryos when exposed to P₄-treated ovariectomized mice (Kirkpatrick, 1971). This effect was more difficult to determine in vivo, as embryos recovered from ovariectomized female mice had significantly fewer cells present compared with embryos recovered from ovariectomized P₄-treated mice, while embryos from both groups had significantly fewer cells compared with embryos recovered from the nontreated control females (Roblero, 1973). These studies illustrate that high levels of P₄ may have a detrimental effect on oviductal stage embryos, although it is still necessary for early development of the oviductal stage embryo.

In contrast, Roblero and Izquierdo (1976) reported that low levels of P₄ (0.1 µg to 1 µg) or serum alone did not affect 4-cell mouse embryo development in vitro whereas, the combination of 1 to 4 µg of P₄ with serum increased the number of cells per blastocyst. The number of cells per blastocyst increased even further when the macromolecular fraction (molecules >1,000 molecular weights) of the serum was used rather than whole serum. Wang et al. (1984) demonstrated that when P₄ was withdrawn (ovariectomy) during oviductal embryo development in the mouse, there was an arrest in
embryo development. However, findings from these experiments may be confounded as early ovariectomy can cause tissue damage (necrosis) and thus confound findings from these experiments.

In an attempt to circumvent this possible confounding effect of ovariectomy, McRae (1994) used a P₄ antagonist (RU-486) on days 1 and 2 post-coitus to isolate the effects of P₄ on oviduct stage mouse embryos. It was demonstrated that treatment with RU-486 significantly reduced the number of day-4 mouse embryos collected from treated females compared with vehicle-treated or untreated control females. In addition, more of the embryos recovered from RU-486-treated females were retarded in development and possessed fewer cells compared with embryos recovered from vehicle and nontreated control females. A similar decrease in embryo development occurred when P₄ antibodies were administered on day-3 and/or day-4 post-coitus to pregnant ferrets (Heap et al., 1989). These studies illustrate that high levels of P₄ may have an inhibitory effect on oviduct-stage embryos, although some P₄ may still be necessary for early development of oviduct-stage embryos.

Age is one maternal factor that can affect circulating levels of P₄ during early pregnancy. It has been reported that aged female rats have an increase in delayed embryo development, as well as, an increase in the number of degenerate embryos present in the uterus by 5 days following mating (LaPolt et al., 1990). This was found to be associated with lower P₄ and higher E₂ concentrations, on day 4, when compared with younger female rats, which did not exhibit delayed development or an increase in degenerate embryos. Furthermore, Binart et al. (2000) have reported that the administration of P₄ 12 hours post-ovulation could rescue embryo development in mice with luteal insufficiency.

It has been proposed that the P₄ levels from the previous cycle in sheep are also important for establishing pregnancy (Wilmut, 1985). In ovariectomized sheep, there was a marked increase in the number of abnormal embryos when a priming P₄ dose (during the luteal phase of the previous estrous cycle) was omitted from the hormone replacement treatment (Miller et al., 1977). Miller and Moore (1983) demonstrated that when priming P₄ was omitted prior to E₂ administration (to induce estrus) uterine secretions were delayed to 4 to 6 days following estrus (~2 days later than normal). However, it was noted that the content of the secretions were not modified. This pattern
was also found by Wilmut et al., (1985), who concluded that adequate P₄ levels in the ovine estrous cycle prior to becoming pregnant was as important as adequate P₄ levels during early pregnancy.

The mare appears to be one species that early P₄ levels may not play a critical part of early pregnancy as has been previously noted for other species. There has been a report that P₄ supplementation during early pregnancy (day 0 to day 6, ovulation = day 0) does not affect embryo developmental rates in the mare (Ball et al., 1992). When embryos were recovered from pregnant mares receiving 450 mg of P₄ on day 0 to day 6 following ovulation there were no differences in embryo developmental stages, embryo diameter or number of cells per embryo when compared with embryos collected from nontreated control mares (Ball et al., 1992).

Contrary to evidence from in vivo embryo development there has also been evidence suggesting that P₄ may not have a great effect on in vitro produced (IVP) bovine embryos (Fukushima and Fukui, 1985; Fukui and Ono, 1989; Goff and Smith, 1998). Fukushima and Fukui (1985) demonstrated that the addition of steroid hormones (including P₄) did not improve developmental rates of IVP bovine embryos. Similarly, Fukui and Ono (1989) and Fukui (1989) reported that the supplementation of P₄ or E₂ in the presence of fetal calf serum or estrous cow serum to IVP embryos co-cultured on oviduct cells did not increase the number of bovine embryos developing to the blastocyst stage. Furthermore, it was demonstrated that co-culture on endometrial stroma cells in the presence of P₄ failed to increase the number of IVP bovine embryos reaching the blastocyst stage compared with nontreated control IVP embryos (Goff and Smith, 1998).

In contrast to these reports, there have been reports of increased blastocyst development in IVP mice, rabbit and bovine embryos cultured in the presence of P₄ (EL-Banna and Daniel, 1972; Wiemer et al. 1987; Lavaranos and Semark, 1989). EL-Banna and Daniel (1972) reported that developmental rates of IVP rabbit embryos were increased when the embryos were cultured with uterine proteins and P₄ supplementation. It was suggested that this increase may have been due to a complex of free P₄ bound to uterine proteins that became available to the embryos. In addition, Wiemer et al. (1987) reported that the presence of P₄ stimulated blastocyst development in bovine embryos co-cultured on fetal uterine cells. Similar findings have been reported in mice (Lavaranos and Semark, 1989).
Effects of P₄ on Embryo Metabolism

Although, P₄ has been shown to increase uterine protein and carbohydrate content in uterine secretions, there is evidence that P₄ may improve metabolism efficiency of these components by the embryo. In mice, P₄ administration to ovariectomized females has been shown to alter the rate of glycogen metabolism, which resulted in increased embryo glycogen metabolism and increased the amount of labeled carbon utilization (Edirisinghe and Wales, 1984). In contrast, it has been reported that E₂ does not affect glycogen metabolism, and this is of interest because an E₂ surge is required in mice for normal implantation to occur (McCormack and Greenwald, 1974). It has also been demonstrated that P₄ administration in the mouse increases the amount of nonglycogen, acid-insoluble macromolecules (Edirisinghe and Wales, 1984). This indicates that P₄ may be involved in channeling carbon from storage compounds (e.g., glycogen) into metabolic pathways, thus increasing the synthesis of macromolecules (Edirisinghe and Wales, 1984). These findings support the theory that P₄ may be the agent responsible for the low levels of glycogen found in in vivo embryos compared with IVP embryos (Edirisinghe and Wales, 1984). It is unknown if P₄ exerts this affect directly on the embryo or if the change in embryo metabolism is caused by P₄ altered uterine secretions.

In the mare, it has been demonstrated that P₄-treated pregnant mares resulted in a four to five-fold increase in the activities of the Embden-Meyerhof Pathway of these embryos compared with embryos from nontreated control pregnant mares (Brück et al., 1997). It is important to note that oviduct-stage embryos utilize the Hexose-Monophosphate Oxidation Pathway, while uterine embryos utilize the Embden-Meyerhof Pathway. The increase in maternal P₄ levels does not need to be greatly increased to increase embryonic glucose metabolism (Brück et al., 1997). However, it was unknown if P₄ directly influences glucose metabolism in the embryo or if this effect was mediated by P₄ induced proteins (Brück et al., 1997).

In addition to protein and carbohydrate metabolism, embryos from several species have been reported to possess the ability to metabolize steroids during early development (Huff and Eik-Nes; 1966; Wu, 1985; Wu and Matsumoto, 1985; Wu, 1987). Huff and Eik-Nes (1966) demonstrated that rabbit blastocysts were capable of metabolizing 20α-dihydroprogesterone, 5β-pregnan-3α-ol-20-one and 5β-prenaediol.
The activity of 17β-hydroxysteriod dehydrogenase was determined to be capable of converting E₂ to estrone (E₁) in in vitro pre-implantation mouse and rat embryos (Wu and Matsumoto, 1985).

Wu (1985) also demonstrated that IVF-produced hamster blastocysts readily metabolize progesterone predominately into 5α-dihydroprogesterone (5α-DHP) and to a lesser degree allopregnanolone (AP). Three β-hydroxysteriod dehydrogenase (3β-HSD) is thought to be the responsible enzyme for the metabolism of P₄ in the hamster (Wu, 1985). It was reported that the mouse blastocyst was also capable of metabolizing P₄ (Wu, 1987). However in the mouse, the main P₄ metabolite was found to be AP while 5α-DHP was found in lesser amounts. It was demonstrated that much of the 5α-DHP was converted to AP. These studies suggest a mechanism by which the embryo may be able to alter uterine secretions and thereby, adjust the uterine environment to better support its stage of development. At this stage, P₄ metabolism by the embryo has not been investigated in the cow and ewe and unfortunately, these species serve as the main models to demonstrate the abilities of embryos to increase or decrease their rate of development to reflect the stage of development of the uterus.

**Effects of P₄ on Post-Implantation Development**

In sheep, Kleeman et al. (1994) demonstrated that early P₄ administration, days 1 to 6 following mating, could subsequently influence the developing fetus later in gestation. The fetuses of pregnant ewes treated with P₄ had significantly greater crown-rump lengths and weights when compared with fetuses of nontreated control pregnant ewes. In addition, Kleenman et al. (2001) proposed that the increase in fetal length and weight were a response to increased heart, skeletal and muscle growth of the conceptus.

P₄ supplementation via serum supplementation to in vitro culture systems in cattle has been suggested as a model for the study of “large calf syndrome” that occurs as a result of in vitro culture of embryos (Barnes et al., 2000). It has been suggested that expression of embryonic insulin-like growth factor 2 (IGF2) and P₄-induced endometrial expression of IGFBP2 may be two of the factors responsible for this over development of calves born.

Although, E₂ has been established as a more important factor in embryo-uterine synchrony in the pig, exposure of porcine embryos to advanced uteri has resulted in
similar developmental effects as noted in sheep (Wilson et al., 2001). Wilson et al. (2001) demonstrated that when day-2.5 porcine embryos were transferred to day-3.5 recipients, significantly heavier fetuses resulted, possessing larger placental surface areas and longer uterine implantation sites in embryo recipient females.

**Embryonic P₄ Synthesis during Early Pregnancy**

There is strong evidence that early stage embryos were capable of synthesizing steroid hormones (Huff and Eik-Nes, 1966; Seamark and Lutwakmann, 1972; Perry et al., 1973; Gadsby et al., 1980; Wilson et al., 1992; Chiappe et al., 2002). Huff and Eik-Nes (1966) have reported that rabbit blastocysts possessed enzymes that would enable them to synthesize cholesterol and pregnenolone from acetate. It was later reported that P₄ and its metabolites, 20α-dihydroprogesterone and 17α-hydroxyprogesterone, were present in the ovine blastocyst and blastocoel fluid (Seamark and Lutwakmann, 1972). P₄ and E₂ are also synthesized in swine blastocysts (Perry et al., 1973). Shemesh et al. (1979) reported that bovine blastocysts (day 13 or greater) were capable of synthesizing P₄, testosterone and E₂ (Shemesh et al., 1979). Subsequently, Gadsby et al. (1980) demonstrated that day-13 bovine blastocysts were capable of synthesizing E₂. It was later reported that day-10 bovine and swine IVP embryos were capable of synthesizing and secreting P₄, E₁ and E₂ (Wilson et al., 1992).

More recently there is evidence that P₄ may act in an autocrine manner on the developing bovine embryo (Chiappe et al., 2002). In this study IVP bovine embryos cultured and it was reported that 3β-HSD activity occurred in mature bovine oocytes and bovine embryos at the 1-cell, 2-cell, 8-cell, morula, blastocyst and hatched blastocyst stages. It was noted that 3β-HSD activity occurred at a high rate from the mature oocyte to the 4-cell stage and then became very active at the blastocyst and 100% active in the hatched blastocyst stage.

The presence of 3β-HSD allows the embryo to synthesize steroids including P₄ (Chiappe et al., 2002). It was also suggested that the reason for a greater increase in activity in the 3β-HSD enzyme was that only embryos that possessed active 3β-HSD were permitted to continue normal development. This study provides evidence that there can be an autocrine function involved in P₄ stimulation of embryo development.
P₄ Levels in Pregnant and Nonpregnant Females

Short (1958a) and Short (1959b) was one of the first to report circulating P₄ levels in the pregnant cow. A decade later, Shemesh et al. (1968) reported that by day-19 post-mating there was significant differences between circulating P₄ levels in pregnant cows compared with nonpregnant cows, however, no differences were detected from day-10 to day-18 post-mating. Pope et al. (1969) reported they failed to detect differences in circulating P₄ between pregnant and nonpregnant cattle prior to day 14 of the estrous cycle (estrus = day 0), however, there was a significant decrease following on day 14 in nonpregnant cows, with this difference attributed to the process of luteal regression.

In addition, Bulman and Lamming (1978) found that there were no differences in P₄ levels prior to day 13 between pregnant and nonpregnant dairy cows when using milk samples to determine P₄ levels. Furthermore, Robertson and Sarda (1971) failed to find a difference in circulating P₄ levels prior to the time of luteolysis between pregnant and nonpregnant cattle. Other researchers have failed to find differences in P₄ levels between pregnant and nonpregnant cows prior to day-16 (Sreenan and Diskin, 1983) or day-17 post-mating (Geisert et al., 1988). The researchers who failed to find a difference prior to day-16 post-mating suggested that the maintained P₄ levels in pregnant cows compared with nonpregnant cows was simply a result of the embryo maintaining CL function.

In contrast, Henricks et al. (1970) reported that differences from day 10 to day 16 of the estrous cycle in circulating P₄ did exist between pregnant and nonpregnant bovine females. It was reported that P₄ increased at a rate of 0.61 ng/ml per day from day 8 to day 14 in pregnant females, while this rate was only 0.15 ng/ml in nonpregnant females. This rate of increase significantly increased circulating P₄ levels from day 10 to day 14 in pregnant females compared with nonpregnant females, however, by day 16 P₄ levels were similar between the two groups. Henricks et al. (1971) reported significantly higher circulating P₄ levels in pregnant cows compared with nonpregnant cows as early as day 6 of the estrous cycle (estrus = day 0), while Erb et al. (1976) reported significantly higher P₄ levels in pregnant cows compared with nonpregnant cows by day 8 (estrus = day 0). Moreover, Lukaszewska and Hansel (1980) demonstrated that P₄ levels were higher from day 10 onward (estrus = day 0) in conjunction with greater CL weights in
pregnant compared with nonpregnant dairy cows. These findings demonstrated that differences in P₄ levels were likely present, however, individual animal differences in P₄ levels or possible blood sampling frequencies could be the reason for the variation reported for P₄ levels between pregnant and nonpregnant cows over the years.

Sexual maturity and age can also affect the systemic level of P₄ in pregnant and nonpregnant cattle. Byerley et al. (1987) reported that pregnant heifers on their first and third post-pubertal cycles had higher P₄ levels compared with nonpregnant heifers at their first or third post-pubertal cycles. When comparing sexually mature and immature ewes, it has been demonstrated that pregnant ewe lambs had lower P₄ levels compared with pregnant mature ewes by day 13 of pregnancy (estrus = day 0) and that this may contribute to the decreased lambing rate noted in ewe lambs (Davies and Beck, 1993). In contrast, cattle tend to follow a reverse pattern related to sexual maturity, as Silva et al. (2002) reported that dairy heifers, which tend to be more fertile than multiparous cows, have increased P₄ levels on day 4 and day 7 of the estrous cycle (estrus = day 0) compared with multiparous cows. These findings are in agreement with those Mann and Lamming (1996), who showed a correlation between the rate of embryo development and levels of P₄ following ovulation. They found that more embryos with abnormally small diameters were recovered from cows with lowered circulating P₄ levels at day-5 post-estrus (estrus = day 0) compared with cows with normal or elevated circulating P₄. These findings suggested that P₄ levels, as affected by age or sexual maturity, can control the growth rate of early stage embryos in vivo.

Remsen and Roussel (1982) reported that in dairy heifers, pregnancy resulting from ET was closely correlated to P₄ levels at the time of ET. They reported that 80% of the females that failed to maintain pregnancy from ET had P₄ levels below 2.0 ng/ml at time of ET. These findings on the day of transfer did not correlate to those reported by Hasler et al. (1980), who reported that circulating P₄ levels from days 3 to 9 of the estrous cycle (day 0 = estrus) between pregnancy and nonpregnant females were not correlated with ET pregnancy rates. A correlation between pregnancy rates and the duration of estrus was found, with cows having a shorter duration of estrus had significantly higher P₄ levels on day 3 of the estrous cycle compared with cows with longer durations of estrus. Similarly, Silva et al. (2002) reported no difference in P₄ levels from day 4 and day 7 between pregnant and nonpregnant ET recipient cows. These
findings suggest that even though a relationship between levels of P₄ at the time of ET the likelihood of a pregnancy being established based on circulating P₄ levels around the time of transfer as a result of ET can not be predicted. However, increased levels of circulating P₄ from day 3 to day 7 (estrus = day 0) may increase the probability of a pregnancy being established from ET in early P₄ deficient cattle.

Although increased levels of circulating P₄ during early pregnancy may increase pregnancy rates, there is likely a threshold that when not reached or surpassed can result in detrimental effects on the establishment of a pregnancy (Henricks et al., 1971; Erb et al., 1976; Bulman and Lamming, 1978; Wilmut et al., 1985). These abnormal circulating P₄ levels or the start of P₄ secretion (start of luteal tissue function) may be a cause of infertility in RPB females. Bulman and Lamming (1978) reported that ~20% of the RPB females sampled had significantly higher P₄ during insemination and immediately afterwards. This finding supports that of Erb et al. (1976), who demonstrated that infertile services were associated with increased P₄ levels immediately following ovulation. This was in addition to a report that females with degenerating embryos ≥2-cell recovered on day-3 post-mating (estrus = day 0) had significantly higher P₄ levels 2 days prior to the onset of estrus than females in which normal embryos ≥4-cell were recovered on day 3 (Henricks et al., 1971).

Correspondingly, Maurer and Echternkamp (1982) reported that females with normal developing embryos had higher P₄ levels on day 3 and day 6 (estrus = day 0) compared with females that had degenerating embryos in vivo.

Furthermore, Wilmut et al. (1985) demonstrated that when ovariectomized ewes did not receive exogenous P₄ until day 4 or later (estrus = day 0), only 18% remained pregnant compared with 70% that remained pregnant when P₄ administration was initiated prior to day 4. Ashworth et al. (1989) demonstrated that more ewes remained pregnant to natural mating when they reached 50% of their luteal phase P₄ levels early in the estrous cycle.

The majority of embryo mortality occurs between day 3 and day 19 of the estrous cycle (estrus = day 0) (Ayalon, 1978). In the normal, fertile cow successful fertilization occurs ~90% of the time from natural mating. However in ~25% of females, embryo loss has occurred by day 11 to day 13 of the estrous cycle (Ayalon, 1978). These findings were similar to those of Diskin and Sreenan (1980), who reported the majority of
embryonic morality in cattle occurred by day 12 following mating. This is quite different when compared with RPB cows where 25% of embryos are lost by day 3 post-mating and an additional 25% lost by day 11 to day 13 (Ayalon, 1978). More recently, it has been reported that AI fertilization rates in dairy cows were ~90%, while ≤50% of inseminated dairy cows calved from a single insemination (Lamming et al., 1989). These results confirm that marked embryo mortality occurs following mating in cattle.

The same embryonic loss pattern has also been reported in sheep, where only 5% to 10% of unfertilized oocytes were recovered from mated ewes with the majority of embryo mortality occurring before the third week of gestation (Wilmut et al., 1986). Furthermore, Ashworth et al. (1989) reported that <6% of early embryo mortality occurred as a result of chromosomal abnormalities. This leaves a large percentage of nonpregnant ewes that are the result of reasons other than fertilization failure and embryonic chromosomal abnormalities.

The most common explanation for high rates of embryo mortality occurring before luteolysis has been that the embryo failed to send a strong enough maternal recognition signal or an inappropriately timed maternal recognition signal. Lukaszwaska and Hansel (1980) suggest that the embryo was in some manner stimulating CL function. When day-13 to day-15 (estrus = day 0) ovine embryo extracts were in vitro cultured with ovine or bovine luteal tissue there was a significant increase in P₄, (Godkin et al., 1978). It was also determined that the embryo was not producing measurable amounts of P₄ at this time. This demonstrated that the conceptus was capable of elevating maternal P₄ levels. This further supports the hypothesis that there was a difference in P₄ levels between pregnant and nonpregnant females prior to luteolysis.

Beal et al. (1981) have reported that day-18 bovine embryos secreted a luteotrophic substance that increased P₄ levels when cultured with dispersed luteal cells in vitro. Also, Thibodeaux et al. (1994) reported that bovine embryos and trophoblastic vesicles when cultured with in vivo collected luteal cells stimulated P₄ secretion in these luteal cells. This embryo-induced luteotropic effect was also found to occur in rabbits (Nowak and Bahr, 1983). When embryos were removed from pregnant rabbits the circulating P₄ levels would decline to the baseline in psuedopregnant rabbits, indicating a direct luteal stimulatory effect from the embryo.
Hwang et al. (1988) demonstrated that day-7 (estrus = day 0) in vivo-produced blastocysts produced PGE$_2$, and as the embryo developed it obtained the ability to synthesize and secrete PGI$_2$ and serotonin (both are luteotrophins). Lewis et al. (1982) reported that bovine blastocysts could convert arachidonic acid into prostaglandins when cultured in vitro with endometrial slices. Using [H$^3$] arachidonic acid it was reported that bovine blastocysts produce considerable amounts of PGF$_{2\alpha}$ and PGE$_2$ (Lewis, 1989). These findings were consistent with reports of greater circulating PGE$_2$ levels in pregnant compared with nonpregnant females (Lewis et al., 1978; Silvia et al., 1984; Vincent et al., 1986; Parkinson and Lamming, 1990). It had been previously reported that PGE$_2$ administered during the luteal phase could extend the lifespan of the CL in the ewe (Pratt et al., 1979). Collectively, these results demonstrate the embryo’s ability to increase P$_4$ levels during the luteal phase in vivo prior to maternal recognition.

P$_4$ supplementation prior to time of embryo transfer has been shown to increase uterine secretions and effectively alter the secretion pattern of proteins and alter embryo development or possibly allow for asynchronous embryo transfer (Wilmut and Sales, 1981; Garrett et al., 1988). In addition, P$_4$ has also been reported to play a part in maternal recognition. Vincent et al. (1985) found that when P$_4$-treated day-6 ewes that received day-10 blastocysts, resulted in an increase in PGE$_2$ beginning on day 8 compared with day 12 in mated ewes. It was not known at the time whether P$_4$ increased uterine PGE$_2$ and/or embryo-derived PGE$_2$. However, it has been reported that P$_4$ administration could prevent early luteal regression caused by an intrauterine device in the ewe which was accompanied by an increase in PGE$_2$ that was believed to be of uterine origin (Warren and Hawk, 1972).

Mann et al. (1994) attempted to correlate the post-ovulatory rise (metestrus) in P$_4$ to the timing of the luteolysis. It was demonstrated that P$_4$ played a role in the synthesis of uterine PGF$_{2\alpha}$ in the cow (Lamming and Mann, 1995a). In addition, Mann and Lamming (1995) have concluded that mated cows with lower P$_4$ levels during early pregnancy had a significantly stronger luteolytic signal than mated cows with higher levels of P$_4$ during early pregnancy. It was also reported that higher P$_4$ levels during the estrous cycle (diestrus) exerted an inhibition on the luteolytic mechanism thereby, increasing the chances of the embryo establishing maternal recognition.
In a subsequent experiment, Mann et al. (1998) demonstrated that an increase of only 0.6 ng/ml during the early luteal phase was sufficient to alter the timing of the luteolytic signal. Furthermore, it was shown that an early P₄ rise could advance the luteolytic signal but elevated levels of P₄ weakened the luteolytic signal (Mann et al., 1998). Embryos that experienced increased levels of P₄ during early pregnancy were capable of synthesizing and secreting larger quantities of interferon-tau (the primary maternal recognition signal in cattle) and in these pregnant females, less interferon-tau was then required to maintain the CL (Mann et al., 1999). An increase in embryonic interferon-tau was also found in embryos recovered from cows administered P₄ from day 1 to day 4, following the onset of estrus (Garrett et al., 1988).

Gawronska et al. (2000) demonstrated that under P₄ domination the uterus would produce more PGE₂ than PGF₂α. This finding was confirmed when Shaham-Albalancy et al. (2001) demonstrated that low concentrations of P₄ during the luteal phase increased in PGF₂α metabolite (PGFM). This could represent a mechanism by which low P₄ during the luteal phase could cause early termination of pregnancy. Mann and Lamming (2001) reported inseminated cows with increased P₄ levels during early pregnancy (~day 4 to day 9) had altered the uterine ratio of PGE₂ to PGF₂α to favor PGE₂. Embryos collected from these females also synthesized more interferon-tau in vitro than embryos collected from cows with lower early P₄. In addition, when interferon-tau was administered to these females, the females with high levels of early P₄ required higher levels of interferon-tau to initiate luteolysis than did females with low levels of early P₄. These results indicate that early P₄ levels control the timing and degree of the luteolytic signal, however, it is unknown if this effect is controlled by ovarian P₄ alone or a combination of embryo-uterine relationship via luteotropins stimulated P₄ levels.

Maintenance of Pregnancy in Ovariectomized and Luteoectomized Females

Hess (1921) and Schmaltz (1921) (cited by McDonald et al., 1952) were the first to report that CL removal in the cow during pregnancy would result in pregnancy termination. Following these findings, Raeside and Turner (1950) attempted to determine the amount of P₄ supplementation required to support pregnancy in dairy cows following CL ablation at 44, 48 or 76 days of gestation. In this study, it was discovered that 25 mg of P₄ per day per head dissolved in olive oil commencing administration on the day of CL ablation was insufficient to support pregnancy, however,
the administration of 50 mg of P₄ per day per head maintained pregnancy in two of four females for 100 days at which time P₄ supplementation was ceased and the two pregnant females aborted within 4 days of the end of P₄ supplementation.

In a subsequent study by Raeside and Turner (1951) it was reported that when CL ablation occurred it was dependant upon the stage of gestation as to whether supplemental P₄ was required to maintain the pregnancy. Using 75 mg and 100 mg of P₄ dissolved in sesame oil and administered daily following CL ablation at 58 to 68 days of gestation in mature dairy cows, it was determined that if P₄ was administered until day 162 to day 237 of gestation and then ceased pregnancy would continue until day 254 to day 282 of gestation (McDonald et al., 1952). However, if daily P₄ administration was ceased at day 76 or 137 of gestation the pregnancy would be terminated. Furthermore, McDonald et al. (1952) reported that of the females that did continue pregnancy to term following CL ablation and P₄ supplementation until ~day 162 of gestation, at parturition there was a high incidence of dystocia and retained fetal membranes.

In a follow-up study, McDonald validated previous findings of Raeside and Turner (1951) that stated the continuation of pregnancy following CL removal without supplementing P₄ was dependant on the stage of gestation. When mature pregnant dairy cows underwent CL removal from 92 days to 236 days of gestation no female that underwent CL removal prior to day 171 of gestation continued pregnancy to term and produced a live calf. If CL removal occurred on or after day 191 of gestation pregnancy continued in all females (n=6), however, these females experience dystocia and a high incidence of retained placental membranes.

Although it was previously demonstrated that the CL of pregnancy could be removed during gestation and depending on the stage of gestation and 50 mg or greater of P₄ supplementation daily pregnancy could continue, however it was unknown how early the CL could be removed and allow pregnancy to continue. Hawk et al. (1960) using ovariectomy at day 5 to day 7 post-estrus maintained pregnancy in six pregnant dairy cows until ~day 60 of gestation when these animals were sacrificed and it was determined the fetuses were live. The ovariectomized pregnant dairy cows were administered 25 mg of P₄ per 100 lbs of body weight and 6.25 mcg of estrone per 100 lbs of body weight. This study demonstrated that pregnancy could be continued in the
absence of ovaries as early as day 5 of gestation and normal implantation could occur in
the absence of ovaries provided that $P_4$ and $E_1$ were supplemented daily.

In an effort to dispense with the difficulty of daily steroid administrations, Johnson
and Erb (1962) tested the efficiency of administering 500 mg of Delatutin (17-alpha-
hydroxyprogesterone-17-n-caproate) or 500 mg repositol progesterone (progesterone in
propylene glycol), progestins and progesterone with a longer half life, weekly. However,
pregnancy could not be maintained in ovariectomized pregnancy dairy cows receiving
only weekly administrations of delatutin or repositol progesterone. It was reported that in
the control group that received daily administration of $P_4$ these females experienced a
high incidence of retained placental membranes.

There have been successful reports of using progestins instead of progesterone
in supporting pregnancy (Zimbelman and Smith, 1966; Kesler, 1997). Using ovariectomy
at ~day 170 of gestation and supplementing 4 mg of MGA daily until ~277 days of
gestation the oral progestin MGA would support pregnancy to term with a lower
incidence of dystocia and retained placental membranes (Zimbelman and Smith, 1966).
Later, it was demonstrated that two 15 mg norgestomet implants administered s.c. into
the ear could maintain pregnancy in pregnant beef heifers ovariectomized at 10 days
following AI (Kesler, 1997). These implants were removed at day 273 of gestation.
Calving rates were lower compared with sham surgery controls and there was a high
incidence of dystocia (67%), retained fetal membranes (67%) and calf survival was low
(33%). These findings demonstrate that pregnancy can be supported in the absence of a
CL or ovaries and with or without supplementing $P_4$ dependant upon stage of gestation
that CL removal or ovariectomy is performed. However, regardless of the method of
maintaining pregnancy partition is difficult and results in a greater probability of dystocia
and fetal placental membrane retention occurring.
CHAPTER 3
THE USE OF ALTRENOGEST TO SYNCHRONIZE ESTRUS IN YEARLING BEEF HEIFERS

Introduction

Altrenogest (ALT) is an orally active progestin that has been developed to control reproductive function in nonruminant domestic animals. During the past two decades ALT has become a commonly used progestin in the mare (Squires et al., 1979; Webel and Squires 1982; Squires et al., 1983) and the sow (Davis et al., 1979; Davis et al., 1987; Martinat-Botte et al., 1985). ALT has been found to safely and effectively suppress behavioral estrus in mares, when administered at 0.044 to 0.220 mg per kg of body weight (Shideler et al., 1983). When administered at a rate of 0.044 mg per kg of body weight, ALT has been effective in the synchronization of estrus in cycling mares (Squires et al., 1979) and transitional mares (Turner et al., 1981; Weber and Squires, 1982). However, there have been several reports that ALT did not completely block ovulation in all cycling or transitional mares treated (Squires et al., 1979; Turner et al., 1981; Weber and Squires, 1982). In addition, ALT has been demonstrated to decrease the number of large follicles and size of the dominant follicle in the mare (Turner et al., 1981; Webel and Squires, 1982; Squires et al., 1983). These decreases from a mean follicle size of 39.9 mm in ALT-treated mares compared with a mean follicle size of 41.5 mm in control-treated mares and a mean of 0.60 follicles that were >40 mm following treatment with ALT compared with a mean of 0.79 follicles >40 mm in the control treatment group were not detected when mares were administered another progestin, norgestomet (Wiepz et al., 1988).

Hinrichs et al. (1985) and Hinrichs and Kenny (1987) demonstrated that bilaterally ovariectomized mares could serve as embryo transfer (ET) recipients when administered ALT for at least 7 days prior to ET. Also, ALT has been reported, in mares, to support pregnancy in the absence of ovarian luteal tissue (McDowell et al., 1988; Daels et al., 1991). In addition, ALT has been reported to support pregnancy in an Okapi with a history of fetal mortality (Schwarzenberger et al., 1999).

Although ALT has not been reported to maintain pregnancy in the absence of luteal tissue in sows, there have been reports that ALT can control the time of parturition (Guthrie, 1985; Varley et al., 1985; Guthrie et al., 1987). Furthermore, it has been
reported that ALT can increase fertility in postpartum sows when administered prior to weaning (Stevenson et al., 1985; Koutsotheodoros et al., 1998). The physiological actions of ALT are comparable with those of other progestins and/or progesterone (P₄) in other domestic animals.

Although ALT has been reported to function as a progestin in the mare and the sow, research indicates that various progestins used in cattle (e.g., norgestomet and megestrol acetate) were not effective in maintaining pregnancy in the mare (Loy and Swan, 1966; McKinnon et al., 2000).

With the removal of norgestomet (Synchro-mate B®) from the commercial market in the United States, fewer progestins are now available to cattle producers for use in estrous synchronization regimens. Melengestrol acetate (MGA) is a commonly available orally active progestin used for estrous synchronization in cattle, however, this protocol is time consuming requiring a 31-day treatment schedule. Also, when MGA was administered for 7 days there has been a noted reduction in fertility following breeding with only 40 to 55% pregnancy rates reported (Beal et al., 1988; Patterson et al., 1989). However, when used in a 31-day estrous synchronization protocol, yearling heifers had pregnancy rates of 64 to 77% following artificial insemination (AI) (Deutscher et al., 1989; Jaeger et al., 1992; Patterson and Corah, 1992). The use of a 31-day estrous synchronization schedule allows an additional estrous cycle prior to AI that may be a factor in the reported improvement in pregnancy rates after synchronization using the 31-day protocol.

The availability of an additional orally active progestin that could effectively synchronize estrus with a shorter treatment period to breeding than MGA would add more flexibility for cattle producers when selecting an estrous synchronization regime. However, as described MGA usage results in reduced fertility rates, in beef cattle, following a 7-day treatment period but these reduced fertility rates were not found when norgestomet was used for 7 days (Patterson et al., 1989). This led to the question, would a 7-day treatment schedule with ALT serve as an effective oral progestin in cattle? Therefore, the objective of this study was to determine if ALT could effectively synchronize estrus in beef heifers without reducing fertility following a 7-day treatment protocol.
Materials and Methods

Experimental Animals

This experiment was conducted between the months of April and June for three consecutive breeding seasons at the Center for Reproductive Biology at Louisiana State University, St. Gabriel, Louisiana. All animals were treated in accordance with guidelines set forth by the Animal Care and Use Committee of the Louisiana State University Agricultural Center. During this 3-year period, 256 crossbred (Red Angus and Simmental based) beef heifers ranging in age from 12.3 to 15.7 months, body weights from 345 to 447 kg and body condition scores from 5 to 6 (scale of 1=emaciated to 9=obese) (Richards et al., 1989) were used in Experiment 3.1. These heifers were maintained on pasture (Coastal bermudagrass) and supplemented with 1.36 kg of ground corn per female per day beginning 30 days prior to and throughout the treatment period (Figure 3.1).

Experimental Design

After 60 days of feeding, all heifers that failed to reach a pre-breeding target weight of 345 kg at the time of PGF$_{2\alpha}$ administration were removed from this study. This target weight was selected based on research that demonstrated that the majority of heifers of these two breed types would not be cycling until this target weight was reached (Thallman et al., 1999). In each of the three breeding seasons, heifers were blocked by age and weight then randomly assigned to one of two treatment groups. In each of the three breeding seasons the ALT treatment group was administered ALT (Regumate, Intervet, Inc., Millsboro, DE) orally (manually mixed into feed ration) at a rate of 0.044 mg per kg of body weight per head per day for 7 consecutive days and then administered 25 mg PGF$_{2\alpha}$ (Lutalyse, Pharmacia and Upjohn Co., Kalamazoo, MI) on the 7$^{th}$ day (Figure 3.1). During each breeding season the MGA treatment group was administered MGA (melengestrol acetate, Animal Science Feeds, Nacodogoches, TX) at a rate of 0.4 to 0.5 mg per head per day in the feed for 14 days and then were administered 25 mg of PGF$_{2\alpha}$ 17 days after MGA withdrawal (Figure 3.1). Even though the duration of treatment was different for females in each treatment protocol, the
Figure 3.1. Estrous synchronization protocols were arranged so that prostaglandin (PGF$_{2\alpha}$) was administered simultaneously in both treatments. This allowed for estrus detection and AI to occur concurrently in both treatment groups.
regimens were arranged so that all heifers received PGF$_{2\alpha}$ and subsequently artificially inseminated during the same time interval (Figure 3.1).

**Experimental Procedures**

Estrus detection was performed with the aid of the HeatWatch System® (DDX, Inc., Boulder, CO), using transponders that were attached to heifers at the time of PGF$_{2\alpha}$ administration. The Heatwatch System® records when a female was mounted by detecting pressure applied to the mount transponder for a minimum of 3 seconds and a minimum of 3 mounts in an hour. The HeatWatch System® was used in addition to two daily observation periods (morning and evening) not less than 45 minutes each.

All inseminations were carried out by experienced technicians (n=2) using semen from one of two Red Angus bulls (Genex Cooperative, Inc., Shawano, WI) of proven fertility. At the time of AI, heifers were given an AI score of 1 to 3, with ‘1’ being the most difficult to AI and ‘3’ the least difficult to AI. The criteria for a score of 1 was that the insemination required greater than 3 minutes to transverse the cervix, while a score of 2 was assigned to an insemination that required 1 to 3 minutes to transverse the cervix with the insemination stylet. An AI score of 3 was assigned to inseminations that required less than 1 minute to transverse the cervix. All inseminated females were examined for pregnancy ~30 days after the onset of estrus via transrectal ultrasonography using an Aloka 500-V ultrasound unit (Corometrics, Wallingford, CT) equipped with a 5 MHz rectal probe. A pregnancy was defined as the presence of a fetus with viable heart beats. Following parturition, calf viability, sex and birth weight were recorded.

**Statistical Analysis**

The heifers that were determined to respond to treatments were those which exhibited behavioral estrus within 7 days following the end of the estrous synchronization protocol. There were no significant differences in estrual response within a treatment group between breeding seasons thus, all data were pooled for further statistical analysis. The pregnancy rate per treatment was determined by dividing the number of heifers pregnant by the number of heifers inseminated. This excluded nine heifers, five of which experienced illness or lameness requiring veterinary intervention prior to 30 day pregnancy examinations and four heifers whose reproductive tract abnormalities prevented uterine deposition of semen. Calving rates were calculated as
the number of heifers calving from the number of pregnant heifers. Estrous response rates, pregnancy rates and calving rates were analyzed using procedures for categorical data, Chi square, in SAS (SAS Institute. Inc., Gary, NC). Independent variables were age, body weight, synchronization protocol, sire, AI technician and all interactions. The Proc GLM procedure was used to determine differences in duration of estrus, number of mounts and pregnancy rates in response to independent variables.

Results

The physical characteristics (i.e., age and weight) of the heifers that reached the 345 kg target weight were similar for each treatment group (Table 3.1). Overall, the number of heifers that exhibited standing behavioral estrus in the ALT-treated group (48 of 69; 70%) was significantly greater (P<0.05) compared with the number in the MGA-treated group (37 of 75; 50%) (Table 3.2). The mean(±SE) interval from PGF$_{2\alpha}$ administration to the onset of estrus was not significantly different (P>0.05) between ALT-treated (55.7±2.6 hours) and for MGA-treated heifers (55.3±4.3 hours). The distribution pattern of the time to onset of estrus post-PGF$_{2\alpha}$ was similar for both treatment groups (Figure. 3.2). However, the duration of estrus and number of mounts per heifer, monitored via the HeatWatch System®, were statistically different between treatment groups. Heifers in the ALT-treated group had a significantly less (P<0.05) mean number of mounts per female of 35±4 with a mean duration of estrus of 8.5±0.6 hours and compared with the mean of 48±6 mounts with a mean duration of estrus of 10.6±0.7 hours for the MGA-treated group (Table 3.2).

There was no effect of treatment on mean AI scores at time of insemination (Figure. 3.3). In this study, there were no significant differences (P>0.05) in time to the onset of estrus and duration of estrus between pregnant and nonpregnant females (Table 3.3). Overall, there was a significant difference (P<0.05) in the mean number of mounts per female between pregnant (48±5.0) and nonpregnant females (33±5.0).

There was no statistical difference in the pregnancy rates for ALT-treated (27 of 41; 66%) compared with MGA-treated heifers (23 of 35; 66%) (Table 3.4). A total of nine heifers (two from the MGA group and seven from the RGM group) that responded to estrous synchronization protocols were removed from the pregnancy data.
Table 3.1. Characteristics of mixed-breed beef heifers that reached the pre-determined target weight (345 kg) and were exposed to two estrous synchronization protocols using either altrenogest (ALT) or melengestrol acetate (MGA) progestin treatments

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. per group</th>
<th>Mean±SE age (day)</th>
<th>Mean±SE weight (kg)</th>
<th>Mean body condition score&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>69</td>
<td>432±3.0</td>
<td>371±2.1</td>
<td>5.9±0.1</td>
</tr>
<tr>
<td>MGA</td>
<td>75</td>
<td>431±2.6</td>
<td>373±2.3</td>
<td>6.0±0.1</td>
</tr>
</tbody>
</table>

<sup>1</sup> Body condition scores (1=emaciated to 9=obese).
Table 3.2. Effects of estrous synchronization treatments on the percentage of heifers exhibiting estrus, interval to the onset of estrus following prostaglandin (PGF$_{2\alpha}$) administration, duration of estrus and number of mounts per female in mixed-breed beef heifers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number in estrus (%)</th>
<th>Onset of estrus post-PGF$_{2\alpha}$ (h)$^1$</th>
<th>Duration of estrus (h)$^1$</th>
<th>Number of mounts/female$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT$^3$</td>
<td>48/69 (70%)$^b$</td>
<td>55.7±2.6$^a$</td>
<td>8.5±0.6$^a$</td>
<td>35±4$^a$</td>
</tr>
<tr>
<td>MGA$^4$</td>
<td>37/75 (50%)$^a$</td>
<td>53.3±4.3$^a$</td>
<td>10.6±0.7$^b$</td>
<td>48±6$^b$</td>
</tr>
</tbody>
</table>

$^{a,b}$ Within a column, means with different superscripts are significantly different (P<0.05).

$^1$ h = hours.

$^2$ Indicates the number of mounts per female for the synchronized estrus.

$^3$ ALT=altrenogest.

$^4$ MGA=melengestrol acetate.
Figure 3.2. Distribution of time to the onset of estrus following prostaglandin (PGF$_{2\alpha}$) administration for altrenogest (ALT)-treated and melengestrol acetate (MGA)-treated heifers.
Figure 3.3. Artificial insemination (AI) scores assigned to heifers that exhibited estrus that were synchronized with either altrenogest (ALT) or melengestrol acetate (MGA).
Table 3.3. Time from prostaglandin (PGF$_{2\alpha}$) administration to the onset of estrus, mean duration of estrus and number of mounts per female during estrus for altrenogest (ALT)-treated and melengestrol acetate (MGA)-treated pregnant and nonpregnant beef heifers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Status</th>
<th>Time to the onset of estrus post-PGF$_{2\alpha}$ (h)$^1$</th>
<th>Duration of estrus (h)$^1$</th>
<th>Number of mounts/♀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Pregnant</td>
<td>58±2.6</td>
<td>8.4±0.6</td>
<td>37±5.8</td>
</tr>
<tr>
<td></td>
<td>Nonpregnant</td>
<td>53±5.7</td>
<td>9.8±1.5</td>
<td>35±6.8</td>
</tr>
<tr>
<td>MGA</td>
<td>Pregnant</td>
<td>56±6.1</td>
<td>11.2±0.9</td>
<td>58±8.4</td>
</tr>
<tr>
<td></td>
<td>Nonpregnant</td>
<td>48±5.1</td>
<td>9.4±1.4</td>
<td>32±9.4</td>
</tr>
<tr>
<td>Total</td>
<td>Pregnant</td>
<td>57±3.0</td>
<td>9.5±0.5</td>
<td>48±5.0$^a$</td>
</tr>
<tr>
<td></td>
<td>Nonpregnant</td>
<td>50±3.6</td>
<td>9.6±0.9</td>
<td>33±5.0$^b$</td>
</tr>
</tbody>
</table>

$^a,b$ Within a column, means with different superscripts are significantly different (P<0.05).

$^1$ h=hours.
Table 3.4. Pregnancy rates, calving rates and mean birth weight of calves produced from heifers synchronized with either altrenogest (ALT) or melengestrol acetate (MGA)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number pregnant (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Number calving (%)</th>
<th>Calf birth weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>27/41 (66%)</td>
<td>23/27 (85%)</td>
<td>33.5±1.0</td>
</tr>
<tr>
<td>MGA</td>
<td>23/35 (66%)</td>
<td>20/23 (87%)</td>
<td>34.1±1.0</td>
</tr>
</tbody>
</table>

<sup>1</sup> Due to the inability to transverse the cervix during insemination four heifers were removed from pregnancy data. Five additional heifers were removed due to severe illness prior to 30-day pregnancy evaluation.
Four of these females (two from MGA and two from RGM) were removed due to an inability to transverse the cervix during insemination while five heifers were removed due to severe illness before the 30-day pregnancy evaluation.

In this study, there were no significant differences (P>0.05) in the calving rates between the ALT-treated (23 of 27; 85%) and the MGA-treated group (20 of 23; 87%) (Table 3.4). In addition, there were no differences in calf birth weights nor were there abnormal effects among offspring produced from heifers synchronized with either ALT or MGA. Furthermore, there was no significant effect of estrous synchronization treatment on the sex ratio of calves from heifers treated with ALT or MGA (Figure 3.4). During the course of three breeding seasons the ratio of male to female calves for the ALT-treated calving females were: 33% to 67%, 40% to 60% and 50% to 50% for years 1, 2 and 3, respectively. The male to female calf ratio for MGA-treated calving heifers were: 45% to 55%, 50% to 50% and 45% to 55% for years 1, 2 and 3, respectively. All calves from both treatments developed normally and heifer calves subsequently came to puberty as expected.

**Discussion**

The objective of this study was to evaluate the ability of a progestin (altrenogest) to serve as an estrous synchronization regimen in beef heifers. These results demonstrated that ALT can be an effective progestin, in cattle, when used for estrous synchronization. Although there was a statistical difference in estrous response rates between ALT-treated and MGA-treated heifers, this is likely not an indication of the superiority of ALT over MGA for estrous synchronization. The likely cause of the statistical difference may be due to the use of a range of 0.4 to 0.5 mg of MGA per female per day in the MGA-treated group. It should be noted that Zimbelman and Smith (1966a) have reported that 0.4 mg was the minimum required dose to prevent ovulation and behavioral estrus in cattle. Therefore, the use of 0.4 to 0.5 mg of MGA resulted in estrous synchronization but may not have been at an optimal level consumed by each heifer, even though the estrous response rate among MGA-treated beef heifers was consistent with ranges reported for MGA estrous synchronization in heifers of similar ages and weights (Selk et al., 1987; Mauck et al., 1988; Jaegar et al., 1992; Lamb et al., 2000). The estrous response rate for the ALT-treated group was similar to that of others.
Figure 3.4. Sex ratio of calves produced from heifers synchronized with either altrenogest (ALT) or melengestrol acetate (MGA) treatments.
who used a different progestin (norgestomet) based synchronization regimen for
yearling beef heifers (Patterson and Corah 1992; Larson et al., 1996; Lamb et al., 2000).
Bloss et al. (1966) reported that at a dose of 0.25 mg to 0.5 mg of MGA per head per
day in beef cattle could effectively prevent behavioral estrus and allow for follicular
development to occur.

At this station, altrenogest was administered to five cycling mature beef cows at
days 16 to 18 of the estrous cycle and behavioral estrus was blocked and follicular
development occurred (Ferguson, unpublished observations). Moreover all females
developed a dominant follicle by the 3rd day of altrenogest treatment and these follicles
began to regress thereafter (Ferguson, unpublished observations) with all females
entering estrus within 5 days following withdrawal of ALT. These observations suggest
that altrenogest and MGA may both have similar affects on suppression of estrus
behavior and altrenogest may not have a deleterious effect on follicular dynamics, as
reported in the mare (Turner et al., 1981; Webel and Squires, 1982; Squires et al.,
1983).

Based on the observation that there were no significant differences in AI scores,
altrenogest does not appear to adversely affect reproductive tract morphology or
oviductal/uterine secretions. Also, with no differences occurring in the duration of estrus
and number of mounts per female between the altrenogest-treated and MGA-treated
heifers, altrenogest appears not to adversely affect behavioral estrus when compared
with a commercially available progestin (i.e. MGA). In evaluating behavioral estrus
between pregnant and nonpregnant females there was a significant increase in the
number of mounts per estrus without a significant increase in the duration of estrus in
pregnant females. This suggests pregnant females had a more intense estrous period
(mounts per hour) at the time of mating when compared with similar heifers that
subsequently did not become pregnant after mating. Therefore this post-treatment estrus
intensity may be correlated to fertility.

AI pregnancy rates achieved from both the altrenogest and MGA protocols in this
study were similar to AI pregnancy rates reported for yearling beef heifers synchronized
using the MGA protocol of 65% (Mauck et al., 1988) and 64% (Patterson and Corah,
1992), but slightly less than pregnancy rates for beef heifers reported by Jaeger et al.
(1992) of 77%, and Lamb et al. (2000) of 76 to 81%. The calving rate from heifers
impregnated following synchronization with altrenogest or MGA was consistent with this stations herd calving rate for a 60-day breeding season. These findings indicate that there are no apparent problems with estrous synchronization and AI, heifers maintaining pregnancy and calving following altrenogest treatment.

The heifer calves produced from altrenogest-treated heifers exhibited normal growth rates and normal estrous behavior upon reaching puberty. This finding is in agreement with similar studies involved in analyzing growth rate of fillies and colts produced from altrenogest-treated pregnant mares (Naden et al., 1990a). Naden et al. (1990a) reported no differences between the age at puberty, characteristics of the estrous cycle or fertility between fillies produced from altrenogest-treated pregnant mares or control pregnant mares. However, it was noted that fillies produced from altrenogest-treated pregnant mares had higher serum LH and FSH early in life (e.g. birth to 7 months-of-age) but was not different in colts. Also, there was no reported difference in age at onset of puberty in colts produced from altrenogest-treated pregnant mares compared with those from control pregnant mares (Naden et al., 1990b). It would be difficult to speculate on the potential differences in gonadotrophins occurring in prepuberal heifer calves produced from altrenogest synchronized females due to the brief pre-breeding treatment with altrenogest compared with gestational treatment with altrenogest in the pregnant mares.

Heifer calves conceived during the first replicate were used in both treatment groups of the third year replicate. When these heifers produced from altrenogest-synchronized pregnancies were synchronized with altrenogest or MGA they responded to progestin treatments, became pregnant and delivered live healthy calves. There appeared to be a trend of more heifer calves produced from altrenogest synchronized females compared with control females; however, more observations may be needed in this study to determine an effect of ALT on altering calf sex ratios.

These results demonstrate that a 7-day ALT treatment followed by 25 mg of PGF$_{2\alpha}$ on the 7$^{th}$ day can be an effective method of estrous synchronization in yearling mixed-breed beef heifers. Although MGA has been a commonly used oral progestin for estrous cycle synchronization in beef heifers, it requires a 31-day treatment schedule. In addition, MGA is less palatable to cattle thereby, requiring it to be pre-mixed with other feedstuffs or top-dressed with molasses to improve its palatability. In contrast, ALT
appears to be palatable and heifers readily accept it injected directly into their mouth or top-dressed on ground corn. In conclusion, this is believed to be the first reported use of ALT for estrous synchronization in cattle. These results indicate that ALT can be effectively used for estrous synchronization in heifers; however, further research is needed to substantiate these findings.
CHAPTER 4
EFFECTS OF ADMINISTRATION OF P₄ OR ALTRENOGEST ON DAYS 3 TO 5 POST-MATING ON PREGNANCY RATES

Introduction

Repeat breeder (RPB) females represent a substantial economic loss to beef producers. Unfortunately, these animals can only be identified in hindsight. Research comparing differences between the physiology of subfertile females and fertile females has provided some insight for improving pregnancy rates among RPB females. Bilateral ovariectomy with subsequent steroid replacement therapy has failed to enhance pregnancy rates among RPB beef females compared with that of fertile females (Hawk et al., 1963). Thus, ovarian dysfunction was not believed to be a primary causative factor involved in subfertile matings (Hawk et al., 1963). It was later demonstrated that differences in ovarian function did exist between fertile and nonfertile beef cattle matings. Bage et al. (2001) reported that RPB females enter estrus with similar follicular morphology as fertile females but generally experience a prolonged estrus resulting from a consistent delay in the pre-ovulatory LH peak (Erb et al., 1976; Gustafsson et al., 1986; Albihn, 1991). Additionally, a higher baseline cortisol level and an increased responsiveness to adrenocorticotropic hormone challenges are characteristic of RPB beef females, supporting the hypothesis that decreased fertility may be a result of stress related delayed ovulation (Bage et al., 2000).

Although stress can be a causative factor for decreased pregnancy rates in RPB females, other factors such as luteal insufficiency can also detrimentally affect pregnancy rates in RPB females. Shelton et al. (1990) determined that the post-ovulatory increase of progesterone (P₄) during metestrus was delayed in RPB females compared with fertile heifers. In vitro culture of metestrous luteal tissue collected from RPB females was less responsive to LH and PGE₂ stimulation (Shelton et al., 1990). Albihn (1991) reported that corpora lutea (CL) collected from RPB females had significantly less luteal tissue volume per CL compared with luteal tissue volume per CL collected from fertile females.

These findings have increased the controversy regarding differences in P₄ levels during metestrus and diestrus between pregnant and nonpregnant cattle (Sreenan and Diskin, 1983). It is possible that a less than normal rise in circulating P₄ levels following
mating may hinder embryonic development rates in cattle (Gustafsson et al., 1986; Shelton et al., 1990). In contrast, Linares et al. (1982) found no correlation between circulating $P_4$ levels and abnormal embryo development in cattle. However, when day-7 embryos were recovered from RPB beef females and transferred to virgin heifers, embryo survival rates were lower compared with embryo survival rates in virgin heifers receiving embryos from other virgin heifers (Gustafsson and Larsson, 1983). In a subsequent study, embryo survival at day 16 or day 17 of the estrous cycle was found to be lower in RPB females compared with virgin heifers and less day-7 embryos recovered from RPB females continued to develop when transferred to virgin heifers compared with embryos collected from virgin heifers and transferred to RPB females (Gustafsson, 1985).

These results indicate that a portion of the RPB females may have deficient uterine secretory function resulting from insufficient ovarian function. Stanchev et al. (1991) reported that virgin heifers have significantly more endometrial $P_4$ receptors than RPB heifers, although no differences in $P_4$ levels were detected in these females. Also, often overlooked in these early studies, there has been a trend for a greater increase in $P_4$ from day 3 to day 6 following the onset of estrus in pregnant females compared with $P_4$ levels in nonpregnant females (Erb et al., 1976; Linares et al., 1982; Gustafsson et al., 1986; Shelton et al., 1990).

There have been attempts to improve pregnancy rates in RPB females by supplementing $P_4$ or luteotrophins, however, these treatments have resulted in contradictory results regarding pregnancy rates. The administration of hCG or GnRH during mid-cycle (day 7 of the estrous cycle) has routinely failed to improve pregnancy rates (Holness et al., 1982; Sreenan and Diskin, 1983; Walton et al., 1990). Also, when $P_4$ was administered to RPB beef females ~day 7 of the estrous cycle, there was no consistent increase in pregnancy rates (Wiltbank, et al., 1956; Johnson, et al., 1958; Van Cleef et al., 1991; Stevenson and Mee, 1991).

The failure of $P_4$ administration to RPB females on day 7 to improve pregnancy rates may be a factor of imprecise timing. Therefore, the objective of this experiment was to determine if an artificially induced rise in $P_4$ by administration of low doses of $P_4$ or altrenogest (ALT), on day 3 to day 5 following the onset of estrus could increase pregnancy rates in RPB females.
Materials and Methods

Experimental Animals

Experiment 4.1

Crossbred beef heifers (predominantly Red Angus and Simmental) that were obtained from the Center for Reproductive Biology at Louisiana State University, St. Gabriel, Louisiana, that ranged in age from 13 to 15 months and body condition scores (BCS) (scale: 1 = emaciated to 9 = obese) from 5 to 7, were used in Experiment 4.1. These females were maintained on Costal bremudagrass pasture while being subjected to estrous synchronization (altrenogest or controlled internal drug releasing device, CIDR, protocols). All heifers were treated in accordance with guidelines set forth by the Animal Care and Use Committee of the Louisiana State University Agricultural Center. Following AI, these heifers were blocked on age, body weight and estrous synchronization protocol, and then randomly assigned to one of two treatment groups.

Experiment 4.2

In Experiment 4.2, crossbred mature beef cows and heifers (predominantly Red Angus and Simmental) that were obtained from the Center for Reproductive Biology at Louisiana State University, St. Gabriel, Louisiana ranged in age from 3 to 10 years and with BCS ranging from 5 to 8 at the time of treatment. These females were maintained on Costal bremudagrass pasture. All animals were treated in accordance with guidelines set forth by the Animal Care and Use Committee of the Louisiana State University Agricultural Center.

Experimental Design

Experiment 4.1 was a preliminary experiment designed to evaluate the use of oral administration of ALT on days 3 to 5 following mating to improve pregnancy rates in heifers following estrous synchronization and artificial insemination (AI). Experiment 4.2 was designed to test the efficiency of P₄ or orally administered ALT supplemented on days 3 to 5 following mating to improve pregnancy rates in RPB females. For this experiment, RPB females were defined as cows that that failed to become pregnant from AI breeding or from embryo transfer during a consecutive 2-year interval (Figure 4.1). All of these females were then exposed to two fertile Red Angus bulls for a 60-day breeding season and failed to become pregnant, as determined by rectal palpation, 90-days following the end of the 60-day breeding season. This breeding
Figure 4.1. Experimental protocol for classifying females as fertile and repeat breeder (RPB) cows.
period was designated as the nontreated breeding period in this study. These females were then classified as RPB females. In the last replicate RPB heifers were also used and were defined as yearling heifers that were exposed to two fertile Red Angus bulls for a 60-day breeding season and failed to become pregnant, as determined by rectal palpation, 90-days following the end of 60-day breeding season.

The control treatment group consisted of cows that were exposed to the same two fertile Red Angus bulls the previous year, as the females classified as RPB females, but became pregnant and produced a live healthy calf. The reason for selecting females mated a year previous to the year that RPB females were exposed to bulls was due to the 285-day gestation period for the females. By selecting females mated the previous year it was possible to identify the “fertile” females in retrospect due to the fact they produced a calf to a 60-day breeding season. The control females then served as a control treatment group for Experiment 4.2, to determine bull fertility during the treatment period.

**Experimental Procedures**

**Experiment 4.1**

In Experiment 4.1, all females underwent estrous cycle synchronization using either a CIDR (EAZI-Breed, InterAg, Hamilton, New Zealand) or ALT (Regumate, Intervet, Millsboro, DE) protocol and were inseminated with semen from fertile beef bulls (n=3, used across the treatment groups) ~12 hours after the onset of estrus. Following AI, these females were blocked on estrous cycle synchronization protocol, age and body weight and then randomly assigned to one of the following treatments. The females allotted to the ALT treatment group, (n=19) were brought to a restraining chute and orally (per os) administered 15 mg of ALT on days 3, 4 and 5 following AI (Figure 4.2). Females selected for the control treatment group (n=36) were brought to the chute on days 3, 4 and 5 following AI and temporarily restrained but received no further treatment. All females were examined for pregnancy, 30 days after onset of estrus via transrectal ultrasonography using an Aloka 500-V ultrasound unit (Corometrics, Wallingford, CT) equipped with a 5 MHz rectal probe. A pregnancy was defined as the presence of fetus with a viable heart beats.
Altrenogest (ALT) Treatment Protocol

Onset of Estrus

15 mg of ALT/day

Pregnancy Check

0 1 2 3 4 5 6 // 30-33

Days

Control Treatment Protocol

Onset of Estrus

No ALT Control

Pregnancy Check

Figure 4.2. The experimental procedure for altrenogest (ALT) treatment of heifers in Experiment 4.1.
Experiment 4.2

In Experiment 4.2, all females were pastured with two fertile Red Angus bulls. To allow for pregnancy determination from only one estrous period the experimental breeding season (fall) was limited to 25 days and pregnancy exams occurred 30 to 33 days following the 1st mating. To aid in estrus detection, females in replicate I were outfitted with HeatWatch® estrus detection transponders (HeatWatch®, DDX, Boulder, CO) prior to exposure to bulls and in replicate II, Estrus Alert patches (Estrus Alert, Apple Valley, MN) were used to aid in estrus detection. Visual observations not lasting less than 45 minutes twice daily were used to aid in estrus detection.

In replicate I, females in the P₄ treatment group (RPB cows) (n=14) were administered 15 mg of P₄ (Progesterone, Sigma Chemical, St. Louis, MO) subcutaneously (sc) dissolved in 3 ml of 100% ethanol on days 3, 4 and 5 after the onset of estrus (day 0=onset of estrus) (Figure 4.3). Females in the control group (fertile cows; n=14) were administered 3 ml of 100% ethanol sc on days 3, 4 and 5 after the onset of estrus.

In replicate II, females (RPB cows, n=6 and RPB heifers n=4) in the ALT treatment were administered 15 mg ALT orally on days 3, 4 and 5 after the onset of estrus (day 0=onset of estrus) (Table 4.1). Females in the control group (fertile cows, n=8) were brought to the chute on days 3, 4 and 5 after the onset of estrus and were restrained but received no further treatment (Table 4.1 and Figure 4.4).

Blood samples were obtained via jugular venipuncture on days 3, 4, 5 and 6 after the onset of estrus and before treatment administration. These samples were stored on ice immediately following collection and were subsequently centrifuged at 300 x g for 10 minutes. The serum was then stored in a freezer ({-4°C}) until analysis was to be performed. In replicate I, serum samples were analyzed following extraction with acetone for P₄ levels using a commercial progesterone radioimmunoassay kit (Diagnostic Systems Laboratory, Webster, TX). In replicate II, serum samples were analyzed using a commercial progesterone radioimmunoassay kit (Coat-A-Count Progesterone, DPC, Los Angeles, CA). These commercial progesterone radioimmunoassays are not cross-reactive with altrenogest. Females were examined for pregnancy ~30 days after the onset of gestation via transrectal ultrasonography.
Figure 4.3. The experimental procedure for cows in replicate I of Experiment 4.2.
Table 4.1. Treatments for experimental groups in Experiments 4.1 and 4.2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment group</th>
<th>Description</th>
<th>n</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>ALT$^1$</td>
<td>Heifers</td>
<td>19</td>
<td>15 mg of ALT</td>
</tr>
<tr>
<td>4.1</td>
<td>Control</td>
<td>Heifers</td>
<td>36</td>
<td>No ALT</td>
</tr>
<tr>
<td>4.2</td>
<td>P$_4$</td>
<td>RPB$^2$ Cows</td>
<td>14</td>
<td>15 mg of P$_4$</td>
</tr>
<tr>
<td>4.2</td>
<td>Control</td>
<td>Fertile Cows</td>
<td>14</td>
<td>Vehicle$^3$</td>
</tr>
<tr>
<td>4.2</td>
<td>ALT</td>
<td>RPB Cows</td>
<td>7</td>
<td>15 mg of ALT</td>
</tr>
<tr>
<td>4.2</td>
<td>ALT</td>
<td>RPB Heifers</td>
<td>6</td>
<td>15 mg of ALT</td>
</tr>
<tr>
<td>4.2</td>
<td>Control</td>
<td>Fertile Cows</td>
<td>8</td>
<td>No ALT</td>
</tr>
</tbody>
</table>

$^1$ Altrenogest=ALT.
$^2$ RPB=Repeat breeder.
$^3$ The carrier for progesterone (P$_4$) was a 3 ml dose of ethanol.
Altrenogest (ALT) Treatment Protocol

Onset of Estrus → 15 mg of ALT/day → Pregnancy Check

Days
0 1 2 3 4 5 6 // 30-33

No ALT Control

Onset of Estrus → Pregnancy Check

Control Treatment Protocol

Figure 4.4. The experimental procedure for cows in replicate II of Experiment 4.2.
using an ultrasound machine (Aloka 500-V, Corometrics, Wallingford, CT) equipped with a 5 MHz rectal probe.

**Statistical Analysis**

Pregnancy rates within Experiments 4.1 and 4.2 were analyzed using procedures for categorical data, Chi-square test in SAS (SAS Institute, Inc., Gary, NC). In Experiment 4.2, serum $P_4$ levels were analyzed by repeated measures analysis of variance (ANOVA). Differences in serum $P_4$ levels among pregnant and nonpregnant females were detected using a general linear model (Proc GLM) and Tukey’s Test. The rise in $P_4$ was determined by subtracting the individual serum $P_4$ value on day 3 from the individual animal serum value on day 4. Statistical differences in rise of $P_4$ were determined using the LS means procedure in SAS.

**Results**

**Experiment 4.1**

In Experiment 4.1, there were no effects of either estrous synchronization protocol on pregnancy rates (Table 4.2). In contrast, there were significantly more (P<0.05) pregnant heifers following AI (9 of 19; 44%) in the post-AI ALT treatment group compared with the number of heifers pregnant in the control (no ALT) treatment group (7 of 36; 19%) (Table 4.2).

**Experiment 4.2**

In Experiment 4.2, there were no differences in pregnancy rates between treatment groups. Pregnancy rates for replicate I were: 6 of 14 (43%) in $P_4$-treated RPB cows and 10 of 14 (72%) in control-treated cows (Table 4.2). In replicate II, the pregnancy rates were: 3 of 6 (50%) for the ALT-treated RPB cows, 3 of 4 (75%) in ALT-treated RPB heifers and 4 of 8 (50%) for control-treated cows. However, overall there were no significant differences (P<0.05) between pregnancy rates during the treatment and nontreatment periods among RPB females. The pregnancy rates for RPB cows, regardless of $P_4$ or ALT treatment, was 9 of 20 females (45%) during the treatment period compared with 0 of 20 (0%) during the nontreatment period. The pregnancy rate for RPB heifers was 3 of 4 females (75%) during the treatment period compared with 0 of 4 females (0%) during the nontreatment period.
Table 4.2. Pregnancy rates for females treated with progesterone (P₄) or altrenogest (ALT) on days 3, 4 and 5 of the estrous cycle

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Category</th>
<th>Treatment group</th>
<th>Number pregnant</th>
<th>Percent pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>AI Heifers</td>
<td>ALT</td>
<td>9/19</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.1</td>
<td>AI Heifers</td>
<td>Control</td>
<td>7/36</td>
<td>19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Replicate I</td>
<td>RPB Cows</td>
<td>P₄</td>
<td>6/14</td>
<td>43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.2</td>
<td>RPB Cows&lt;sup&gt;1&lt;/sup&gt;</td>
<td>No P₄</td>
<td>0/14</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.2</td>
<td>Fertile Cows</td>
<td>Control</td>
<td>10/14</td>
<td>72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Replicate II</td>
<td>RPB Cows</td>
<td>ALT</td>
<td>3/6</td>
<td>50</td>
</tr>
<tr>
<td>4.2</td>
<td>RPB Cows&lt;sup&gt;1&lt;/sup&gt;</td>
<td>No ALT</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>4.2</td>
<td>RPB Heifers</td>
<td>ALT</td>
<td>3/4</td>
<td>75</td>
</tr>
<tr>
<td>4.2</td>
<td>RPB Heifers&lt;sup&gt;1&lt;/sup&gt;</td>
<td>No ALT</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>4.2</td>
<td>Fertile Cows</td>
<td>Control</td>
<td>4/8</td>
<td>50</td>
</tr>
<tr>
<td>Pooled</td>
<td>RPB Pooled</td>
<td>P₄ or ALT</td>
<td>12/24</td>
<td>50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pooled</td>
<td>RPB Pooled</td>
<td>No P₄ or ALT</td>
<td>0/24</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pooled</td>
<td>Fertile Cows</td>
<td>Control</td>
<td>14/22</td>
<td>64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Indicates the nontreatment 60-day breeding season that was 5 to 6 months prior to the P₄ treatment (25-day) breeding season and included the same two fertile bulls used during treatment period, for repeat breeders (RPB).

<sup>a,b</sup> Means within a column with different superscripts are significantly different (P<0.05).
The mean(±SE) serum P₄ levels for RPB females exposed to bulls in the P₄ treatment group were: 0.49±0.09 ng/ml (day 3), 1.7±0.34 ng/ml (day 4), 1.86±0.34 ng/ml (day 5) and 2.21±0.21 ng/ml (day 6). The serum P₄ levels for RPB females in the ALT treatment group were: 0.67±0.27 ng/ml (day 3), 0.85±0.20 ng/ml (day 4), 2.04±0.44 ng/ml (day 5) and 2.35±0.69 ng/ml (day 6). Due to mechanical failure of the restraining chute, blood samples were not collected on all RPB heifers. In both replicates there were no significant differences (P>0.05) in mean serum P₄ levels among control females therefore, the data from both replicates were pooled for this group. Overall, the mean serum P₄ levels for fertile females in the control group were 0.45±0.07 ng/ml (day 3), 0.81±0.08 ng/ml (day 4), 1.59±0.18 ng/ml (day 5) and 1.95±0.16 ng/ml (day 6). There were no significant differences (P>0.05) among serum P₄ levels between P₄ or ALT treatment groups.

In Experiment 4.2 replicate I, mean serum P₄ value was significantly lower (P<0.05) on day 3 (0.25±0.01 ng/ml) in RPB females treated with P₄ that were diagnosed as pregnant compared with P₄-treated RPB females on day 3 (0.67±0.01 ng/ml) diagnosed as nonpregnant (Table 4.3 and Figure 4.5). Also, on day 4, the mean serum P₄ value was significantly higher (P<0.05) for P₄-treated RPB females diagnosed as pregnant (2.30±0.7 ng/ml) compared with P₄-treated RPB females diagnosed as nonpregnant (1.22±0.1 ng/ml) (Table 4.3 and Figure 4.5). In addition, there was a significantly (P<0.05) greater increase (rise) of mean serum P₄ levels from day 3 to day 4 in pregnant RPB females (2.07±0.7 ng/ml) compared with nonpregnant RPB females (0.55±0.12 ng/ml). There were no differences in mean serum P₄ levels on day 5, (1.67±0.15 ng/ml compared with 2.01±0.60 ng/ml) and day 6 (2.22±0.17 ng/ml compared with 2.20±0.34 ng/ml) between pregnant and nonpregnant P₄-treated RPB females, respectively (Table 4.3 and Figure 4.5).

In Experiment 4.2 replicate II, mean serum P₄ levels were not significantly different (P>0.05) for pregnancy status (pregnant vs. nonpregnant) in ALT-treated RPB females on day 3 (0.81±0.52 ng/ml compared with 0.54±0.30 ng/ml), day 4 (0.87±0.35 ng/ml compared with 0.85±0.27 ng/ml), day 5 (1.96±0.23 ng/ml compared with 2.12±0.95 ng/ml) or day 6 (1.92±1.08 ng/ml compared with 2.78±1.02 ng/ml).
Figure 4.5. Serum progesterone (P₄) levels in pregnant (preg) and nonpregnant (nonpreg) repeat breeder (RPB) cows administered 15 mg of P₄ on days 3, 4 and 5 after the onset of estrus.
Table 4.3. Serum progesterone (P_{4}) levels in progestin-treated (altrenogest, ALT) and nontreated females on days 3, 4, 5 and 6 after onset of estrus in Experiment 4.2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Pregnancy status</th>
<th>n</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{4} pregnant</td>
<td>6</td>
<td>0.25±0.0\textsuperscript{a}</td>
<td>2.30±0.7\textsuperscript{a}</td>
<td>1.67±0.1</td>
<td>2.21±0.1</td>
<td></td>
</tr>
<tr>
<td>P_{4} nonpregnant</td>
<td>8</td>
<td>0.67±0.1\textsuperscript{b}</td>
<td>1.22±0.1\textsuperscript{b}</td>
<td>2.01±0.6</td>
<td>2.20±0.3</td>
<td></td>
</tr>
<tr>
<td>ALT pregnant</td>
<td>3</td>
<td>0.81±0.5</td>
<td>0.87±0.3</td>
<td>1.96±0.2</td>
<td>1.93±1.1</td>
<td></td>
</tr>
<tr>
<td>ALT nonpregnant</td>
<td>3</td>
<td>0.54±0.3</td>
<td>0.85±0.3</td>
<td>2.16±0.9</td>
<td>2.78±1.0</td>
<td></td>
</tr>
<tr>
<td>Control pregnant</td>
<td>12\textsuperscript{2}</td>
<td>0.44±0.1</td>
<td>0.89±0.1</td>
<td>1.87±0.2\textsuperscript{c}</td>
<td>1.98±0.2</td>
<td></td>
</tr>
<tr>
<td>Control nonpregnant</td>
<td>6</td>
<td>0.43±0.1</td>
<td>0.61±0.2</td>
<td>0.89±0.2\textsuperscript{d}</td>
<td>1.89±0.3</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Number of days after the onset of estrus.
\textsuperscript{2} In this group, blood samples were not collected on one female.
\textsuperscript{a,b} Means within a treatment and column that have a different superscripts are significantly different (P<0.05).
\textsuperscript{c,d} Means within a treatment and column that have a different superscripts are significantly different (P<0.06).
respectively (Table 4.3). There were no differences in the rise in mean serum P₄ levels from day 3 to day 4 between pregnant (0.05±0.19 ng/ml) and nonpregnant (0.51±0.09) RPB females. In the control group, there was no significant difference (P>0.05) between pregnant and nonpregnant females for mean serum P₄ levels on day 3 (0.44±0.08 ng/ml compared with 0.43±0.14 ng/ml) or day 4 (0.89±0.09 ng/ml compared with 0.61±0.19 ng/ml), respectively (Table 4.3 and Figure 4.6). However, the rise in mean serum P₄ levels from day 3 to day 4 was significantly greater (P<0.05) in the pregnant females (0.44±0.06 ng/ml) compared with nonpregnant females (0.20±0.09 ng/ml). There was a statistical difference (P<0.05) in the mean(±SE) serum P₄ levels between pregnant and nonpregnant control females on day 5 (1.87±0.22 ng/ml compared with 0.89±0.16 ng/ml), respectively but not different on day 6 (1.98±0.17 ng/ml compared with 1.89±0.39 ng/ml), respectively (Table 4.3 and Figure 4.6).

**Discussion**

The overall pregnancy rate (28%) for heifers in Experiment 4.1 was lower than expected. The likely cause of this low overall pregnancy rate was that many of the heifers were probably inseminated during their first or second estrous period after the onset of puberty. It has been previously reported that pregnancy rates were lower when heifers were mated during their first or second estrous cycle compared with their third estrous cycle after the onset of puberty (Byerley et al., 1987). However, treatment with a low dose of ALT on days 3 to 5 following the onset of estrus did significantly increase pregnancy rates (44% vs. 19%). This is believed to be the first report investigating the effects of altrenogest treatment from day 3 to day 5 following the onset of estrus and AI, on subsequent pregnancy rates.

There have been reports of various attempts at administration of P₄, progestins or luteotrophic substances during the early to mid-luteal phase to increase pregnancy rates but these reports are conflicting and the treatment protocols vary greatly across laboratories (MacMillan et al., 1991; Van Cleeff et al., 1991; Larson et al., 1995; Smith et al., 1996; Mann et al., 1998). Usually, P₄ or luteotrophic substances, such as hCG or GnRH are administered at ~day 7 of the estrous cycle possibly due to the fact that the majority of embryo transfers occur at day 7 of the estrous cycle. None of these research groups have evaluated the effects of a progestin treatment from day 3 to day 5 following estrus.
Figure 4.6. Serum progesterone (P₄) levels in pregnant (preg) and nonpregnant (nonpreg) “fertile” control (cont) cows on days 3, 4 and 5 after the onset of estrus.
In Experiment 4.2, we can not compare the serum $P_4$ levels between RPB females and “fertile” control females because the RPB females in replicate I received $P_4$ that was >99% cross-reactive with the $P_4$ assay used in this study. The rise in serum $P_4$ from day 3 to day 4 was greater in pregnant females compared with nonpregnant females in the control group. We propose that this increase in $P_4$ levels is beneficial to in vivo embryo development. Shelton et al. (1990) reported a delayed increase of $P_4$ levels following ovulation in subfertile cows compared with the levels in fertile heifers. Among $P_4$-treated RPB cows there was a significantly greater rise of $P_4$ levels in the pregnant females compared with the levels in nonpregnant females. Since the administered $P_4$ was highly cross reactive (>99%) with the $P_4$ assay, an artificially induced rise in $P_4$ from day 3 to day 4 after the onset of estrus was noted. Those RPB females with a marked increase in serum $P_4$, following $P_4$ administration often were those females diagnosed as pregnant 30 days after the onset of estrus. Although the mechanism of this response is unknown it is possible that the nonpregnant RPB females metabolized the administered $P_4$ more quickly than pregnant RPB females.

In replicate II, the RPB females were treated with ALT and because ALT does not cross react with the $P_4$ assay, the endogenous serum $P_4$ levels could be monitored. The rise in serum $P_4$ (day 3 to day 4) was not different between pregnant and nonpregnant females. This was possibly due to a low number of observations in this group. However, these results must be interpreted with caution because the altrenogest administered was not cross reactive with the $P_4$ assay, although it appears to be biologically active. Interestingly, the rise in $P_4$ (day 3 to day 4) from the nonpregnant ALT-treated RPB females was greater than the rise in pregnant control females. It should be noted, however, that the day 3 serum $P_4$ level, in these ALT-treated RPB females were higher than in the pregnant control females. This has been reported to be characteristic of subfertile dairy cows (Erb et al., 1976; Wilmut et al., 1980).

Erb et al. (1976) and Wilmut et al. (1980) have reported that females tended to have higher $P_4$ levels immediately following ovulation during an infertile mating than during a fertile mating. Also, above normal physiological $P_4$ levels during ovulation have been suggested as a cause of delayed ovulation, and result in decreased fertilization and pregnancy rates (Hill et al., 1971; Erb et al., 1976; Ireland and Roche, 1982). As a result, even though the rise in $P_4$ in nonpregnant ALT-treated RPB females was
numerically greater than the rise in the pregnant control females, the premature elevated
\( P_4 \) levels could have disrupted fertilization preventing the formation of a viable embryo.

The mean serum \( P_4 \) levels on day 5 after the onset of estrus were different in the
pregnant and nonpregnant control females. This suggests that the natural increase in \( P_4 \)
may normally occur between day 4 and 5 in fertile females although it is interesting that
pregnancy rates were markedly increased when \( P_4 \) or ALT were administrated on day 3
in RPB females. Furthermore, in the \( P_4 \)-treated RPB there was a significant difference in
serum \( P_4 \) on day 3 and 4 in the pregnant and nonpregnant females. Historically,
differences in serum \( P_4 \) levels have not been reported between pregnant and
nonpregnant females prior to day 10 after mating (Shemesh et al., 1968; Henricks et al.,
1970; Bulman and Lamming, 1978; Lukaszewska and Hansel, 1980). However, in the
present study blood sampling was conducted at 24-hour intervals, as well as the use of
defined fertile (control group) and nonfertile females (RPB group), which could have
allowed for the detection of these differences in \( P_4 \) levels.

The pregnancy rate for control females is in agreement with the reported
literature for fertile females from a single mating (Ayalon, 1978). However, treatment of
RPB females with \( P_4 \) or ALT significantly improved pregnancy rates comparable with
those of fertile control females. There have been previous attempts to improve
pregnancy rates in RPB cattle (dairy cows, dairy heifers and beef cows) using a variety
of regimes that utilized GnRH, GnRH analogues, hCG, \( P_4 \) and progestins. Lee et al.
(1983) reported that GnRH administration at time of insemination significantly increased
pregnancy rates in RPB dairy cows. However, Phatak et al. (1986) failed to find a
significant increase in pregnancy rates among RPB dairy cows using GnRH at time of
AI. It has been found that RPB females characteristically exhibit a longer duration of time
from the pre-ovulatory LH peak until ovulation (Erb et al., 1976; Gustafsson et al., 1986).
Therefore, the inconsistency of the GnRH treatment at time of insemination probably
results from the given number of RPB females that experience delayed ovulation within
a given herd. This same inconsistency of GnRH treatment at the time of AI has also
been reported in normal beef and dairy cattle (Macmillan et al., 1986; Stewart et al.,
1987; Ryan et al., 1991; Mee et al., 1993; Drew and Peters, 1994).

Attempts to increase pregnancy rates in RPB females by stimulating endogenous
\( P_4 \) production with luteotrophins during diestrus has failed to significantly increase
pregnancy rates; hCG administration on days 4 to 19 (Holness et al., 1982), hCG administration on day 5.5 (Walton et al., 1990) and GnRH administration on day 11 (Sheldon and Dobson, 1993). Additionally, these findings are confounded by the reports that GnRH or hCG can significantly increase P₄ levels when administered during this phase of the estrous cycle in cattle (Rajamahendran and Sianangama, 1992; Schmitt et al., 1995).

The administration of P₄ or progestins after day 6 post-mating has failed to increase pregnancy rates in fertile beef and dairy cattle (Munro and Bertram, 1990; Van Cleef et al., 1991; Stevenson and Mee, 1991; Mann et al., 1998). However, when P₄ or progestins are administered prior to day 6 after breeding there was an increase in pregnancy rates in normal beef and dairy cows (Johnson et al., 1958; Robinson et al., 1989; Macmillan et al., 1991; Larson et al., 1995). The increased success of P₄ supplementation prior to day 6 after breeding compared with luteotrophin administration prior to day 6, may be the result of differences in luteal tissue development between fertile and nonfertile females. Albihn (1991) reported that RPB heifers had significantly smaller luteal tissue volumes compared with those of fertile heifers. This difference may prevent administered luteotrophins from increasing endogenous P₄ to beneficial levels or may delay the increase of P₄ to an inappropriate time. In addition, the luteal tissue from RPB cows has been reported to be less responsive in vitro to luteotrophins compared with luteal tissue of fertile females (Shelton et al., 1990). These observations suggest that direct supplementation of P₄ has been more effective in RPB cows than administration of luteotrophins.

The mechanism of early P₄ supplementation on improved embryo developmental rates and increased pregnancy rates is still conflicting and unclear. In embryo transfer (ET) recipients, there have been reported increased pregnancy rates with P₄ supplementation beginning on ~day 7 (Salgado and Donaldson, 1984; Broadbent et al., 1992; Nishigai et al., 2002), although, Moore, (1985) and Smith et al. (1996) reported findings to the contrary. However, administration of hCG at the time of embryo transfer has been reported to consistently fail to increase pregnancy rates in embryo transfer recipients (Looney et al., 1984; Ellington et al., 1991). The failure of P₄ supplementation beginning at day 6 of the estrous cycle or thereafter to increase pregnancy rates in ET
recipients is in agreement with results of \( P_4 \) supplementation in naturally mated or AI females from other studies.

In sheep, early \( P_4 \) administration (days 1 to 6 after mating) has significantly increased fetal mass and crown rump length at 72 days of gestation (Kleemann et al. 1994). In addition, it has been reported that exposure of ovine embryos to advanced stage uteri resulted in increased pregnancy rates (Wilumt and Sales, 1981). In cattle, Garrett et al. (1987) reported that early \( P_4 \) supplementation on days 1.5 to 4 after the onset of estrus to naturally mated females resulted in a significant increase in developmental rates of embryos recovered on day 7 or day 14 compared with control females. Moreover, early \( P_4 \) supplementation on days 1.5 to 4 after the onset of estrus, allowed for the continued development of day-7 to day-8 blastocysts transferred to day-5 recipient females (Geisert et al. 1991a). EL-Banna and Hafez (1970) and Crisman et al. (1980) have reported that early \( P_4 \) administration stimulated the rate of migration of bovine embryos from the oviduct to the uterus. This corresponds with reports that early \( P_4 \) supplementation stimulated uterine secretions and function in supporting embryonic development (Garrett et al., 1988; Geisert et al., 1992; Barnes, 2000). Collectively, it is likely that early \( P_4 \) supplementation hastens the transport of the embryo into the stimulated uterus providing a more hospitable environment for the developing embryo.

In conclusion, early supplementation of \( P_4 \) or ALT to RPB females resulted in increased pregnancy rates after AI and bull exposure. The increased pregnancy rates are likely the result of an induced rise in \( P_4 \) between days 3 to 5 after the onset of estrus. These data suggest that supplementation of low doses of \( P_4 \) should be considered as a practical and effective treatment for subfertile cattle.
CHAPTER 5
EFFECT OF PROGESTERONE ON BOVINE EMBRYO DEVELOPMENT IN VITRO

Introduction

Progesterone (P₄) has been shown to be the principal hormone of pregnancy in the cow (McDonald et al., 1952). Although, the necessity of P₄ during early pregnancy has been accepted, there have been various studies that attempted to determine the effects or mechanism of action that P₄ exerts during this interval. A few of these studies have focused on the effects P₄ exerts on pregnancy through the uterus (Garret et al., 1988).

There has been much controversy over the efficiency of P₄ supplementation to improve pregnancy rates when administered to mated females (for review see Sreenan and Diskin, 1983). A few studies have indicated that used P₄ supplementation during early pregnancy (prior to day 7) has demonstrated positive effects on pregnancy rates or embryo developmental rates (Garret et al., 1988; Geisert et al., 1991a). In the cow, P₄ levels begin to increase from day 3 following the onset of estrus (Henricks et al., 1970; Henricks et al., 1971; Kindahl et al., 1976) at which time, the embryo is about to enter the uterus (EL-Banna and Hafez, 1970). However, when P₄ is administered to the pregnant cow prior to this time, there has been reported a hastened rate of transport of the embryo from the oviduct into the uterus (Crisman et al., 1980). This increase in transport following P₄ administration occurs in other species, such as the mouse (Roblero and Garavagno, 1979), the rat (Forcelledo et al., 1982), the sheep (Moore et al., 1983) and the pig (Day and Polge, 1968). In addition, if P₄ is administered prior to fertilization the number of cleavage stage embryos is then decreased in the hamster (Hunter, 1972), the rabbit (Chang, 1967; Chang, 1969; Nutting and Mares, 1970), the sheep (Johnsson et al., 1974), the pig (Day and Polge, 1968) and the cow (Hill et al., 1971; Imwalle et al., 2002).

After the embryo migrates from the oviduct into the uterus, P₄ prevents uterine myometrial contractions (Rhodes and Nathanielsz, 1990), which allows for a ‘quiet’ environment for embryo development to occur. Concomitantly, P₄ stimulates the production and release of many uterine proteins and growth factors (Dixon and Gibbons,
1979; Garrett et al., 1988; Geisert et al., 1988; Rider et al., 1998; Barnes, 2000) as well as immunosuppressive proteins (Fisher et al., 1985).

It has also been demonstrated that low P₄ levels during early pregnancy can increase the intensity of the luteolytic signal (and increased prostaglandin F₂α metabolite) on days 12 to 16 of the estrous cycle (Mann and Lamming, 1995). Conversely, it was later demonstrated that high levels of P₄ during early pregnancy could decrease the intensity of the luteolytic signal by decreasing PGF₂α levels though inhibitory effects on oxytocin receptors (Lamming and Mann, 1995a) and alter the timing of the luteolytic signal (Mann et al., 1998). Elevated P₄ levels during early pregnancy also resulted in increased embryo production of interferon tau, increased uterine PGE₂ levels and decreased uterine PGF₂α levels as well as a need for less interferon tau to prevent luteolysis from occurring (Niswender et al., 1994; Mann and Lamming, 2001). It is unknown by which mechanism P₄ stimulates increased interferon tau production from the conceptus and a direct effect on developing embryos has not been established.

Although the necessity of P₄ during early pregnancy is accepted and there have been several studies relating to P₄ supplementation in pregnant females, there have been few attempts to determine the effects of P₄ supplementation on embryos in an in vitro culture (IVC) system (Whitten, 1957, Kirkpatrick, 1971, Roblero and Izquierdo, 1976, Wiemer et al., 1987). Early studies have suggested that P₄ supplemented media had an inhibitory effect on early stage mouse embryos (Whitten, 1957; Kirkpatrick, 1971) and rabbit (Daniel, 1964) embryos. Other studies have indicated that elevated levels of P₄ prior to or at time of fertilization have detrimental effects in vivo (Day and Polge, 1968; Chang, 1969; Fraser and Maudlin, 1979; Hill et al., 1971).

Fukui et al. (1982) demonstrated that P₄ supplementation to in vitro oocyte maturation culture (IVM) systems decreased the rate of bovine oocyte maturation and that the addition of P₄ to fertilization culture medium did not improve the number of cleavage stage embryos (Fukushima and Fukui, 1985). Similarly elevated P₄ concentrations at the time of fertilization are generally a characteristic of infertile females or repeat breeder females (Erb et al., 1976, Gustaffason et al., 1986). Thus, addition of P₄ during fertilization or immediately thereafter in vitro would be at a time that P₄ is not readily available to the ovum or embryo and may be a major problem with some approaches to hormone supplementation of embryo culture systems.
IVC with P₄ supplementation in conjunction with oviduct or uterine cell co-culture systems has not increased the number of bovine embryos developing to the blastocyst stage (Wiemer et al., 1987; Fukui, 1989; Goff and Smith, 1998, Reggio et al., 1998), although it was shown to increase blastocyst yield in vitro in the mouse (Lavaranos and Seamark, 1989).

The main drawbacks of the studies to determine the effect of P₄ on embryo development have apparently been addition of P₄ at an inappropriate time or the use of co-culture systems that prevent the detection of direct effects because of supplementary cell interactions. Therefore, the objective of these experiments were to determine if P₄ exerts a direct effect on the developing IVC bovine embryo when supplemented at appropriate stages of embryo development, in the absence of a monolayer co-culture system.

**Materials and Methods**

**Media Preparation**

Oocyte maturation medium was prepared and equilibrated at 39˚C and 5% CO₂ at a commercial company processing center (BOMED, Inc., Madison, WI or OvaGenix LP., San Angelo, TX). Other media was prepared at the Center for Reproductive Biology at Louisiana State University, St. Gabriel, LA and equilibrated at 39˚C and 5% CO₂. For the fertilization medium, Brackett-Oliphant (BO) stock medium was prepared as described in Appendix A and B and for embryo culture, CR1aa stock medium was prepared as described in Appendix C (Rosenkrans and First, 1994).

The oocyte washing and fertilization media were prepared by the addition of 38 ml of BO-A stock solution to 12 ml of BO-B stock solution at which time 0.685 mg of pyruvic acid (P-4562, Sigma Chemcial Co., St. Louis, MO), 50 µl of Gentamicin (15750-060, Gibco, Grand Island, NY) and 25 µl of 10,000 IU/ml heparin sodium were added. BO-caffeine (semen washing and fertilization medium) was prepared by the addition of 24.3 mg of caffeine sodium (C-4144, Sigma Chemical Co., St. Louis, MO) to 25 ml of BO-AB solution. BO-BSA 0.3% medium (oocyte washing) was prepared by the addition of 30 mg of bovine serum albumin (BSA; A-7511, Sigma Chemical Co., St. Louis, MO) to 10 ml of BO-AB solution. The BO-BSA 0.6% medium was prepared by the addition of 30 mg of BSA to 5 ml of the BO-AB solution.
**In Vitro Fertilization**

For insemination, semen from a fertile Holstein bull (CSS 7H5918, Genex Cooperative Inc., Shawano, WI) was thawed in a water bath at 40°C for 15 to 45 seconds and then was placed in a 15 ml conical tube (Corning 15-ml conical tubes; Myriad Industries; San Diego, CA) with 9 ml of BO-caffeine solution and centrifuged at 200 x g for 6 minutes. After completion of the 6 minute cycle, the supernatant was removed while leaving the semen pellet and 9 ml of BO-caffeine solution was added to the semen pellet. This solution was then centrifuged again at 200 x g for 6 minutes. The supernatant was removed again and the semen was resuspended in 4 ml of BO-caffeine solution and 4 ml of BO-BSA 0.6% medium. Then 100-µl droplets of this solution were made in 35 mm Falcon™ plastic petri dishes (Becton and Dickinson, Lincoln Park, NJ) under mineral oil (Sigma M-8410, Sigma Chemical Co., St. Louis, MO) and then placed in an humidified incubator at 39°C and 5% CO₂.

Following a 24-hour oocyte maturation period, oocytes were removed from the maturation medium and washed through two 35 mm Falcon™ plastic petri dishes each containing 3.5 ml of BO-BSA 0.3% medium. Once the oocyte washing was completed, 15 to 20 oocytes were placed per 100-µl fertilization droplet and all dishes were returned to the incubator for 5 to 6 hours.

**In Vitro Culture**

After a 5 to 6 hour insemination period, oocytes were removed from the insemination droplets and washed through two 35 mm Falcon™ plastic petri dishes each containing 3.5 ml of 10 ml TCM-199 (Gibco Laboratories, Grand Island, NY) and 30 mg of fatty acid-free bovine serum albumin (FAF-BSA) (Fatty Acid Free-BSA, A-4503, Sigma Chemical Co., St. Louis, MO) solution. Once oocytes were washed, cumulus cells were removed by vortexing (Vortex-genie mixer, Scientific Products, McGraw Park, IL) the inseminated oocytes for 3 minutes in 1 ml of TCM-199 and 1 mg of hyaluronidase (H-3506, Sigma Chemical Co., St. Louis, MO).

The inseminated oocytes were then washed through two dishes of 3.5 ml of TCM-199 and 30 mg of fatty acid-free bovine serum albumin FAF-BSA. Following the washing steps, inseminated ova were then placed in 50-µl droplets of modified CR1aa [9.5 ml CR1aa medium (Appendix C), 200 µl of BME amino acid solution (B-6766, Sigma Chemical Co., St. Louis, MO), 100 µl of MEM amino acid solution (11140-050, Gibco]
Laboratories, Grand Island, NY), 10 µl of Gentamicin (Gibco Laboratories, Grand Island, NY), 1.46 mg of L-glutamine (G-5763, Sigma Chemical Co., St. Louis, MO) and 30 mg FAF-BSA]. The dishes were then placed in an air-tight modular incubator (Billups-Rothenberg®, Del Mar, CA) that was filled with 5% CO\textsubscript{2}, 5% O\textsubscript{2} and 90% N\textsubscript{2} gas for 4 minutes at 2 psi and then placed in a humidified 5% CO\textsubscript{2} incubator at 39°C.

At 72 hours post-insemination, sperm exposed ova were removed from embryo culture medium and placed in fresh CR1aa embryo culture medium that was modified by the supplementation of 500 µl of 10% heat-inactivated fetal bovine serum (FBS, SH30070.02, Hi Clone Laboratories, Logan, UT).

**Experimental Design**

In Experiment 5.1, modified CR1aa supplemented with ~15 ng/ml of P\textsubscript{4} (Progesterone, Sigma Chemical Co., St. Louis, MO) dissolved in 100% ethanol, was the designated P\textsubscript{4}-treatment group for embryo culture (Treatment A), and the standard modified CR1aa medium served as the control treatment (Control) for embryo culture (Treatment B). At 72 hours post-insemination, morphologically good quality 8-cell embryos were randomly allotted to Treatments A and B. The cumulus cells were removed from all inseminated ova, allowing an embryo culture environment that precluded somatic cell involvement. This experimental design allowed the detection of direct effects of treatments on embryo developmental and development rates.

In Experiment 5.2, Treatments A and B in Experiment 5.1 were continued; however, a carrier-vehicle treatment (Vehicle) of modified CR1aa supplemented with 10 µl of 100% ethanol was added to the experimental design. In this experiment, 173 IVF-derived embryos were allotted to the P\textsubscript{4} treatment group (Treatment A), 170 embryos were allotted to the vehicle treatment group (Treatment B) and 168 embryos were allotted to the control treatment group (Treatment C).

In Experiments 5.1 and 5.2 embryos were placed in treatment medium and returned to the airtight modular incubator inoculated with 5% CO\textsubscript{2}, 5% O\textsubscript{2} and 90% N\textsubscript{2} gas for 4 minutes at 2 psi and then placed in a humidified 5% CO\textsubscript{2} incubator at 39°C. Medium was changed at 7 days post-insemination and embryo culture was discontinued at 9 days post-insemination.
Embryo Evaluation

All embryo evaluations were conducted by using an inverted microscope (Nikon Diaphot, Tokyo, Japan). At 72 hours post-insemination, embryos were evaluated to determine those that had reached the 8-cell or greater stages, at which time the embryos at less than the 8-cell developmental stage were discarded. Additional embryo evaluations were completed at 6, 7, 8 and 9 days post-insemination and embryos at the morula, early blasocyst (EBLST), blastocyst (BLST), expanded blastocyst (EXBLST), hatching blastocyst (HTBLST) or hatched blastocyst (HBLST) stages were recorded for each of these observational days. On day 7, blastocysts were randomly selected from each treatment to measure embryo diameter. Embryo diameter measurements were collected by use of an inverted microscope equipped with a video camera connected to an S-VHS VCR and a color monitor and used to evaluate the embryos using a micrometer slide. The embryos were placed on the inverted scope and their projected image was measured on the television screen. In addition to these measures embryos were assigned a red score based on the stage of embryo development and morphological quality as developed by Ryan et al. (1992).

Statistical Analysis

In Experiments 5.1 and 5.2, statistical differences between the mean number of embryos that reached the morula stage on day-6 post-insemination, the blastocyst stage on day-7 post-insemination and the hatched blastocyst stage on day-9 post-insemination were determined by using procedures for categorical data, with Chi square analysis. Statistical differences between mean embryo diameters on day-7 post-insemination were determined by Chi square analysis. The effects of treatments on embryo developmental rates (mean number of embryos at each developmental stage on various days post-insemination) were determined by the analysis of variance (ANOVA) procedure of the general linear model procedure and statistical differences were determined by the least squares method. All statistical procedures were performed using the SAS statistical package (SAS Institute, Inc., Gary, NC).
Results

Experiment 5.1

In Experiment 5.1, a total of 280 bovine oocytes (in 3 replicates) were inseminated and 221 (79%) developed to the 2-cell stage and 206 (74%) reached the 8-cell stage or greater by 3 days post-insemination. On day-6 post-insemination, there tended to be more (P<0.07) 8-cell embryos developing to the morula stage in the P_4 treatment group (70 of 104; 67%) compared with the control group (52 of 102; 51%) (Table 5.1). On day-7 post-insemination, there were significantly more (P<0.05) blastocysts in the P_4-treated group (61 of 104; 59%) compared with the control treatment group (41 of 102; 40%). On day-9 post-insemination, the P_4 treatment group produced significantly more (P<0.05) hatching and hatched blastocyst (41 of 104; 39%) compared with the control treatment group (20 of 102; 20%).

The rate of in vitro embryo development was accelerated by treatment with P_4 in this experiment. On day-6 post-insemination there were significantly more (P<0.05) embryos at the early blastocyst stage (42 of 98; 40%) and blastocyst stage (4 of 98; 1%) in the P_4 treatment group compared with the control group with (18 of 98; 18%) of developed embryos at the early blastocyst stage and (0 of 98; 0%) at the blastocyst stage, respectively (Table 5.2). On day-8 post-insemination there was a significantly greater (P<0.05) proportion of all developed embryos at the hatched blastocyst stage (29 of 98; 28%) in the P_4 treatment group compared with (10 of 98; 11%) hatched blastocysts in the control treatment group (Table 5.3).

From the blastocysts randomly selected from the P_4 (n=23) and control (n=9) treatment groups on day-7 post-insemination, the P_4-treated embryos had a significantly larger mean±SE diameter (P<0.05) (183±2.1 µm) than the control treatment embryos (172±4.3 µm) (Table 5.4). More embryos were measured in the P_4 treatment group than the control treatment group because more blastocysts were present in the P_4 treatment groups at the time of the measurements.

Experiment 5.2

In Experiment 5.2, a total of 881 bovine oocytes (in 5 replicates) were inseminated with 749 (85%) developing to the 2-cell stage and 511 of these oocytes (58%) developing to the 8-cell or greater stage embryos by day-3 post-insemination. On day-6 post-insemination, one replicate was not evaluated for development and thus, the
Table 5.1. Number of P₄-treated and control 8-cell bovine embryos in Experiment 5.1 that reached the morula, blastocyst and hatched blastocyst developmental stages in respective treatments

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. morulae¹ (%)</th>
<th>No. blastocysts² (%)</th>
<th>No. hatched blastocysts³,⁴ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄⁵</td>
<td>70/104 (67)a</td>
<td>61/104 (59)b</td>
<td>41/104 (39)c</td>
</tr>
<tr>
<td>Control</td>
<td>52/102 (51)b</td>
<td>41/102 (40)b</td>
<td>20/102 (20)d</td>
</tr>
</tbody>
</table>

¹ Morulae were evaluated at 6 days post-insemination.
² Blastocysts were evaluated at 7 days post-insemination.
³ Hatched blastocysts were evaluated at 9 days post-insemination.
⁴ Hatched blastocysts represents embryos developing to the hatching or hatched blastocyst stage.
⁵ P₄ = progesterone.

Within a column, numbers with different superscripts are significantly different (P<0.10).
Within a column, numbers with different superscripts are significantly different (P<0.05).
Table 5.2. Number of P₄-treated and control 8-cell bovine embryos in Experiment 5.1 cultured in respective treatments that reached morula, early blastocyst, blastocyst or expanded blastocyst stages on day-6 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. morulae (%)</th>
<th>No. early blastocysts (%)</th>
<th>No. blastocysts (%)</th>
<th>No. expanded blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄ⁱ</td>
<td>23/98 (22)</td>
<td>42/98 (40)ᵃ</td>
<td>4/98 (1)</td>
<td>0/98 (0)</td>
</tr>
<tr>
<td>Control</td>
<td>34/98 (33)</td>
<td>18/98 (18)ᵇ</td>
<td>0/98 (0)</td>
<td>0/98 (0)</td>
</tr>
</tbody>
</table>

ᵃᵇ Within a column, numbers with differing superscripts are significantly different (P<0.05).

ⁱ P₄ = progesterone.
Table 5.3. Number of P₄-treated and control 8-cell bovine embryos in Experiment 5.1 cultured in respective treatments that reached early blastocyst, blastocyst, expanded blastocyst, or hatched blastocyst stages on day-8 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. early blastocysts (%)</th>
<th>No. blastocysts (%)</th>
<th>No. expanded blastocysts (%)</th>
<th>No. hatched (^1) blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄ (^2)</td>
<td>2/98 (2)</td>
<td>10/98 (10)</td>
<td>27/98 (26)</td>
<td>29/98 (28) (^a)</td>
</tr>
<tr>
<td>Control</td>
<td>6/98 (6)</td>
<td>14/98 (15)</td>
<td>20/98 (21)</td>
<td>10/98 (11) (^b)</td>
</tr>
</tbody>
</table>

\(^1\) Hatched blastocysts represents embryos developing to the hatching or hatched blastocyst stages.
\(^2\) P₄=progesterone.
\(^a,b\) Within a column, numbers with different superscripts are significantly different (P<0.05).
Table 5.4. Mean diameter of blastocyst stage bovine embryos in Experiment 5.1 cultured in respective treatments on day-7 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. blastocysts</th>
<th>Mean (±SE) embryo diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_4^1</td>
<td>23</td>
<td>183±2.1^a</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>172±4.3^b</td>
</tr>
</tbody>
</table>

^1 P_4=progesterone.  
^a,b Within a column, means with different superscripts are significantly different (P<0.05).
number of morula is based only on the number of embryos evaluated at this time which was 98 embryos for per treatment group.

As noted in Experiment 5.1, the rate of embryo development in vitro was advanced by P4 treatment. Among the treatment groups, significantly more (P<0.05) P4 treatment embryos (8 of 98; 8%) developed to the blastocyst stage by day-6 post-insemination compared with vehicle treatment embryos (3 of 98; 3%) and the control group (0 of 98; 0%) (Table 5.5). There was a trend (P<0.10) for more 8-cell bovine embryos developing to the morulae stage in the P4 treatment group (73 of 98; 75%) and the control group (70 of 98; 71%) compared with the vehicle group (57 of 98; 58%) (Table 5.6). Also, on day-7 post-insemination, there was a trend for more (P<0.10) blastocysts in the P4-treated group (123 of 173; 69%) compared with the control group (104 of 168; 62%). In addition, there were significantly more (P<0.05) blastocysts in the P4 and control groups compared with the vehicle group (90 of 170; 53%) (Table 5.5). On day 9 post-insemination, the P4 treatment group produced significantly more (P<0.05) hatching and hatched blastocyst (57 of 173; 33%) compared with the vehicle group (37 of 170; 22%) and the control group (36 of 168; 21%) (Table 5.5).

On day 7 post-insemination, there were significantly more (P<0.05) Grade-1 blastocysts in the P4 treatment group (43 of 173; 25%) compared with the vehicle group (26 of 170; 15%) and the control group (28 of 168; 17%) (Table 5.6). In contrast, there were no statistical differences (P>0.05) in the number of Grade-2 blastocysts on day 7 post-insemination between any of the treatment groups.

From the blastocysts randomly selected from the P4 treatment (n=34), vehicle treatment (n=28) and control treatment (n=30) groups on day 7 post-insemination, the P4-treated embryos had a significantly larger mean(±SE) diameter (187±4.1 µm) than the vehicle-treated embryos (174±3.3 µm) and the control embryos (176±3.4 µm) (Table 5.7). In addition, on day-8, post-insemination, there was a significantly greater proportion (P<0.05) of all developed 8-cell embryos at the hatched blastocyst stage in the P4 treatment group (36 of 173; 21%) compared with the vehicle treatment group (21 of 170; 12%) and the control treatment group (14 of 168; 8%) (Table 5.8). Over the course of the 4-day evaluations there was a significant increase in the mean embryo red score for embryos cultured in P4 compared with vehicle-treated embryos or control embryos for day 6 and day 9 (Table 5.9). The mean embryo red score was significantly lower for
Table 5.5. Number of P₄-treated and control 8-cell bovine embryos in Experiment 5.2 that reached the morula, blastocyst and hatched blastocyst developmental stages in respective treatments.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. morulae¹ (%)</th>
<th>No. blastocysts² (%)</th>
<th>No. hatched blastocysts³,⁴ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄⁵</td>
<td>73/98 (75)a</td>
<td>123/173 (71)a</td>
<td>57/173 (33)d</td>
</tr>
<tr>
<td>Vehicle⁶</td>
<td>57/98 (58)b</td>
<td>90/170 (53)b</td>
<td>37/170 (22)e</td>
</tr>
<tr>
<td>Control</td>
<td>70/98 (71)a</td>
<td>104/168 (62)c</td>
<td>36/168 (21)e</td>
</tr>
</tbody>
</table>

¹ Morulae were evaluated at 6 days post-insemination.
² Blastocysts were evaluated at 7 days post-insemination.
³ Hatched blastocysts were evaluated at 9 days post-insemination.
⁴ Hatched blastocysts represents embryos developing to the hatching or hatched blastocyst stage.
⁵ P₄ = progesterone.
⁶ Vehicle = ethanol.

a,b,c Within a column, numbers with differing superscripts are significantly different (P<0.10).
d,e Within a column, numbers with differing superscripts are significantly different (P<0.05).
Table 5.6. Number of grade 1 or 2 bovine blastocyst staged embryos in Experiment 5.2 on day-7 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. grade-1 blastocysts (%)</th>
<th>No. grade-2 blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄¹</td>
<td>43/173 (25)a</td>
<td>53/173 (31)a</td>
</tr>
<tr>
<td>Vehicle²</td>
<td>26/170 (15)b</td>
<td>43/170 (25)a</td>
</tr>
<tr>
<td>Control</td>
<td>28/168 (17)b</td>
<td>53/168 (32)a</td>
</tr>
</tbody>
</table>

¹ P₄=progesterone.
² Vehicle=ethanol.

a,b Within a column, numbers with different superscripts are significantly different (P<0.05).
Table 5.7. Mean diameter of blastocyst staged bovine embryos in Experiment 5.2 cultured in respective treatments on day-7 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. blastocysts</th>
<th>Mean (±SE) embryo diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>34</td>
<td>187±4.1a</td>
</tr>
<tr>
<td>Vehicle</td>
<td>28</td>
<td>174±3.3b</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>176±3.4b</td>
</tr>
</tbody>
</table>

P4 = progesterone.
Vehicle = ethanol.

a,b Within a column, numbers with different superscripts are significantly different (P<0.05).
Table 5.8. Number of \( \text{P}_4 \)-treated and control 8-cell bovine embryos in Experiment 5.2 cultured in respective treatments that reached early blastocyst, blastocyst, expanded blastocyst, or hatched blastocyst stage on day-8 post-insemination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. early blastocysts</th>
<th>No. blastocysts (%)</th>
<th>No. expanded blastocysts (%)</th>
<th>No. hatched(^1) blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{P}_4 )(^2)</td>
<td>0/173 (0)</td>
<td>36/173 (21)</td>
<td>42/173 (24)</td>
<td>36/173 (21)(^a)</td>
</tr>
<tr>
<td>Vehicle(^3)</td>
<td>0/170 (0)</td>
<td>30/170 (18)</td>
<td>39/170 (23)</td>
<td>21/170 (12)(^b)</td>
</tr>
<tr>
<td>Control</td>
<td>3/168 (2)</td>
<td>41/168 (24)</td>
<td>47/168 (28)</td>
<td>14/168 (8)(^b)</td>
</tr>
</tbody>
</table>

\(^1\) Hatched blastocysts represents embryos developing to the hatching or hatched blastocyst stages.
\(^2\) \( \text{P}_4 \)=progesterone.
\(^3\) Vehicle=ethanol.

\(^a\)\(^b\) Within a column, numbers with different superscripts are significantly different (\(P<0.05\)).
Table 5.9. The mean red score per embryo per treatment over the four observatory periods of embryo development

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P&lt;sub&gt;4&lt;/sub&gt;</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.92±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.88±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.56±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.26±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vehicle&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.55±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.43±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.21±0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.48±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.68±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.49±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.88±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.52±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> P<sub>4</sub>=progesterone.
<sup>2</sup> Vehicle=ethanol.
<sup>a,b</sup> Within a column, numbers with different superscripts are significantly different (P<0.05).
vehicle-treated embryos on day 7 compared with P₄-treated or control embryos (Table 5.9).

**Discussion**

In Experiments 5.1 and 5.2, the supplementation of P₄ to the CR1aa culture medium significantly increased the number of 8-cell bovine IVF-derived embryos that developed to the blastocyst stage on day 7 and the hatched blastocyst stage on day 9, post-insemination. These positive findings were not in agreement with those of Fukui et al. (1982), Wiemer et al. (1987), Fukui (1989), Reggio (1997) and Goff and Smith (1998), who added P₄ to a co-culture embryo culture system for bovine embryos. Furthermore, these studies supplemented P₄ to the culture medium at 20 hours post-insemination (Fukui et al., 1982; Wiemer et al., 1987; Fukui, 1989 and Goff and Smith, 1998). These results may have occurred because pre-cleavage stage embryos would not have been exposed to moderate or elevated levels of P₄ as they would have been if in vivo. Moreover, elevated P₄ levels following fertilization has been a characteristic of repeat breeder cattle and/or infertile matings to fertile bulls (Erb et al., 1976; Gustafsson, 1986). The results of Reggio (1997) may have been confounded by the use of water soluble P₄ supplementation in the embryo culture medium. The levels of P₄ used in these studies, with the exception of Goff and Smith (1998), are much greater than the circulating levels in the cow during the early stages of the estrous cycle. Also these experiments (Fukui et al., 1982; Wiemer et al., 1987; Fukui, 1989; Goff and Smith, 1998) utilized co-culture systems using bovine uterine cells, which could have confounded the results on the direct effects of P₄ on the developing embryo.

Using water soluble P₄, Reggio (1997) reported that P₄ supplementation in the absence of a co-culture system did not significantly increase embryo developmental rates. However, when water soluble P₄ was used in the presence of a somatic cell co-culture system P₄ supplementation of the medium increased the number of embryos developing to the blastocyst stage. An increase in the number of embryos developing to the blastocyst stage has been previously reported with lipid soluble P₄ supplementation in the culture medium for mouse embryos (Kirkpatrick, 1971; Lavranos and Seamark, 1989) and rabbit embryos (EL-Banna and Daniel, 1972). In the present study, the decrease in the number of embryos developing to the blastocyst stage in the P₄ vehicle
(ethanol) treatment group was unexpected. However, these findings indicate that \( P_4 \), not ethanol, must have a beneficial effect on the developing IVC embryos to increase the number of embryos developing to the blastocyst stage in vitro.

Stachecki et al. (1994) reported that the exposure of murine embryos to ethanol stimulated the rate of expansion of blastocyst stage embryos. In addition, Wiebold and Becker (1987) reported that ethanol supplementation in the medium of murine embryos increased the number of embryos that implanted when transferred but no significant increase was observed in the number of pups born. In the present study, there was no difference in the number of hatched blastocysts on day-9 post-insemination between the vehicle treatment and control groups. The results of our experiment suggest the opposite effect of ethanol on IVF-derived bovine IVC embryos compared with the effect of ethanol reported for mouse embryos. It is possible that ethanol is subsequently detrimental to pre-blastocyst staged bovine embryos but beneficial to blastocyst staged bovine embryos. Additional experiments that utilize different carrier vehicles would be beneficial.

Results from the current study indicate that the addition of a physiological level of \( P_4 \) (~15 ng/ml) to the culture medium of developing 8-cell bovine embryos increased the rate of embryo development. In the \( P_4 \)-treatment group, on day-6 post-insemination, there were significantly more early blastocysts and blastocysts compared with that of the control culture medium in Experiment 5.1, and significantly more blastocysts compared with that of the vehicle and control groups in Experiment 5.2. On day 8 post-insemination, there were significantly more hatched blastocysts in the \( P_4 \) treatment group compared with the control group in Experiment 5.1 and significantly more hatched blastocysts compared with the vehicle and control groups in Experiment 5.2.

This increase in embryo developmental rates following \( P_4 \) supplementation was reported to occur in vivo (Garret et al., 1987; Garret et al., 1988). When ovine embryos were transferred to an advanced stage uterus (asynchronous embryo transfer) the embryos were found to hasten their developmental rate to match the stage of the uterus (Wilmut and Sales, 1981). Furthermore, this accelerated rate of development was more prevalent in early staged ovine embryos (prior to the blastocyst stage) than in later staged ovine embryos (Lawson et al., 1983). Although, there are limits to the degree of
asynchrony that ovine embryos can withstand and asynchrony greater than 4 days can result in rapid development but without continued pregnancy (Lawson and Cahill, 1983).

This accelerated embryo developmental rate has also been reported in P₄ supplemented cattle. Garret et al. (1988) demonstrated that rapid bovine embryo development occurred when inseminated females were supplemented with P₄ from day 1.4 to 4 (day 0 = estrus). An increased rate of uterine secretions was detected with P₄ administration to cows during the first 4 to 5 days of the estrous cycle, and this treatment was hypothesized to stimulate the rate of embryo development in vivo (Garrett et al., 1988; Geisert et al., 1992). The increase in developmental rates have been reported to have lasting effects on the ovine conceptus where fetuses from P₄ supplemented ewes on days 1 to 6 following mating had a significantly greater crown-rump length compared with fetuses from control ewes (Kleemann et al., 1994; Kleemann et al., 2001). Geisert et al. (1988) have reported that the increased bovine embryo developmental rates in vivo were caused by P₄, IGF-II and IGFBP-2 signaling in the uterine endometrium. However, it remains to be determined if the increased embryo development in these studies is due to the advanced uterine secretory state or due to a direct effect of P₄ on the developing embryo.

The accelerated embryo growth rate in our study was also evident in the diameter differences of the developing IVC bovine embryos. Embryos cultured in the presence of P₄ had a significantly greater mean diameter than embryos in the vehicle and control groups. This increased diameter is reflective of embryos undergoing expansion earlier than embryos in the vehicle and control groups. This accelerated development rate was also reported by Reggio (1997) when IVF-derived bovine embryos were cultured in the presence of water soluble P₄ and somatic cell co-culture.

In the present study, there were more grade-1 quality embryos on day-7 post-insemination produced in the P₄ treatment group compared with the vehicle-treated and control groups. In contrast, there was no difference in the number of grade-1 embryos produced between the vehicle and the control group. This demonstrates that the increased rate of development in the presence of P₄ was not detrimental to embryo quality. Furthermore, these results demonstrated that P₄ may be a viable option for supplementing IVC systems to increase the number of transferable bovine IVF-derived
embryos. The next step would be to transfer embryos cultured in the presence of P₄ to verify that pregnancies can be established.

In conclusion, the addition of physiological levels of P₄ to embryo culture medium on day 3 post-insemination was beneficial to embryo development in several ways. First, P₄ supplementation increased the number of IVC embryos developing to the blastocyst stage by day-7 post-insemination, as well as the number of hatched blastocysts on day-9 post-insemination. Second, P₄ supplementation hastened the rate of embryo development and increased the number of grade-1 blastocysts on day 7 post-insemination. By supplementing the IVC culture medium with P₄ at the same stage of development that the embryo would normally be exposed to increasing levels of P₄ it was demonstrated that P₄ can exert a positive direct effect on the developing IVC bovine embryo.
CHAPTER 6
AN ATTEMPT TO DETECT PROGESTERONE RECEPTORS IN EARLY STAGES OF IN VITRO-PRODUCED BOVINE EMBRYOS

Introduction

During metestrus in a pregnant cow, the 8-cell bovine embryo begins to undergo genomic activation prior to its migration into the uterus (Barnes and First, 1991; Crissman et al. 1980). Coincidently, the corpus luteum (CL) is beginning to synthesize and secrete progesterone in measurable quantities into the circulatory system. It has been established that P$_4$ contributes to the control of the rate of embryo transport into the uterus in the cow (EL-Banna and Hafez, 1970; Crisman et al., 1980). However, it is unknown if endogenous P$_4$ plays a direct role in regulating genomic activation in the cow.

Benefits to increased P$_4$ levels during early metestrus in cattle have been demonstrated in vivo (see Chapter 4) and in vitro (see Chapter 5). When P$_4$ is administered to early pregnant bovine females there is an increase in embryo developmental rates (Garrett et al., 1987; Garrett et al., 1988). In sheep, this increase in development correlates to greater fetal growth later in gestation (Kleemann et al., 1994; Kleemann et al., 2001). Geisert et al. (1988) have demonstrated that the supplementation of P$_4$ during metestrus stimulated the rate of maturation of the uterus resulting in an increase in stromal glands within the uterus and uterine protein secretion profiles that were similar to females at later days post-estrus. It has been hypothesized that this increase in embryo development was likely the result of increased uterine proteins and growth factors acting on the embryo (Garret et al., 1988). However, in vitro co-culture systems that utilize uterine or oviductal cells and P$_4$ for embryo culture have produced variable results (Fukui et al., 1982; Fukushima and Fukui, 1985; Weimer et al., 1987; Goff and Smith, 1998). Because the uterine peptide/protein secretion pattern changes with stages of the bovine estrous cycle (Geisert et al., 1992) this may account for the variation in reports on P$_4$ supplementation of IVF-derived embryos such as the use of uterine or oviductal cells harvested from cattle on different days of the estrous cycle.
Alternatively, the variation found with in vitro co-culture systems may be a result of the use of cells from females of different ages, as age of the female has also been demonstrated to affect fertility (Byerley et al., 1987, Quirke and Hanrahan, 1983). Byerley et al. (1987) have reported that heifers experience low fertility during their first two estrous cycles after puberty. Also in sheep, fertility following the mating of ewe lambs is lower compared with mature ewes (Quirke and Hanrahan, 1983). Thus, if uterine or oviductal tissue were harvested from mature fertile cows during metestrus it would not be possible to determine whether $P_4$ effects on the early embryo development were directly on the embryo or via uterine peptide/protein secretions. In Chapter 5, it was demonstrated that $P_4$ could directly affect embryo development in vitro. However, the mechanism by which $P_4$ acted on the bovine embryo is still unknown. Therefore, the objective of this study was to determine if early developing IVP bovine embryos had $P_4$ receptors.

Materials and Methods

Media Preparation

Oocytes were purchased from BOMED, Inc. (Madison, WI) or OvaGenix LP (San Angelo, TX) and arrived in maturation medium between 38°C and 39°C. The oocytes were allowed to remain in maturation medium for 24 hours. Oocyte and sperm washing medium was prepared from Brackett-Oliphant (BO) stock medium (Appendix A and B). The working stock solution of oocyte and sperm washing medium (BO-AB stock solution) was prepared by the addition of 38 ml of BO-A stock solution to 12 ml of BO-B stock solution and supplemented with 3.85 mg of pyruvic acid (P-4562, Sigma Chemical Co., St. Louis, MO), 50 µl of gentamicin (15750-060, Gibco, Grand Island, NY) and 25 µl of 10,000 IU/ml heparin sodium. The final sperm washing medium (BO-Caffeine) was prepared by the addition of 24.3 mg of caffeine sodium (C-4144, Sigma Chemical Co., St. Louis, MO) to 25 ml of BO-AB stock solution.

The final oocyte washing solution (BO-BSA 0.3%) was prepared by the addition of 30 mg of bovine serum albumin (BSA; A-7511, Sigma Chemical Co., St. Louis, MO) to 10 ml of BO-AB and the final sperm stock solution (BO-BSA 0.6%) was prepared by the addition of 30 mg BSA (A-7511, Sigma Chemical Co., St. Louis, MO) to 5 ml of BO-AB. All fertilization, culture and wash media was prepared at the Center for Reproductive Biology at Louisiana State University, St. Gabriel, LA and equilibrated at 39°C and 5%
CO₂. The embryo culture medium, CR1aa stock solution was prepared as described in Appendix C (Rosenkrans and First, 1994).

In Vitro Fertilization

For the in vitro fertilization procedure, semen from a fertile Holstein bull (CSS 7H5918, Genex Cooperative Inc., Shawano, WI) was thawed in a water bath at 40°C for 15 to 45 seconds. The straw was wiped clean of residue water and expelled into a 15 ml conical tube (Corning 15 ml conical tubes, Myriad Industries, San Diego, CA), extended with 9 ml of BO-Caffeine and centrifuged at 200 x g for 6 minutes. Following the completion of the first washing the supernatant was removed and 9 ml of fresh BO-Caffeine medium was added to resuspend the sperm pellet and the wash procedure was repeated. After completion of the second sperm washing the supernatant was removed and the semen was resuspended in 4 ml of BO-Caffeine medium and 4 ml of BO-BSA 0.6%. Then 100 µl droplets of this solution were prepared in 35 mm Falcon™ plastic petri dishes (Becton and Dickinson, Lincoln Park, NJ) under mineral oil and then placed in an humidified incubator at 39°C and 5% CO₂.

Following a 24-hour oocyte maturation period, oocytes were removed from the maturation medium and washed through two 35 mm Falcon™ each containing 3.5 ml of BO-BSA 0.3% medium. Following completion of the two oocyte washings 15 to 20 oocytes were placed in 100 µl fertilization droplets and all dishes were returned to the incubator for 5 to 6 hours.

In Vitro Culture

When the 5- to 6-hour insemination period was completed the inseminated oocytes were removed from the insemination droplets and washed through two 35 mm Falcon™ plastic Petri dishes that contained 3.5 ml of 10 ml TCM-199 (Gibco Laboratories, Grand Island, NY) and 30 mg of fatty acid-free bovine serum albumin (FAF-BSA) (Fatty Acid Free-BSA, A-4503, Sigma Chemical Co., St. Louis, MO) solution.

Following the oocyte washing the cumulus cells were removed from inseminated oocytes by vortexing (Vortex-genie mixer, Scientific Products, McGraw Park, IL) for 3 minutes in 1 ml of TCM-199 and 0.1 mg of hyaluronidase (H-3506, Sigma Chemical Co., St. Louis, MO) solution. After the vortexing was complete the oocytes were removed from vortexing solution and washed through two dishes of 3.5 ml of TCM-199 and 30 mg of fatty-acid-free bovine serum albumin (FAF-BSA). Following the final oocyte washing,
the inseminated oocytes were then placed in 50 µl droplets of modified CR1aa [9.5 ml of CR1aa (Appendix C), 200 µl of basal medium eagle (BME) (B-6766, Sigma Chemical Co., St. Louis, MO), 100 µl of modified eagle medium (11140-050, Gibco Laboratories, Grand Island, NY), 10 µl of gentamicin (Gibco Laboratories, Grand Island, NY), 1.46 mg of L-glutamine (G-5763, Sigma Chemical Co., St. Louis, MO) and 30 mg of FAF-BSA]. Once oocytes were placed in CR1aa droplets the dishes were then placed in an airtight modular incubator (Billups-Rothenberg®, Del Mar, CA) that was filled with 5% CO2, 5% O2 and 90% N2 gas for 4 minutes at 2 psi and then placed in a humidified 5% CO2 incubator at 39°C.

At 3 days post-insemination, embryos were removed from embryo culture medium and placed in fresh CR1aa embryo culture medium that was modified by the supplementation of 500 µl of 10% heat-inactivated fetal bovine serum (FBS, SH30070.02, Hi Clone Laboratories, Logan, UT).

**Tissues Samples**

Morphologically good quality embryos (n=47) were randomly removed from in vitro culture at the 4-cell (n=3), 8-cell (n=5), 16-cell (n=3), morula (n=4), early blastocyst (n=5), blastocyst (n=7), expanding blastocyst (n=8), hatching blastocyst (n=4) and hatched blastocyst stage (n=8). In addition, adult bovine uteri (n=2) were collected from an abattoir and histologically sectioned. Uterine samples collected were from the distal portion of the uterine horn ipsilateral to the ovary bearing a mature CL.

**Uterine Tissue Processing**

Uteri were collected from a local abattoir (Hyde’s Slaughter Plant, Hammond, LA). These uteri were collected from mature dairy cows and only reproductive tracts that possessed a mature CL were collected. The reproductive tracts were transported (at 25°C) to the Embryo Biotechnology Laboratory in physiological saline and once at the laboratory ~1.5 cm sections were excised from the distal portion of the uterine horn (near the utero-tubal junction) ipsilateral to the ovary bearing the CL. These sections were then placed in 2% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) solution and transported to the Louisiana State University School of Veterinary Medicine (LSU SVM) Pathology Laboratory.

The uterine sections were trimmed and then dehydrated by passing samples through a series of alcohol concentrations and then alcohol was removed from the
sections by placing the sample in a xylene solvent. Following this process, the sample was infiltrated and impregnated with paraffin wax prior to embedding. The sample was embedded with a Leica TP1050 (Leica Microsystems, Deerfield, IL). Samples are sectioned (1 mm thickness) and mounted on a microscope slide for further staining.

**Experimental Design**

Uterine sections from the ipsilateral uterine horn to the CL collected from adult cows served as positive controls to verify that the staining procedure was correctly performed. For a negative control, embryos were used that underwent the fixation process but did not undergo the staining process. All embryos except those at the hatching or hatched blastocyst stages had the zona pellucida opened with a flexible glass needle using a Nikon inverted microscope (Nikon Instech Co. Ltd., Kanagawa, Japan).

**Experimental Procedures**

When embryos were randomly removed from in vitro culture on day 3 through day 9, they were placed in a fixing solution of 1 ml of 2% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and 7 ml of TCM-199 (Sigma Chemical Co., St. Louis, MO). To ensure fixation of these selected embryos they remained in this 2% paraformaldehyde solution, at room temperature, for 2 to 24 hours and afterwards they were placed back into TCM-199 and refrigerated for later use. At time of P₄ receptor staining, embryos and uterine sections were washed with a washing solution of 50 ml of PBS and 0.025 g of BSA (A-7511, Sigma Chemical Co., St Louis, MO). Embryos and uterine sections were washed three times for 5 to 10 minutes per washing in a Falcon™ 4-well dish (BD Biosciences, San Jose, CA). Embryos were then placed in a blocking solution of 10 ml of TCM-199, 200 µl of goat serum (Equitech-BIO, Kerrville, TX), 2 mg of BSA (A-7511) and 1µl of Triton-X (X-102, Sigma Chemical Co., St. Louis, MO) for 1 hour at room temperature.

Following removal from blocking solution, embryos and uterine sections were then placed in 100-µl droplets of the primary antibody (AB) solution (Zymed Laboratories Inc. (San Francisco, CA). Embryos and uterine sections were allowed to stay in the primary AB droplet for 1 to 2 hours. Following this step, embryos were then washed in washing solution 3 times for 10 to 15 minutes each in a Falcon™ 4-well dish. After the embryos were washed they were then placed in a 1:1000 dilution of secondary AB (goat-
anti-mouse Fc-conjugated to FITC, Sigma Chemical Co., St. Louis, MO). This step was performed in the dark. The dish was covered with a piece of foil and embryos and uterine sections were allowed to sit in the secondary AB for 1 to 2 hours (at room temperature). Following removal from the secondary AB, the embryos and uterine sections were washed in the washing solution two times for 10 to 15 minutes per washing then once for 30 minutes.

Embryos and uterine sections were removed from the washing medium and placed in an anti-avidin anti-fade solution (Vector Laboratories Inc., Burliname, CA). The embryos and uterine sections were then evaluated for fluorescence using a Leica sp1 laser scanning confocal microscope equipped with a 20X objective. Stained embryonic and uterine cells were excited with a 488 nm line from an argon laser and scanned images were captured with an adjusted barrier wavelength of 520 to 560 nm. These images were contrast enhanced in Adobe Photoshop 4.0 (Adobe System, Mountain View, CA) for evaluation of fluorescence.

**Results**

Stained uterine tissue (positive controls) fluoresced with the appropriate green pattern as expected, indicating the presence of P₄ receptors in the endometrium (Figure 6.1). However, IVF-derived embryos from the 8-cell to the hatched blastocyst stages, as well as, the unstained embryos (negative controls) only exhibited autofluorescence (Figure 6.2, 6.3). The appropriate apple green fluorescence pattern was not detected in embryos of any stage of development in this experiment.

**Discussion**

The presence of the correct fluoresce in the uterine tissue indicated that this staining method was able to detect the presence of P₄ receptors. However, IVP bovine embryos unexpectedly exhibited only an autofluorescence. Due to this autofluorescence, the procedure used to determine P₄ receptors in IVP bovine embryos may not be the most appropriate method. In unpublished attempts to detect P₄ receptors in embryos using this method, it was determined that the zona pellucida had to be perforated for the stain to enter and exit the perivitelline space (Ferguson, unpublished data).
Figure 6.1. Fluorence of sectioned bovine uterine tissue. Note the specific staining of P₄-receptors along the luminal lining of uterine tissue.
Figure. 6.2. Autofluorescence of unstained IVF-derived bovine hatched blastocysts. This indicates the staining procedure is not applicable in bovine embryos.
Figure. 6.3. Autofluorescence of stained IVF-derived bovine hatched blastocysts. Note the nonspecific staining pattern that precludes the determination of the presence of P₄ receptors.
Due to the difficulty in the histological sectioning of bovine embryos, alternative approaches, such as detection of mRNA for the P₄ receptor may be an alternative approach. Using this approach in mice, it was determined that mRNA for the P₄ receptor was not detected until the blastocyst stage (Hou and Gorski, 1993). In porcine embryos, however, mRNA for the P₄ receptor was detected at the 2-cell stage but levels had declined by the 4-cell stage and were not detectable at any other stage of development (Ying et al., 2000). Although P₄ receptor mRNA was detected in the mouse and pig embryo, there have been no reports of the expression of the P₄ receptor in early developmental stage bovine embryos.

Recent reports have shown that interferon-tau (INF-t) can improve IVF-derived bovine embryo development (Takahashi et al., 2003). It was previously believed that the bovine embryo did not express the INF-t receptor until day 15 of development (Han et al., 1997) but Takahashi et al. (2003) reported the presence of a INF-t type I receptor in IVF-derived bovine embryos from day 5 (morula) through day 8 (expanded blastocysts). Interestingly, an increase in embryo development was noted when interferon alpha (INF-α) was supplemented in place of INF-t, however, the receptor for INF-α was not detected (Takahashi et al., 2003). It was hypothesized that the INF-α utilizes the INF-t receptor, although with a lower binding affinity due the findings that a larger concentration of INF-α was required to equal the proliferative effects on embryonic development noted with INF-t supplementation. Therefore, is possible that the P₄ receptor is present but an effective method of detection was not been developed in our study.

There maybe an alternative approach to explain the improved development of IVF-derived bovine embryos cultured in the presence of P₄. There has been limited evidence that some cells do not express classical genomic progesterone receptors (Bramley, 2003). Indirect evidence from P₄ supplementation in cell cultures of treated cells that lacked a cell nucleus, the effect of P₄ on the nucleated and enucleated cells was the same indicating an ability to affect a cell without having a nuclear receptor present. There is limited evidence for the nonnuclear P₄ receptors, however, this remains as a possibility to describe a mechanism by which P₄ can affect an IVF-derived bovine embryo without nuclear P₄ receptors present.
In conclusion, using this antibody conjugated-FITC P₄ receptor staining procedure indicated that P₄ receptors were not present in IVC-produced bovine embryos. However, the autofluorescence occurring in the IVC-produced bovine embryos may prevent this method from being a viable method of P₄ receptor staining. It seems logical that the receptor staining method would generate more valuable data than detection of mRNA for P₄ receptors because the mRNA detection method does not demonstrate the expression of the receptor that would be required to bind P₄.
CHAPTER 7
RESCUE OF PREGNANCIES IN BEEF CATTLE AFTER A LUTEOLYTIC DOSE OF PROSTAGLANDIN F$_{2\alpha}$

Introduction

It is well known that PGF$_{2\alpha}$ can induce luteolysis in cycling cattle and at various times during pregnancy in cattle. When PGF$_{2\alpha}$ is administered to cycling females, luteolysis is usually accompanied by the emergence of a dominant follicle, estrus and ovulation ~72 hours following PGF$_{2\alpha}$ administration. Although PGF$_{2\alpha}$ administration is not always followed by 100% of pregnant females aborting (Lauderdale, 1972; Sloan, 1977; Wright and Kiracofe, 1988), it has been reported to be very effective when administered between 50 to 150 days of gestation (Jackson and Cooper, 1977).

It is also accepted that once PGF$_{2\alpha}$ induced luteolysis is initiated in cattle the corpus luteum (CL) can not be ‘rescued’. In early studies to determine effect of CL ablation on the bovine pregnancy, researchers administered progesterone (P$_4$) prior to (Raeside and Turner, 1950a, 1950b; Zimbelman and Smith, 1966c) or simultaneously (Raeside and Turner, 1951; McDonald et al., 1952; Tanabe, 1966; Kesler, 1997; Bridges et al., 2000) to CL ablation. In these cases, pregnancy was maintained when P$_4$ was supplemented until near the end of gestation. However, it remains unknown if pregnancy can be maintained when PGF$_{2\alpha}$ is administered prior to the administration of maintenance P$_4$ and if this is possible, what are the time limitations from the time of administration of PGF$_{2\alpha}$ to the administration of maintenance P$_4$. This is a pertinent question due to the common use of PGF$_{2\alpha}$ on farms and cattle ranches that utilize estrous synchronization regimes and embryo transfer. In these situations, opportunities exist for erroneous administration of PGF$_{2\alpha}$ to pregnant females.

Although the prospect of saving a pregnancy of a prostaglandin-treated gestating female may exist theoretically, there are other problems to overcome involving saving an ongoing pregnancy. It has been demonstrated that cattle experience a higher incidence of dystocia and premature calving when parturition is initiated in the absence of a CL (Raeside and Turner, 1951; McDonald et al., 1952; Estergreen et al., 1967; Chew et al., 1979; Kesler, 1997). This dystocia is usually accompanied by increased incidence of retained placental tissues, increased cow and calf mortality and insufficient milk production (during lactation) post-calving. In addition, Kesler (1997) reported reduced or
absent maternal instinct behavior in beef females calving in the absence of a CL. An increase in dystocia occurring in the absence of a CL has also been described in swine (First and Staigmiller, 1973; Nara et al., 1981), however, it was later demonstrated that the CL were the primary source of relaxin and suggested this to be the primary cause of this dystocia problem in swine (Nara et al., 1981).

There have been reports of induction of luteal tissue during early pregnancy in beef cattle (Lulai et al., 1994; Wright et al., 1994; Bridges et al., 2000). However, it was demonstrated that if the induced CL was not present ipsilateral to the gravid uterine horn, the induced luteal tissue was only transient (Lulai et al., 1994). Although, Wright et al. (1994) and Bridges et al. (2000) demonstrated that induced luteal tissue, on the ovary ipsilateral to the gravid uterine horn, could support pregnancy in the absence of P₄ therapy, neither of these studies reported if these females calved or whether parturition was normal.

These reports have provided insight into a possible mechanism to prevent problems that commonly occur in cattle with the absence of a CL. Therefore, the objectives of these experiments were to: (1) determine if pregnancy could be maintained in the absence of a CL with administration of P₄ or altrenogest (ALT) after PGF₂α treatment and (2) determine if induced luteal tissue could successfully support the maintained pregnancy to term and result in normal parturition producing a viable offspring.

**Materials and Methods**

**Experiment 7.1**

**Experimental Animals**

During the fall breeding season, 12 mature crossbred beef cows (Angus, Charolais, Red Angus and Simmental) ranging in age from 4 to 8 years of age and with body condition scores (BCS) of 5 to 7 (range 1=emaciated to 9=obese) were maintained on Costal bermudagrass pastures at the Center for Reproductive Biology at Louisiana State University, St. Gabriel, LA. All animals were treated in accordance with guidelines set forth by the Animal Care and Use Committee of the Louisiana State University Agricultural Center.

These females were pasture mated with two crossbred beef bulls and diagnosed pregnant at 30 days of gestation (range = 32 to 42 days) via ultrasonography using an
Aloka 500-V ultrasound unit (Corometrics, Wallingford, CT) equipped with a 5 MHz rectal probe. A pregnancy was defined as the presence of a fetus with a viable heart beat.

**Experimental Design**

Once females were diagnosed as pregnant (n=12) at 32 to 42 days of gestation they were randomly assigned to receive one of five treatments (Part I of this study). All females received a luteolytic dose (25 mg) of PGF$_{2\alpha}$ (Lutalyse®, Pharmacia & UpJohn, Kalamazoo, MI) (i.m.) at time 0 hour. Following PGF$_{2\alpha}$ treatment, females received 100 mg of P$_4$ (Progesterone, Sigma Chemical Co., St. Louis, MO) (i.m.) dissolved in 3 ml of 100% ethanol (Aaper Alcohol, Shelbyville, KY) at 2 hours post-PGF$_{2\alpha}$ (Treatment A) (n=2), 6 hours post-PGF$_{2\alpha}$ (Treatment B) (n=3), 10 hours post-PGF$_{2\alpha}$ (Treatment C) (n=3), 14 hours-post PGF$_{2\alpha}$ (Treatment D) (n=2) or 18 hours post-PGF$_{2\alpha}$ (Treatment E) (n=2) in this experiment.

**Experimental Procedure**

During P$_4$ treatments, blood samples were collected via jugular venapuncture immediately prior to PGF$_{2\alpha}$ administration and then at 2-hour intervals thereafter until the time of the respective P$_4$ treatment. Blood samples were not collected from an animal shortly after P$_4$ administration due to the cross reactivity of the P$_4$ administered with that of endogenous P$_4$. Blood samples were stored at -20°C until the P$_4$ radioimmunoassay was performed. Blood serum samples were analyzed following extraction with acetone for P$_4$ levels using a commercial progesterone radioimmunoassay kit (Diagnostic Systems Laboratory, Webster, TX). The intra- and inter-assay coefficients of variation and assay sensitivities were 5%, 9%, and 0.05 ng/ml, respectively.

Following PGF$_{2\alpha}$ treatment, females received daily administration of 100 mg of P$_4$ for 7 days in an effort to maintain pregnancies. A successfully saved pregnancy was defined as a viable (fetal heartbeats detected) pregnancy that was maintained for 7 days in females that underwent successful luteolysis, as determined by ultrasonography.

Three females from those that remained pregnant were selected for induction of luteal tissue (Part II of this study). These females were monitored three times weekly (via ultrasonography) for the development of a ≥10 mm diameter follicle on the ovary ipsilateral to the gravid uterine horn. Once a ≥10 mm follicle was detected, 2,500 IU of hCG (Chorulon®, Intervet Inc., Millsboro, DE, USA) was administered (i.m.) to the pregnant females. Females were monitored three times weekly until luteal tissue was
detected, via ultrasonography. After luteal tissue was detected, the maintenance dose of 
$P_4$ was decreased to 50 mg of $P_4$ per day for 7 days then 25 mg of $P_4$ for 3 days and 
then the daily treatment was terminated.

**Experiment 7.2**

**Experimental Animals**

During the spring breeding season, 7 crossbred beef cows (Angus, Red Angus and 
Simmental) ranging in age from 4 to 8 years of age and with BCS of 5 to 6 (range 
1=emaciated to 9=obese) were pastured on Costal bermudagrass at the Center of the 
Reproductive Biology at Louisiana State University, St. Gabriel, LA. All animals were 
treated in accordance with guidelines set forth by the Animal Care and Use Committee 
for Louisiana State University Agricultural Center. These females were pasture mated 
with two crossbred beef bulls and diagnosed pregnant at 80 days of gestation (range = 
74 to 90 days) via ultrasonography using an Aloka 500-V ultrasound unit equipped with a 
5 MHz rectal probe. A pregnancy was defined as the presence of a fetus with viable 
heartbeats.

**Experimental Design**

Females at 70 to 90 days of gestation were randomly assigned to receive one of 
three treatments. All pregnant females received a luteolytic dose (25 mg) of PGF$_{2\alpha}$ (i.m.) 
at time 0 hour. Following PGF$_{2\alpha}$ treatment, females received 100 mg of altrenogest 
(ALT) (Regu-mate, Intervet, Millsboro, DE) (orally) at 2 hours post-PGF$_{2\alpha}$ (Treatment A) 
(n=3), 6 hours post-PGF$_{2\alpha}$ (Treatment B) (n=2) or 12 hours post-PGF$_{2\alpha}$ (Treatment C) 
(n=2). At 24 hours-post ALT treatment; all females received two 15 mg norgestomet 
implants (s.c.).

**Experimental Procedure**

Blood samples were collected via jugular venipuncture at 0 hours, 2 hours, 6 
hours, 12 hours and 24 hours post-PGF$_{2\alpha}$. These samples were stored at -20°C until 
assayed for $P_4$. The serum samples collected from blood were analyzed using an 
extraction process with acetone. The concentration of $P_4$ was determined by using a 
commercial progesterone radioimmunoassay kit (Diagnostic Systems Laboratory, 
Webster, TX). The intra- and inter-assay coefficients of variation and assay sensitivities 
were 5%, 9%, and 0.05 ng/ml, respectively.
Females were monitored (via ultrasonography) 3 and 7 days post-PGF$_{2\alpha}$ treatment to determine fetal viability. A successfully maintained pregnancy was defined as a viable fetus present at day 7 in females that underwent luteolysis, as determined by ultrasonography. These females (n=3) were then monitored once or twice weekly for the development of a 10 mm follicle or greater on the ovary ipsilateral to the gravid horn. Once a follicle of this criterion was detected 2,500 IU of hCG was administered until new luteal tissue could be detected via ultrasonography. After luteal tissue was detected via ultrasonography for 2 consecutive weeks then the norgestomet implants were removed.

**Experiment 7.3**

**Experimental Animals**

During the fall breeding season, 10 mature crossbred beef heifers (Red Angus and Simmental) of 3 years of age that ranged in BCS of 5 to 6 (BCS 1=emaciated to 9=obese) were maintained on a Costal bermudagrass pasture at the Center for Reproductive Biology at Louisiana State University, St. Gabriel, LA. All animals were treated in accordance with guidelines set forth by the Animal Care and Use Committee of the Louisiana State University Agricultural Center. These females were treated with altrenogest for estrous synchronization (as described in Chapter 2) and PGF$_{2\alpha}$ after 7 days of altrenogest treatment. Following PGF$_{2\alpha}$ administration, females were introduced to two fertile crossbred beef bulls (one was common to all breeding seasons) and mating was detected by visual observation and Estrus Alert (Estrus Alert, Apple Valley, MN) estrus detection aids. Then 30 to 40 days after PGF$_{2\alpha}$ administration, females (n=7) were diagnosed as pregnant (range = 30 to 40 days) via ultrasonography using an Aloka 500-V ultrasound unit equipped with a 5 MHz rectal probe were used. A pregnancy was defined as the presence of a fetus with viable heartbeats.

**Experimental Design**

Pregnant females (30 to 40 days of gestation) were randomly assigned to receive one of three treatments. All pregnant females received a luteolytic dose (25 mg) of PGF$_{2\alpha}$ (Lutalyse, Pharmacia & UpJohn, Kalamazoo, MI) (i.m.) at time 0 hour. Following PGF$_{2\alpha}$ treatment, females received 100 mg of altrenogest (Regu-mate, Intervet, Millsboro, DE) orally at 6 hours post-PGF$_{2\alpha}$ (Treatment A) (n=2), 12 hours post-PGF$_{2\alpha}$ (Treatment B) (n=2) or 18 hours post-PGF$_{2\alpha}$ (Treatment C) (n=3). At 24 hours post-ALT treatment two 15 mg norgestomet implants were inserted (s.c.) into each female.
Experimental Approach

Blood samples were collected via jugular venipuncture at 0 hours, 6 hours, 12 hours, 18 hours, 30 hours and 72 hours post-PGF$_{2\alpha}$. These samples were stored at -20°C until P$_4$ radioimmunoassay was performed. The blood serum samples were analyzed for P$_4$ concentration using a commercial progesterone radioimmunoassay kit (Coat-A-Count Progesterone, DPC, Los Angeles, CA). The intra- and inter-assay coefficients of variation and assay sensitivities were 5%, 9% and 0.05 ng/ml, respectively.

Females were monitored (via ultrasonography) 3 and 7 days post-PGF$_{2\alpha}$ to track fetal viability. A successfully maintained pregnancy was defined as a viable fetus present at day 7 in females that underwent successful luteolysis, determine by ultrasonography. These females were then monitored once weekly for the development of a follicle 10 mm or greater in size on the ovary ipsilateral to the gravid horn. Once a follicle of this criteria was detected, 2,500 IU hCG or 150 mcg GnRH (Fractrel®, Fort Dodge Animal Health, Fort Dodge, IA) was administered until luteal tissue could be detected via ultrasonography and serum P$_4$ radioimmunoassayed. After luteal tissue was detected, via ultrasonography and by circulating serum P$_4$ levels for two consecutive weeks then the norgestomet implants were removed.

Results

Experiment 7.1

In Part I of this experiment, 11 of 12 females (92%) responded to PGF$_{2\alpha}$ administration as determined by ultrasonography and verified by serum P$_4$ levels. However, since the P$_4$ that was administered to save the pregnancy was highly cross reactive with the P$_4$ assay, only blood samples recovered from females prior to the administration of P$_4$ was used to determine luteal regression. The mean(±SE) serum P$_4$ levels (number of animals) at 0, 2, 6, 10, 14 and 18 hours were: 5.5±0.7 ng/ml (n=11), 4.8±0.3 ng/ml (n=7), 4.0±0.5, ng/ml (n=4), 1.6±0.7 ng/ml (n=6), 0.9±0.2 ng/ml (n=3) and 1.4±0.26 ng/ml (n=2), respectively (Figure 7.1). The mean P$_4$ levels did not decline below 2 ng/ml until 10 hours (4 of 5 females) and 14 hours (3 of 3 females). Pregnancy was maintained in 2 of 2 (100%), 3 of 3 (100%), 2 of 3 (67%), 1 of 2 (50%) and 0 of 1 (0%) females in Treatments A (2 hour), B (6 hour), C (10 hour), D (14 hour) and E (18 hour), respectively. It was determined that one female in the 18-hour
Figure 7.1. The circulatory progesterone ($P_4$) pattern constructed from mature crossbred beef cows ($n=12$) 30 to 40 days of gestation following prostaglandin ($\text{PGF}_{2\alpha}$) treatment (Experiment 7.1).
treatment group did not undergo complete luteolysis following PGF$_{2\alpha}$ and was excluded from the data set.

Of the nine females in which pregnancy was maintained, three pregnant females were used to induce luteal tissue (Part II of this study) in an effort to support pregnancy in the absence of exogenous P$_4$ treatment. In female No. 1 (from Treatment A), a follicle greater than 10 mm in diameter appeared on day 10 after PGF$_{2\alpha}$ and two follicles greater than 10 mm appeared on day 15 after PGF$_{2\alpha}$. By days 19 and 24 post-PGF$_{2\alpha}$, luteal tissue was detected via ultrasonography (Figure 7.2). This female (given PGF$_{2\alpha}$ at ~30 days of gestation) gave birth to a healthy bull calf at 284 days of gestation (Figure 7.3) with no incidence of dystocia. The cow exhibited normal maternal behavior and lactation. With female No. 2 (from Treatment B), a 10 mm follicle appeared on day 10 after PGF$_{2\alpha}$. This follicle luteinized in response to hCG by day 23 after PGF$_{2\alpha}$, however, this female lost the pregnancy at ~60 days after PGF$_{2\alpha}$. With female No. 3 (from Treatment B), a follicle greater than 10 mm appeared on day 10, however, by 3 days later (following PGF$_{2\alpha}$ administration) the fetus was lost.

**Experiment 7.2**

In Experiment 7.2, 5 of 6 females responded to PGF$_{2\alpha}$ treatment as determined by ultrasonography and P$_4$ blood samples. The mean serum P$_4$ levels at 0, 2, 6, 12 and 24 hours were: 8.5±1.6 ng/ml, 9.5±2.1 ng/ml, 4.6±1.0 ng/ml, 2.7±0.3, 1.5±0.4 ng/ml, respectively (Figure 7.4). Mean serum P$_4$ levels did not decline below 2 ng/ml until 12 hours (2 of 7, 29% of females) and 24 hours (6 of 7, 86% of females). Pregnancy was maintained in 2 of 3 (67%), 1 of 1 (100%) and 0 of 2 (0%) of females in 2-hour treatment, 6-hour treatment and 12-hour treatment group, respectively. These results are excluding the one female that did not respond to PGF$_{2\alpha}$.

In the three females that pregnancy was maintained, induction of luteal tissue was attempted in two of these females. The remaining female was selected to serve as a control to assure that if a pregnancy was lost it was from the delayed P$_4$ treatment and not from a failure of the norgestomet implant to maintain pregnancy. Of the two females in which induction of luteal tissue was attempted, both had a follicle >10 mm on day 7 following PGF$_{2\alpha}$ on the ipsilateral ovary to the gravid uterine horn. Both of these females received hCG at this time. One female received a second dose of hCG 15 days later because luteal tissue could not be verified via ultrasonography and a follicle 10 mm or
Figure 7.2. Development of follicles ≥10 mm in pregnant crossbred beef cows and their subsequent luteinization after the administration of 2,500 IU of hCG following the initial prostaglandin (PGF$_{2\alpha}$) treatment, as recorded via ultrasonography.
Figure 7.3. First calf (bull calf weighing 35 kg) produced from a crossbred beef cow (No. 1) that was administered a luteolytic dose of prostaglandin and supplemented progesterone (P₄) starting 2 hours following prostaglandin administration for 24 days. The pregnancy was continued on hCG-induced luteal tissue at ~45 days of pregnancy.
Figure 7.4. The circulatory progesterone (P₄) patterns constructed from mature crossbred beef cows (n=12) at 80 to 90 days of gestation following prostaglandin (PGF₂α) treatment (Experiment 7.2).
greater was present on the ipsilateral ovary to the gravid uterine horn.

Following hCG treatment(s) both females formed luteal tissue as determined by ultrasound and a serum P₄ radioimmunoassay. These two females continued to maintain their pregnancies in the absence of supplemental P₄. One hCG-treated female (No. 1) calved at 271 days of gestation and gave birth to a live heifer calf, however; this calf died from heat stress 3 days following birth (Figure 7.5). The second hCG-treated cow (No. 2) gave birth to a healthy bull calf at 286 days of gestation. This calf was healthy and viable at birth and subsequently exhibited a normal growth pattern (Figure 7.5). The norgestomet implant control female (No. 3), became ill requiring veterinary intervention and subsequently lost the pregnancy (~25 days after receiving norgestomet implant). For the two hCG-treated females that calved exhibited normal maternal behavior and subsequent lactation. The surviving calf was raised by the dam without any intervention needed.

**Experiment 7.3**

In Experiment 7.3, all pregnant females (30 to 40 days of gestation) responded to PGF₂α treatment based on ultrasound and circulating serum P₄ levels. The mean serum P₄ levels at 0, 2, 12, 18, 30 and 72 hours were: 12.4±1.5 ng/ml, 4.2±0.5 ng/ml, 2.6±0.6 ng/ml, 1.3±0.0 ng/ml, 1.3±0.1 ng/ml and 0.7±0.2 ng/ml, respectively (Figure 7.6). Mean(±SE) serum P₄ levels did not decline below 2 ng/ml in 3 of 7 (43%) females until 12 hours and finally in 7 of 7 (100%) females until 18 hours after PGF₂α.

Pregnancy was maintained in 100% of females for all treatments and thus all of these females were used to attempt to induce luteal tissue. Cumulatively, on day 7 (4 of 7, 57%), day 14 (5 of 7, 71%) and by day 21 (7 of 7, 100 %), following PGF₂α administration, pregnant-females had a follicle 10 mm or greater on the ovary ipsilateral to the gravid uterine horn.

Luteinization had occurred in (3 of 5 hCG/GnRH-treated females) by day 21, (3 of 4 hCG/GnRH-treated females) by day 28 and (1 of 1 hCG/GnRH-treated females) by day 49 following PGF₂α administration. When compared by treatment groups there were no differences in the time from PGF₂α administration to luteal tissue formation. The 3 females forming luteal tissue by day 21 were represented by 2 females from the
Figure 7.5. The second calf (heifer calf weighing 23 kg) and third calf (bull calf weighing 34 kg) produced from cows (No. 1 and 2) that were administered altrenogest 2 hours after a luteolytic dose of prostaglandin (PGF$_{2\alpha}$) was administered. The pregnancies were maintained on exogenous progesterone (P$_4$) until luteal tissue was induced by hCG administration. At 19 days, post-PGF$_{2\alpha}$ administration, exogenous P$_4$ was discontinued and the pregnancies were continued to term from hCG-induced luteal tissue.
Figure 7.6. The circulatory progesterone ($P_4$) pattern constructed from mature crossbred beef heifers 30 to 40 days of gestation following prostaglandin (PGF$_{2\alpha}$) treatment (Experiment 7.3).
18-hour treatment group and 1 female from the 6-hour and 12-hour treatment groups. For the 2 females forming luteal tissue by 28 days following PGF\(_{2\alpha}\) one was from the 6-hour and 12-hour treatment groups. The final female forming luteal tissue by 49 days following PGF\(_{2\alpha}\) administration was from the 12-hour treatment group. The mean number of hCG or GnRH administrations required to induce luteal tissue was 2.2 per female and the mean time required to induce luteal tissue was 40.2 days per female. Excluding the last females that required 49 days to respond to hCG/GnRH administration the mean time required from luteotrophin administration to luteal tissue formation was 21 days.

One female (No. 1, from the 12-hour treatment group) lost her pregnancy by 44 days following PGF\(_{2\alpha}\) and one female (No. 2, from the 18-hour treatment group) lost her pregnancy by 79 days following PGF\(_{2\alpha}\). One of these females (No. 2, 18-hour treatment group heifer) had induced luteal tissue without a norgestomet implant while the other (No. 1, 12-hour treatment group heifer) had induced luteal tissue and the norgestomet implants at time of pregnancy loss. The remaining five females (2 from 6-hour, 1 from 12-hour and 2 from 18-hour treatment groups), with induced luteal tissue, remained pregnant with norgestomet implants removed following the confirmation of induced luteal tissue.

Of the five remaining pregnant females, four gave birth to live healthy calves at ~ 285 days of gestation (Figure 7.7). However, one female (No. 3) died during parturition and the carcass was disposed without a necropsy being performed, and this prevented determining the cause of death of the cow and the calf. Of the four heifers that calved normally; one heifer (No. 4) from the 6-hour treatment group produced a healthy bull calf that weighed 35 kg, one heifer (No. 5) from the 12-hour treatment group produced a healthy heifer calf that weighed 36 kg, and two heifers (No. 6 and No. 7) from the 18-hour treatment group produced a healthy heifer calf that weighed 33 kg, and a healthy bull calf that weighted 38 kg. Parturition was normal in the four surviving heifers as well as maternal instinct and lactation. All calves experienced normal growth rates and were raised by the dam without intervention.
Figure 7.7. The fourth calf (heifer calf weighing 36 kg at birth) and fifth calf (bull calf weighing 38 kg at birth) (A) sixth calf (heifer calf weighing 33 kg at birth) (B) and seventh calf (bull calf weighing 35 kg at birth) (C) produced from cows that were administered altrenogest 6 hours (1 calf) 12 hours (1 calf) and 18 hours (2 calves) after a luteolytic dose of prostaglandin (PGF$_{2\alpha}$) was administered. The pregnancies were maintained on exogenous progesterone (P$_4$) until luteal tissue was induced by hCG administration. At 21 to 49 days post-PGF$_{2\alpha}$ administration, exogenous P$_4$ was discontinued and the pregnancies were continued to term on induced luteal tissue.
**Discussion**

In these experiments, a total of 25 of 27 (93%) cows (between 30 and 90 days of gestation) responded to the PGF$_{2\alpha}$ administration with a decline in circulating P$_4$ (levels less than 2 ng/ml). This is consistent with other reports for administration of PGF$_{2\alpha}$ to pregnant cows (50 to 150 days of gestation) (Jackson and Cooper, 1977; Wright and Kiracofe, 1988). In Experiments 7.2 and 7.3, cows responding to the PGF$_{2\alpha}$ administration had serum P$_4$ levels below 2 ng/ml by 12 to 18 hours after PGF$_{2\alpha}$. Other reports have shown that P$_4$ levels decline below 2 ng/ml by 24 hours after PGF$_{2\alpha}$ in pregnant beef cattle (Jackson and Cooper, 1977; Guilbault et al., 1988; Wright and Kiracofe, 1988) but unfortunately, these studies did not report P$_4$ values during the post-administration intervals (12 to 24 hours).

In the present study, pregnancy was maintained in 86% of females that received P$_4$ prior to 10 hours after PGF$_{2\alpha}$ treatment compared with 60% of females maintaining pregnancy when P$_4$ was administered ≥12 hours post-PGF$_{2\alpha}$ treatment. These observations suggest that when progestin treatment occurred prior to the time of prostaglandin-induced P$_4$ levels declining below 2 ng/ml, there is an increased chance of maintaining the pregnancy. However, in cows at 80 to 90 days of gestation, in which there were no pregnancies maintained when progestin administration occurred at 12 hours (12-hour treatment group) the two females had 2.5 and 2.9 ng/ml serum P$_4$ levels at 12 hours post-PGF$_{2\alpha}$ treatment. This indicates that the stage of gestation at which PGF$_{2\alpha}$ is administered may affect the efficiency of rescuing pregnancy by utilizing this protocol.

For heifers at 30 to 40 days of gestation, 3 of 3 females maintained pregnancy with progestin treatment starting at 18 hours following prostaglandin administration. These females had a serum P$_4$ level of 1.3, 1.5 and 1.4 at 18 hours post-PGF$_{2\alpha}$. Overall, these results suggest that serum P$_4$ levels at time of progestin treatment may not directly affect the chances of fetal survival during the early implantation period (30 to 40 days of gestation) compared with those treated with PGF$_{2\alpha}$ at a post-implantation period (e.g., 80 to 90 days of gestation).

Part II of these experiments was designed to prevent loss of calves and hopefully decrease dystocia at parturition. This problem has been previously well documented in
cattle (beef and dairy) calving without a CL (Raeside and Turner, 1951; McDonald et al., 1952; Estergreen et al., 1967; Chew et al., 1979; Kesler, 1997). It has been demonstrated that induced luteal tissue was capable of supporting pregnancy in luteoctomized cattle (CL removed before day 10 of gestation) in the absence of maintenance progestin (Lulai et al., 1994; Wright et al., 1994; Bridges et al., 2000). However, there was a low success rate in these cases if the induced luteal tissue was on the contralateral ovary to the gravid uterine horn (Wright et al., 1994; Bridges et al., 2000).

Also, there have been no reports of females calving as a result of continued pregnancy on induced luteal tissue. The successful maintenance of pregnancy in this fashion was not reported to result in calving (Bridges et al., 2000) however, this procedure provided a mechanism by which a ‘saved pregnancy’ could be maintained without the use of long-term progestin treatment and possibly result in normal parturition.

In Experiment 7.1, of two females with induced luteal tissue, one female required two separate hCG administrations 5 days apart, while the remaining female only required one hCG administration. In these two females, one female (receiving a single injection of hCG) aborted by 90 days of gestation, while the other produced a healthy bull calf at 286 days of gestation. This female did not exhibit dystocia or have retained placental membranes. The cause of the lost of pregnancy at 90 days is unknown. The pregnancy was not lost until 23 days following P₄ removal and this was confirmed by pregnancy checks weekly until ~ 21 days following P₄ removal. Unlike the third female in Experiment 7.1 that aborted during the attempt to induce luteal tissue it is possible that the increased frequency of rectal palpation could have resulted in the loss of this pregnancy. Also, this lost pregnancy may be one that is normally observed in non-manipulated cattle (Vaillancourt et al., 1979; Thurmond and Picanso, 1993). However, in the female which lost the pregnancy at 60 days following the induction of luteal tissue, it is possible that the newly formed CL was not capable of supporting the pregnancy to term, although it was successful in the female that calved. Although, there were no differences in the size of follicles that luteinized in these two females, the female that calved may have formed more luteal tissue in response to two administrations of hCG. It
should be noted that one of the live calves produced from Experiment 7.2 was from a female that received only a single administration of hCG.

The reason for the loss of one heifer during parturition in Experiment 7.3 is unknown, since a necropsy was not performed. However, this heifer required 49 days following prostaglandin administration to induce luteal tissue and it is possible that the luteal tissue was short-lived however, the pregnancy continued to ~270 days of gestation. For this physiological model to be valid, the adrenal-placental axis would had to support the pregnancy from ~90 days of gestation until 210 days of gestation. This is unlikely based on reports that pregnancy in the cow can not be maintained without a CL or maintenance P₄ until ~210 days of gestation and generally resulting in parturition beginning ~265 days of gestation (Raeside and Turner, 1951; McDonald et al., 1952; Tanabe, 1966). The other females (No. 4, 5, 6, 7) calved normally and readily accepted and raised their respective calves without outside intervention. This further supports the results from Experiment 7.1 and 7.2.

In these experiments, females that calved following hCG-induced luteal tissue displayed normal maternal recognition and subsequent lactation following parturition. Previous descriptions of beef and dairy cows that pregnancy was maintained to term with exogenous P₄, in the absence of a CL, displayed a lack or absence of maternal behavior and insufficient lactation to raise calf (Kelser, 1997). However, the females in this study had normal births, raised their own calves without assistance and only with two calves lost shortly after birth. The reason(s) for this loss of these two calves is believed to be unrelated to pregnancy rescue treatments.

Woody et al. (1967) have reported administration of exogenous P₄ administered during the first 6 days of CL development (1 to 7 days of estrous cycle) results in premature luteal regression (8 to 10 days of the estrous cycle). Thus it would be difficult to speculate on the mechanism responsible for bovine luteal tissue development under elevated exogenous P₄ for maintenance of pregnancy. However, it has been reported that the bovine embryo produces luteotrophins (PGE₂ and PGI₂) during early development (Hwang et al., 1988) and others have reported luteotrophic proteins in the allanotic fluid prior to day 37 of gestation (Hickey et al., 1989; Hansel and Blair, 1996). In addition, Ryan et al. (1992) demonstrated that the placenta produced luteotrophic
substances during the first trimester in beef cattle. These findings provide a potential model for how the induce luteal tissue continues to persist under elevated exogenous P₄ and is capable of supporting pregnancy to term in the absence of maintenance P₄. It is possible that these factors could stimulate the induced luteal tissue to continue to function throughout gestation. Ryan et al. (1992) reported that pregnant females which received PGF₂α in conjunction with induced fetal death returned to estrus in a mean of 6.2 days while females that underwent induced fetal death without PGF₂α did not return to estrus until a mean of 13.5 days. This finding suggests that placental tissue could prolong luteal function in the absence of a viable fetus.

In conclusion, these data illustrate that a valuable pregnancy can be salvaged following an erroneous administration of PGF₂α without having to administered progesterone for an extended interval during gestation. In addition to saving the pregnancy, the pregnancy can be supported to term and result in normal parturition, normal maternal behavior and normal lactation. This procedure could provide a valuable means to prevent financial losses that can occur on farms/ranches by utilizing this approach of preventing abortions from occurring in valuable embryo transfer recipient females that may have erroneously receive PGF₂α. Also, this procedure can exclude unnecessary veterinary expenses and increased chances of cow or calf mortality due to parturition occurring in the absence of a CL.

To our knowledge this is the first report of successful births of calves from prostaglandin-treated pregnant beef cows that were administered P₄ or altrenogest following the prostaglandin administration. Also, to our knowledge this is the first report of calves produced from cows allowed to continue to term with induced luteal tissue maintaining the pregnancy. Finally, to our knowledge this is also the first report of a bovine pregnancy being maintained with altrenogest in the absence of a luteal tissue.
CHAPTER 8
SUMMARY AND CONCLUSIONS

In these experiments the primary objective was to determine if altrenogest (ALT) used for monogastric animals would act as a biological progestin in cattle (a ruminant). A secondary objective was to evaluate the effect of progestins on early embryo development in vivo and in vitro. The effects of ALT in cattle were unknown due to the fact it had not been assessed as a progestin for use in cattle.

In Chapter 3, it was demonstrated that ALT could serve as a progestin in beef cattle. It was proposed that ALT would synchronize the estrous cycle in beef heifers similar to that of MGA, which has been long known to synchronize beef cattle. ALT successfully synchronized beef females in this study and there were no detectable detrimental effects of ALT on subsequent fertility or on the health parameters of the calves produced from ALT-treated beef females.

In Chapter 4, ALT effectively improved pregnancy rates in repeat breeder females (RPB) when administered on days 3 to 5 following the onset of estrus. In the course of this experiment it was found that mated cattle that have a greater increase in circulating progesterone from day-3 to day-4 of the estrous cycle were more likely to become pregnant as a result of the mating. By administering a low dose of \( P_4 \) or ALT during this interval the \( P_4 \) levels in RPB females mimicked that of control fertile females increasing the pregnancy rate to greater than expected in RPB females.

In Chapter 5, the ability of \( P_4 \) to exert a positive direct effect on the developing in vitro produced (IVP) bovine embryo was evaluated. When \( P_4 \) was administered to the 8-cell stage IVP bovine embryos, significantly more developed to the blastocyst and hatched blastocyst stages on both day-7 and day-9 post-insemination, respectively. Also, more \( P_4 \)-treated IVP bovine embryos developed to grade-1 quality blastocysts on day 7 post-insemination. In addition, \( P_4 \)-treatment hastened the developmental rate of IVP bovine embryos while in in vitro culture and this could ultimately increase transfer pregnancy rates. These findings demonstrated that \( P_4 \) exerts a direct effect on developing IVP bovine embryos.

In Chapter 6, it was determined that using an antibody staining method that \( P_4 \) receptors were not present in developing IVP bovine embryos. Because IVP bovine
embryos exhibited an autofluorence, however, this finding needs further verification.
Additional PCR based experiments to determine the presence of mRNA for the P₄ receptor will also be needed to support these findings.

In Chapter 7, the ability to ‘rescue’ a pregnancy in beef females between 30 and 90 days of gestation following the administration of a luteolytic agent (PGF₂α) was attempted. In these experiments, a maintenance dose of P₄ was administered at set time intervals of 2 to 18 hours following prostaglandin administration. It was determine that pregnancy could be salvaged in females with administration of P₄ initiated as late as 18 hours following PGF₂α administration. In addition to salvaging pregnancy, these females were induced to ovulate to prevent the continued need to administer a maintenance dose of P₄. From these experiments, seven calves were produced from prostaglandin-treated pregnant beef cows and heifers. To our knowledge this is the first report where calves were produced from prostaglandin-treated (30 to 90 days of gestation) pregnant cattle. Also, this is the first report to our knowledge by anyone of beef cattle calving as a result of induced luteal tissue maintaining the pregnancy to term. Parturition, maternal instinct and subsequent lactation were normal in all but one of these cows.

In conclusion, it was demonstrated that ALT could serve as a progestin in cattle. In addition, it was determined that ALT administration during early pregnancy could enhance pregnancy rates in RPB females. Also, P₄ may also have a direct effect on the developing embryo in vitro. The next step would be to evaluate if this response can be generated by ALT.

Furthermore, ALT can maintain pregnancy in a pregnant beef cow given a luteolytic dose of prostaglandin while the beef cow is in the process of having induced luteal tissue form. Calves produced from pregnant beef cow treated with ALT appear to be normal and reproductively sound following puberty. These findings provide beef producers with a new progestin that can be used for estrous cycle synchronization, improving pregnancy rates in problem breeder females and maintaining pregnancy in pregnant beef cows that experience luteal regression.
REFERENCES


O'Reilly, P. J., R. McCormack, K. O'Mahony, and C. Murphy. 1979. Estrus synchronization and fertility in gilts using a synthetic progestagen (allyl trenbolone) and inseminated with fresh stored or frozen semen. Theriogenology 12:131-137.


Pollard, J. W., J. M. Scodras, L. Plante, W. A. King, and K. J. Betteridge. 1991. Definition of the cleavage stage(s) at which oviductal epithelial cells enable bovine embryos to pass through the in vitro 8-16-cell block. Theriogenology 35:256 (abstr.).


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## BO-A STOCK SOLUTION

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1 Solution was prepared in a 500-ml bottle and stored in refrigerator for up to 3 months.
## BO-B STOCK SOLUTION\(^1\)

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\(^1\) Solution was prepared in a 250-ml bottle and CO\(_2\) was introduced into bottle for 1 to 2 mintues (until coloration becomes peach-colored). This solution was stored in refrigerator for up to 3 months.
CR1aa STOCK SOLUTION

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1 Solution was prepared in a 100-ml bottle filtered with a 0.04 µm filter and stored in refrigerator for up to 3 months.
VITA

Clarence Edward Ferguson was the second child born to Dixie and Ronnie Ferguson on January 10, 1973, in Beaumont, Texas. Edward was raised on a racehorse farm in southeast Texas. He attended the school district where his mother taught 2nd grade for over 40 years, Hamshire-Fannett ISD. Following graduation from high school in May of 1991, he enrolled in Sam Houston State University. After spending two years at Sam Houston he transferred to McNeese State University where he earned a bachelor of science degree in biological sciences in December 1996. Upon graduating from McNeese, Edward was employed as a substitute teacher for six months until enrolling in the graduate program in animal sciences at Stephen F. Austin State University in May 1997. After completing his research and class requirements by 1998 he accepted a position as the rodeo coach and research assistant to Dr. Dennis Schmidt at Southwest Missouri State University. There he enrolled in a second master’s program (Master of Natural and Applied Sciences) while completing his thesis for Stephen F. Austin University ultimately receiving a master of science degree in 1999. During the same year he completed all his requirements for the Master of Natural and Applied Science and was accepted to begin a doctoral degree in animal sciences at Louisiana State University under the tutelage of Dr. Robert A. Godke. He received his master of natural and applied science degree in the year of 2000. He is expected to receive his doctorate in December 2004 and has since accepted a position as post-doctoral researcher at the University of Illinois with Dr. Matthew B. Wheeler.