Post-Transfer Outcomes in Cultured Bovine Embryos Supplemented with Epidermal Growth Factor, Fibroblast Growth Factor 2, and Insulin-Like Growth Factor 1

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Abstract

The high incidence of pregnancy loss is a major issue facing the cattle industry. Use of in vitro fertilized (IVF) bovine embryos has become increasingly popular to help alleviate several of these reproductive issues and provide a means to enhance genetic gain for production traits. An uterine paracrine factor cocktail containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor 1 (IGF1) (collectively termed EFI) was recently identified as a means for improving *in vitro* derived bovine embryo development and trophectoderm cell numbers. The objectives of this work were to determine if EFI treatment during *in vitro* bovine embryo culture improves transferable embryo quality and post-transfer placental and fetal development. For each replicate (3 total), slaughterhouse-derived bovine oocytes were matured and fertilized *in vitro*. At day 4 post-fertilization, ≥8 cell embryos were harvested, pooled, and exposed to either the EFI treatment (10ng/ml EGF, 10ng/ml FGF2, 50ng/ml IGF1) or carrier only (1% Bovine Serum Albumin). At day 7, individual embryos were transferred to estrous synchronized beef cattle. Artificial insemination (AI) was completed on a subset of cows. The EFI treatment increased ($P<0.05$) the percentage of transferable embryos. Pregnancy rate at day 28 post-estrus was similar among treatments. Circulating concentrations of pregnancy-associated glycoproteins (PAGs) were determined from plasma harvested at day 28, 42 and 56. Transrectal ultrasonography was used to measure fetal crown-rump length (CRL) at day 42 and 56 and to determine fetal sex at day 60. There were no main effect differences observed across days for PAG concentration. Fetus sex by ET/AI group interactions were absent at day 28 but existed at days 42 and 56 ($P<0.05$). At both days, this interaction reflected fetus sex-dependent changes within the ET control group, where PAG concentrations were greater ($P<0.05$) in male fetuses than female fetuses. No CRL differences or interactions existed among fetal sex and pregnancy group. In summary, addition of the EFI cocktail during bovine embryo culture improved the quality of transferable embryos, but did not affect placental function or embryonic/fetal development. Increasing the numbers of transferable embryos is of value given the cost of in vitro embryo production, but no apparent increases in embryo or placental competency were detected. The EFI treatment increased ($P<0.05$) the percentage of transferable embryos.

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General Audience Abstract

The high incidence of pregnancy loss is a major issue facing the cattle industry. Use of in vitro fertilized (IVF) bovine embryos has become increasingly popular to help alleviate several of these reproductive issues and provide a means to enhance genetic gain for production traits. An uterine paracrine factor cocktail containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor 1 (IGF1) (collectively termed EFI) was recently identified as a means for improving *in vitro* derived bovine embryo development and trophectoderm cell numbers. The objectives of this work were to determine if EFI treatment during *in vitro* bovine embryo culture improves transferable embryo quality and post-transfer placental and fetal development. For each replicate, slaughterhouse-derived bovine oocytes were matured and fertilized *in vitro* and at day 4 post-fertilization, embryos were exposed to either the EFI treatment (10ng/ml EGF, 10ng/ml FGF2, 50ng/ml IGF1) or carrier only (1% Bovine Serum Albumin). Artificial insemination (AI) was completed on a subset of cows and the remaining cattle receive embryos at day 7. The EFI treatment increased ($P<0.05$) the percentage of transferable embryos. Pregnancy rate at day 28 post-estrus was similar among treatments. Circulating concentrations of pregnancy-associated glycoproteins (PAGs) were determined from plasma harvested at day 28, 42 and 56. Transrectal ultrasonography was used to measure fetal crown-rump length (CRL) at day 42 and 56 and to determine fetal sex at day 60. There were no main effect differences observed across days for PAG concentration. Fetus sex by ET/AI group interactions were absent at day 28 but existed at days 42 and 56 ($P<0.05$). At both days, this interaction reflected fetus sex-dependent changes within the ET control group, where PAG concentrations were greater ($P<0.05$) in male fetuses than female fetuses. No CRL differences or interactions existed among fetal sex and pregnancy group. In summary, addition of the EFI cocktail during bovine embryo culture improved the quality of transferable embryos, but did not affect placental function or embryonic/fetal development. Increasing the numbers of transferable embryos is of value given the cost of in vitro embryo production, but no apparent increases in embryo or placental competency were detected. The EFI treatment increased ($P<0.05$) the percentage of transferable embryos.

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List of Abbreviations

AI	Artificial insemination
ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
CIDR	Controlled internal drug release
CNL	Crown-nose length
COC	Cumulus-oocyte complex
CRL	Crown-rump length
EGA	Embryonic genome activation
EGF	Epidermal growth factor
ET	Embryo transfer
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FGF2	Fibroblast growth factor 2
FGF7	Fibroblast growth factor 7
FSH	Follicle stimulating hormone
GLM	General linear model
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnRH	Gonadotropin releasing hormone
ICM	Inner cell mass
IFN	Interferon
IFNT	Interferon-tau
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
ISG	Interferon stimulating genes
IVF	*In vitro* fertilization
IVP	*In vitro* produced
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MET	Maternal to embryonic transition
MRP	Maternal recognition of pregnancy
OPU	Ovum pickup
P4	Progesterone
PAG	Pregnancy associated glycoproteins
PBL	Peripheral blood leukocytes
PDIFF	Probability of difference
PGF2α	Prostaglandin F2 alpha
SAS	Statistical analysis system
SOF	Synthetic oviduct fluid
TE	Trophectoderm
TGF-β	Transforming growth factor-beta
UGKO	Uterine gland knockout
ZP	Zona pellucida

Chapter 1 - Literature Review

Introduction

Pregnancy loss is one of the most predominant issues facing the dairy and beef cattle industries, causing reduced production efficiency and economic loss. Failure of lactating dairy cows to establish and carry a pregnancy to term results in an average loss of $555 per pregnancy [1]. Various environmental and physiological factors contribute to pregnancy loss, with uterine function being one of the major contributors to infertility. The use of various assisted reproductive techniques has been implemented to evade the damaging effects suboptimal uterine environments and other environmental and physiological factors have on embryonic development. *In vitro* production (IVP) of bovine embryos and embryo transfer (ET) is gaining popularity globally as a means for improving genetic potential in beef and dairy cattle [2].

There are three primary stages of pregnancy loss established by the Committee on Bovine Reproductive Nomenclature (1972). The first stage is early embryonic death, which encompasses the period of undetected losses that occur within the normal range of an estrous cycle (16-24 days) in cattle [3]. The second stage of pregnancy loss is termed late embryonic death which occurs between 25 and 42 days of gestation [3]. During this period, corpus luteum (CL) function is maintained beyond a normal estrous cycle length. The third period of pregnancy losses, which occurs after day 42 when a fetus is distinguishable, are termed fetal losses [3]. Of the three stages, the majority of pregnancy losses occur during the early embryonic stage [4]. Improvements in early embryonic development to reduce these early pregnancy losses must, therefore, be explored. The following literature review will go into further detail of the stages of

pregnancy loss, economic impacts of losses, events of pregnancy success, major contributors to pregnancy loss and potential ways to reduce these losses.

Stages of Pregnancy Losses in Cattle

Pregnancy loss is loosely defined as losses in pregnancies that occur throughout gestation immediately after sygamy of the male and female pronuclei and creation of a new life form. Infertility in cattle is typically reflected in embryonic or fetal mortality, which is seen through poor fertilization rates and improper pregnancy maintenance. Fertilization rates have not changed much over the past several decades, therefore problems with fertility usually are caused by increases in embryonic or fetal losses. Losses are more prevalent in lactating cows due to the metabolic stresses of milk production. Cows will normally experience a negative energy balance in early lactation due to the metabolic demands of lactation, and this has detrimental effects on embryonic development. Pregnancy losses also are influenced by various environmental factors. For example, fertilization rate in lactating cattle is reduced to 55% during heat stress periods [5].

There are three primary stages of pregnancy loss in the cow. These stages have been established by the Committee on Bovine Reproductive Nomenclature (1972). The first stage is early embryonic death. This occurs between days 1 and 24 of pregnancy. This period encompasses undetected losses that occur within the normal range in estrous cycle lengths in cattle (16 to 24 days) [3]. These pregnancy losses occur prior to the time of corpus luteum (CL) maintenance, or maternal recognition of pregnancy, which will be described later in this review. Periods of early embryonic loss include pre-embryonic genome activation (EGA), post-EGA, and maternal recognition of pregnancy.

The bovine EGA becomes activated 3-4 days post-fertilization, therefore any alterations in this process may cause pregnancy failure [6]. Additional early embryonic losses occur between EGA and maternal recognition of pregnancy. Maternal recognition of pregnancy, which will be discussed in further detail in subsequent sections, occurs at day 16 of pregnancy.

Lactation is a prominent cause for early embryonic losses. In moderate-producing cows, early embryonic losses are about 40% based on a 90% fertilization rate. Also, it is estimated that 70-80% of these early embryonic losses occur between 8 and 16 days after insemination [4]. Embryo viability studies completed in dairy cows found approximately 50.0, 57.9, and 71.9% of potential pregnancies in parous lactating, parous nonlactating, and nulliparous heifers, respectively, to be viable by days 5-6 post-insemination [3]. A similar pattern is noted in beef cows where viability of embryos collected from parous lactating, parous nonlactating, and nulliparous heifers is 57.5, 79.5, and 77.6%, respectively [3]. These data confirm the impact lactation has on escalating early embryonic losses and limiting pregnancy maintenance.

The second stage of pregnancy loss is termed late embryonic death. This occurs between 25 and 42 days of gestation. This is the period where CL function is maintained beyond a normal estrous cycle length. It also contains the initiation of implantation, placentome formation and completion of much of embryonic differentiation [7]. Lactating dairy cows display extensive late embryonic death compared to beef cattle, with an average loss of 12.8% during this period [3, 8]. Greater late embryonic losses could be attributed to the increased demand of lactation on the body in the dairy

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cow. Additionally, placental insufficiencies such as reduced blood flow or inadequate nutrient supply can contribute to late embryonic losses.

At day 42-45 of gestation, the embryo has fully attached to the uterus and a fetus has become distinguishable. Thus, pregnancies failures after day 42, the third period of pregnancy losses, are termed fetal losses [3]. Fetal losses are less frequent in both beef and dairy cattle. The causes for these losses generally go undetermined if not caused by known infectious agents [3]. In beef and dairy cows, fetal losses are generally low averaging 2.5 and 4.2%, respectively.

Economic Impacts of Pregnancy Loss

Substantial economic losses result from pregnancy failures in cattle. This has been well described in dairy cattle. The value of pregnancy varies depending on stage of lactation, lactation number, milk yield, and reproductive status. Cattle that have a longer interval between post-calving and re-breeding are more costly to the producer, therefore retaining pregnancies is economically crucial [9-11]. The average loss per pregnancy in lactating dairy cows is $555, while the average added value of a new pregnancy is $278 [1]. For example, a 100-head dairy operation with a 40% early and late embryonic loss rate will lose $22,200 in revenue from pregnancy losses. By contrast, a 100-cow dairy with only a 15% early and late embryonic loss rate reduce total economic losses to $8,325 and gain $6,950 with added pregnancies. The economic impact of pregnancy loss outweighs the value of a new pregnancy, so it is crucial to reduce early pregnancy losses and minimize economic losses.

The negative economic impact of early and late embryonic losses also exists in the beef industry. The timing of calving is coupled with conception rates, thus the ability to get cows pregnant in a short timespan has an important influence on economic return. Most of the income cow-calf operations generate is determined by the number of calves sold and by the weight of these calves when they are sold. Cows that achieve pregnancy after first insemination and avoid pregnancy loss contribute to improved calf crop and greater weaning weights. Producers can increase net return by $204 per cow by simply improving net calf crop by 10% and increasing weaning weight by 200 pounds [12].

Events Associated with Pregnancy Success in Cattle

Various time-sensitive events must occur to ensure pregnancy success in cattle. The following paragraphs will go into these specific developmental events in more detail. Among these events, heat detection has improved timing of fertilization and pregnancy rates. Also, advancements in estrous synchronization protocols have eliminated the heat detection component and improved conception rates when implemented. Oocyte maturation, fertilization, and cleavage events are all necessary for a normal pregnancy. Early embryonic development and pregnancy maintenance is regulated by maternal recognition of pregnancy and subsequent implantation and placentation. Progression through these events is imperative for pregnancy success in cattle.

<u>Estrous Synchronization and Detection</u>

Based on several sources, heat detection has drastically improved throughout recent history, and especially over the past 15-20 years. This has reduced days to first

conception and increased the number of inseminations within a set breeding season. Both of these outcomes have increased resulting pregnancy rates. Prior to the use of drug-based ovulation strategies, detecting estrus was a major contributor to infertility in operations that artificially inseminated cattle. Only 30-70% of cows that exhibit heat are detected in heat, which leads to inadequate timing of fertilization and failure to maintain pregnancy [13]. High producing dairy cows are more likely to have decreased heat expression due to the high metabolic hormone turnover.

The development of synchronization protocols has alleviated the heat detection issue. The first timed artificial insemination (AI) protocol developed targeted the synchronization of ovulation using gonadotropin releasing hormone (GnRH) and prostaglandin F2 alpha (PGF2α) [14]. Estrus detection is associated with greater fertility when using a timed AI protocol. Current estrous synchronization protocols focus on synchronizing CL function and follicular waves. Progesterone inserts, or Controlled Internal Drug Release (CIDR) allow emergence of a synchronized follicular wave around 4 days after initiation of protocol [15, 16]. Timed AI protocols are used in conjunction with intravaginal inserts to remove the heat detection component and result in reasonable conception rates.

Oocyte Maturation

Oocyte competency is defined as the ability of an oocyte to complete maturation, undergo successful fertilization and cleavage, develop into a blastocyst, and achieve a viable offspring [17, 18]. Oogenesis, the process of developing oocytes, begins during pre-natal development and is completed by birth in cattle [18]. Primordial germ cells proliferate through mitotic divisions before birth, producing primary oocytes. At birth, the

nucleus of the primary oocytes become dormant, a phenomenon termed nuclear arrest. This nuclear arrest continues until fertilization occurs. However, cytoplasmic development continues. This is especially evident during folliculogenesis, when maternal mRNA and proteins are loaded and transferred into oocytes [19]. Cytoplasmic reserves of RNA accumulate until oocyte becomes full size, and this material will sustain the oocyte and early-developing embryo until the embryonic genome is activated [20].

Oogenesis progresses in synchrony with folliculogenesis through the interdependence of cellular communications between the oocyte and follicle [18]. Folliculogenesis begins with primordial follicles and continues development though primary, secondary, tertiary, and antral follicles. The Graafian, or antral, follicle contains theca and granulosa cells which have receptors for luteinizing hormone (LH) and follicle stimulating hormone (FSH), respectively. Follicle growth is stimulated by FSH and the LH surge causes ovulation. Waves of follicle growth and atresia occur continuously throughout the reproductive period and contribute to oocyte competency.

Fertilization

Fertilization rates in cattle are generally high. Lactating beef cattle have an average fertilization rate of 75%, likely due to the prevalence of post-partum anestrous periods, whereas non-lactating beef cattle have an average rate of 98.6% [3, 21-23]. Following a similar pattern, fertilization rates in lactating and non-lactating dairy cattle are around 75% [3]. Lactating dairy cows have much lower conception rates due to the increased nutritional demand for milk production, which negatively affects embryo

quality [5]. Lactating beef cattle can recover body condition and resume cyclicity, with conception rates ranging from 50-65% [3].

Successful fertilization in the cow requires proper sperm travel and capacitation processes in the oviduct, concurrent with oocyte ovulation. After sperm capacitation has occurred in the uterus or oviduct, the sperm encounters the egg and attaches to the zona pellucida (ZP) initiating the acrosomal reaction. This reaction releases hydrolytic enzymes packaged within the acrosome, which digest the ZP and allow spermatozoa penetration of the ZP. The fusion of the female and male pronuclei, or syngamy, results in formation of a zygote. The first cleavage events occur 23-31 hours post fertilization, producing a 2-cell stage embryo [24]. Cleavage to the 4- and 8-cell stage occurs 36-50 hours and 56-64 hours post-fertilization, respectively. The final cleavage event, which yields a 16-cell stage embryo, occurs 80-86 hours post-fertilization. Cells produced by cleavage events are referred to as blastomeres. A single cell (blastomere) can give rise to a complete individual and is considered totipotent at these initial stages of development. Cell number increases post-fertilization, but the cytoplasm mass remains the same. The embryo travels through the uterine horn during cleavage events to the uterine body to continue development.

Early Embryonic Development

The oocyte and early developing embryo must rely on stored mRNA and protein for survival and developmental signals. Thus, the activation of the embryonic genome is essential for continued survival of embryos. In cattle, this event occurs around the 8- or 16-stage cell embryo, or 3-4 days post-fertilization [6]. At this time, the embryo begins to experience maternal to embryonic transition (MET). The depletion of maternal

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transcripts, replacement of maternal transcripts by embryonic transcripts, and generation of new embryo specific transcripts are involved in MET [25]. By day 5, maternal stores are usually depleted and the embryo controls its own development. The embryonic genome transition is imperative to survival, as it allows the embryo to respond to environmental stresses and any perturbations that may harm its development [6].

As blastomere cleavages continue, the embryo begins a process referred to as compaction. Compaction occurs when blastomeres increase contact with neighboring cells, forming a compact sphere of cells. This change in cellular behavior, in which number of cells becomes difficult to count, is known as the beginning of the morula stage. As the morula progresses through development, a cavity is formed by a process known as cavitation. Cavitation and formation of the internal fluid cavity, termed blastocoel, marks the progression of the morula to the blastocyst stage of development. This process typically occurs around day 7 post-fertilization in cattle.

After cell divisions have concluded, two distinct cell populations are formed. The outer cells are termed trophectoderm (TE) and the inner cells are termed inner cell mass (ICM) [26]. The ICM will give rise to the embryo proper, or body of the embryo which eventually forms the fetus. Additionally, extraembryonic membranes including the yolk sac, amnion, and allantois will be formed from the ICM cells. Trophectoderm cells give rise to the chorion, ultimately forming the placenta. Extraembryonic membrane development is essential for embryonic development and subsequent uterine attachment.

The developing embryo reaches the blastocyst stage by day 7, begins hatching from the ZP, and emerges as a spherical conceptus. Following the blastocyst stage, bovine TE cells undergo extensive proliferation termed conceptus elongation. Simultaneously with TE proliferation, the ICM-derived yolk sac continues growth and reaches peak development by day 20 of pregnancy. After peak development is reached, the yolk sac regresses as its nutritive function is taken over by the emerging allantois [27]. This elongation process occurs between day 14 and 21 of gestation in cattle [28]. By day 16 of pregnancy, the bovine conceptus reaches a length of 10-30 cm. By day 21, or time of uterine attachment, the conceptus reaches a length of 50-200 cm. Elongation is essential to increase surface area which aids conceptus apposition, attachment, and adhesion to the uterine wall [29].

Maternal Recognition of Pregnancy

Pregnancy recognition and maintenance requires cross talk between the conceptus and maternal system. Signaling from the embryo between days 16-25 determines if pregnancy is prolonged, or a new estrous cycle begins. Improper signaling results in embryonic death and pregnancy loss.

During elongation, the conceptus signals its presence to prolong CL life, thus termed the period maternal recognition of pregnancy (MRP). Interferon tau (IFNT) is a member of the Type 1 interferon (IFN) family and is the maternal recognition of pregnancy factor in ruminants [30-32]. Type 1 IFNs are involved in immune responses and anti-proliferative activities [30, 33]. However, unlike other Type 1 IFNs, IFNT has evolved to serve a key role in pregnancy recognition in ruminant species.

Interferon tau is secreted from the mononuclear cells of the trophectoderm. Interferon tau mRNA is present during the preimplantation stage of development, with the earliest detection around day 7, or the morula/early blastocyst period [34-37]. During elongation, IFNT secretions increase with maximal secretion around day 12-13 of gestation in sheep and day 16-19 in cattle [30, 38]. The conceptus of ruminants transforms through spherical, tubular, and filamentous stages with increases in trophoblast cell numbers, corresponding to higher IFNT secretion and attachment preparation [31, 39, 40].

The primary action of IFNT is to function as an antiluteolytic agent so CL regression can be prevented [30-32]. Through paracrine signaling, IFNT acts locally on the endometrium prevent CL regression [30, 31, 40]. Estrogen and oxytocin receptor expression is suppressed by IFNT, and this blocks the pulsatile release of PGF2α and its ability to induce CL regression [30, 31, 41]. Ovine trophoblast protein-1, the ovine specific IFNT, extends the lifespan of the CL and prevents luteal regression when injected into the uterine lumen of non-pregnant ewes [42]. Similarly, bovine trophoblast protein-1 significantly increases the inter-estrous interval when infused through the cervix of cyclic cows [43].

Additionally, IFNT induces expression of classical interferon-stimulating genes (ISG) in the uterus, including ISG15 and OAS [44, 45]. Increased expression of these and other ISGs are correlated with regulation of uterine receptivity, conceptus elongation, and implantation [31, 45]. Consequently, impaired conceptus development, insufficient production of IFNT, and MRP inhibition will be translated in reduced or no ISG expression in maternal circulation. Expression of ISG in maternal blood circulation

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can be used as an indicator of early embryonic loss, specifically around MRP and implantation events [46]. Interferon stimulating genes will be discussed in further detail in subsequent sections.

Implantation

Approximately 10 to 12 days after hatching and 7 to 9 days after elongation begins, the conceptus begins making contact with the uterine luminal epithelium. This occurs on or shortly after day 19 of pregnancy in cattle [47]. At this time, the conceptus typically spans the length of the uterine horn ipsilateral to the CL, and extends into the contralateral horn. The steps that precede implantation include apposition and adhesion of conceptus trophectoderm to uterine and luminal epithelium [29, 48].

Placental Development

As implantation is underway, post-implantation placental development commences. One major event that occurs in cattle and other ruminants is the formation and migration of bincucleate cells from the chorion into the maternal endometrial epithelium. As apposition and adhesion occur, the trophectoderm begins producing a specialized, invasive cell termed the binucleate cell. These cells will migrate to fuse with the endometrial luminal epithelium [48, 49].

This invasive cell type is one way that a placental unit is established. The other way this is established is by the great surface area contact developed in these placentae. Cattle have a cotyledonary placenta, and thus the uterus contains numerous aglandular nodules termed cotyledons. These serve as placental attachments with cotyledons, structures that contain the ability to produce highly interdigitated, villous

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networks with caruncles. These attachments are collectively called placentomes. Around day 25, the chorion initiates attachment to the caruncles of the uterus. By day 40, placentomes are well established [26]. Placentome growth occurs throughout gestation and cotyledons increase many-fold, measuring up to 6 inches in diameter in late pregnancy. This growth provides greater surface area to support placental transfer of nutrients from the maternal side and metabolic wastes from the fetus. Fetal development through the remainder of gestation is completely reliant on nutrients delivered through the placenta.

Major Contributors to Pregnancy Loss in Cattle

There are many contributors to pregnancy failure in cattle. Among these are oocyte competency, uterine development and function, MRP, and implantation/placental development. As has already been discussed, the collective functioning of these factors is essential to establish and maintain pregnancy. Therefore, any perturbation along the way can result in pregnancy loss. The following section will describe the major contributors to pregnancy loss in greater detail.

Oocyte Competency

Follicle size is one factor that has a direct effect on oocyte competence. Higher blastocyst rates are observed in oocytes retrieved from large follicles (2-6mm) and oocytes surrounded by more cumulus cell layers collected from large follicles [50, 51]. Additionally, higher cleavage rates (early embryonic cell division) and blastocyst number are noted when retrieving oocytes from >4-8mm (large) and >2-4mm (medium) follicles [52]. Conversely, small follicles had no day 7 blastocysts and low cleavage rates [52]. Oocytes collected from small follicles have reduced quality, validating the relationship

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between follicle size and oocyte quality [51, 53, 54]. There is a likely factor inherent in oocytes from small follicles that limits development, but the mechanism responsible for these differences are unclear. However, ultrastructural changes in the oocyte and estradiol limit achievement of developmental competence, thus maturation of the oocyte and exposure to estrogens seems to facilitate competency [51-53].

The specific phase of follicle development is another factor affecting oocyte competence. There is improved development and increased quality of transferable blastocysts from oocytes in the growth phase (day 2 and 10) compared to the dominant phase (day 7 and 15) of folliculogenesis [17, 54]. In addition, large follicles collected during the growth phase (day 3) have higher blastocyst development rates compared to oocytes from the dominant phase (day 7), which demonstrate lesser developmental potential [17].

Atretic follicles can impact oocyte competency positively or negatively, depending on the stage of atresia. Oocytes showing beginning signs of degeneration (Class 3), are more likely to develop than late atretic follicles [53]. Class 3 oocytes appear to be more differentiated and therefore are still able to obtain developmental competence. A small degree of cumulus expansion still allows a competent oocyte to form [55]. There are higher blastocysts rates in early atretic follicles with minor cumulus expansion, but not late atretic follicles [56]. The level of atresia and cumulus expansion is highly correlated with oocyte developmental potential, as demonstrated by the findings of these studies.

One major determinant of oocyte competency is the level of P4 that exists during follicle selection and dominance. Oocyte quality, rate of embryo development, and

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uterine activity is altered by low P4 levels [57, 58]. One time that low P4 exists is during

anestrous periods. Postpartum cows producing large amounts of milk may not be able

to support the early embryo as a result of elevated steroid metabolism and lower P4

concentrations, though a large volume of luteal tissue is present [59, 60]. Thus, lower

blastocyst rates are noted. Oocytes transferred to heifers have a higher blastocyst rate

of 33.9±3.6% compared to 18.3±7.9% blastocyst rate in postpartum cows, but no

difference in blastocyst quality was reported [60].

Uterine Development and Function

The positive interaction between the conceptus and uterus is necessary for

maintenance of pregnancy in the cow. Although most female reproductive organs are

fully developed at birth, the uterus is differentiated and developed postnatally in cattle

[61, 62]. Ruminants have a bicornuate uterus with a common corpus and single cervix

[63]. The endometrium in cattle and sheep consists of aglandular caruncular areas and

glandular intercaruncular areas, which provide nutrients to the developing embryo

through fusion of cotyledons, forming a placentome [64]. Adenogenesis, or the process

whereby uterine glands develop, involves the differentiation of lumen epithelium to

glandular epithelium and coiling and branching of germinal epithelium through stroma

[62]. Uterine glands synthesize and secrete or transport a variety of enzymes, growth

factors, cytokines, hormones, and proteins that provide histotrophic nutrition to the

conceptus [65].

Proper functioning uterine glands are essential for uterine receptivity, blastocyst

implantation, and embryonic growth in sheep and mice [62, 66-68]. In uterine gland

knock out (UGKO) ewes, blastocysts are present and development is normal, however

conceptuses are not detected in day 14 uteri flush [62]. Fertilization and transport defects were eliminated by transferring day 7 sheep embryos from normal, non-UGKO donor sheep thus confirming the importance of uterine glands for establishment of pregnancy.

The Progesterone-Uterus Link

Progesterone during the peri-implantation stage is critical for establishment and maintenance of pregnancy in ruminants. Early conceptus growth and development, and specifically elongation, is regulated by P4 acting on the uterus [57, 69-71]. Progesterone regulates endometrial receptivity and secretions by changing gene expression, which alters the composition of histotroph essential for conceptus survival and growth [72]. Histotroph is comprised of several hormones, cytokines, growth factors, lipids, amino acids and nutrients which are vital for embryonic development [31]. Larger day 14 conceptuses were found in P4-treated cows than in non-treated cows [57, 73]. Also, providing P4 early after ovulation can hasten conceptus development. Vaginal insertion of a P4-releasing device at day 3 of gestation enhanced P4 concentrations four- to five-fold and increased conceptus length in vivo. This treatment induced changes in the uterine environment that advanced elongation and increased interferon-tau concentrations [72, 74].

It may be possible to use this knowledge about P4's role in early pregnancy to improve pregnancy outcomes. One study also found that early P4 exposure increased pregnancy rates, but others found no such effect of this treatment [75, 76]. This type of treatment likely does not work effectively because although an advancement in conceptus development occurs, uterine events also are advanced, and cattle return to

estrus earlier than normal. Thus, any advantages seen in conceptus development likely will be offset by the shorted window for maternal recognition of pregnancy. However, it may be possible to enhance pregnancy retention by providing supplemental P4 later in early pregnancy. In one recent study, P4 supplementation beginning at day 4, and again at day 7 post-AI improved pregnancy per AI rates when cows were inseminated following estrus detection [77]. When a timed-AI protocol was implemented, P4 supplementation beginning at day 4, or during metestrus, improved pregnancy per AI rates. Supplemental P4 is beneficial to improving pregnancy rates, but the magnitude of benefit depends on stage of the cycle and days post-AI when supplementation occurs.

Maternal Recognition of Pregnancy

Improper IFNT signaling during MRP leads to pregnancy loss, with losses during this period averaging around 30% [78]. There are several potential reasons as to why MRP might fail. First, the developing embryo must fend off the maternal immune response that attempts to terminate pregnancy because of the paternally inherited proteins [78]. Irregular elongation, insufficient histotroph, and disruption in trophectoderm formation can also restrict IFNT signaling by the embryo. Finally, suboptimal circulations of P4 during the early luteal phase, which suppress embryonic growth, reduce pregnancy success during the MRP period [72].

One measurable means for determining how well IFNT is acting in early pregnancy is to examine how it influences the expression of ISGs in peripheral leukocytes [46, 79]. Transcripts for these ISGs are measured in the maternal peripheral blood leukocytes (PBL) and are used to identify non-pregnant cattle [46, 80]. Increases in ISG mRNA abundance can be detected as early as day 17-18 of pregnancy, but the

greatest amount of ISG are seen on days 20-22 of pregnancy, likely due to the conceptus secreting larger quantities of IFNT at this time [80]. Mx1, Mx2, and ISG-15 are a few of the most common ISG detected in PBL [46, 81, 82].

Implantation and Placental Failures

Poor placental development during gestation will ultimately cause pregnancy failures. This impaired development can result from improper implantation, poor fetal/maternal interaction, and reduced nutrient exchange. The best way to examine placental fitness and fetal well-being is to look at PAG concentrations.

Pregnancy associated glycoproteins are aspartic proteases that are secreted into the bovine maternal bloodstream throughout pregnancy from binucleate cells of the placenta [83-86]. Measured through presence of pregnancy specific proteins in plasma, PAG are used as pregnancy predictors by day 24 of pregnancy. Plasma PAG concentrations increase around day 24, peak at approximately day 30 of gestation, and decline through the second month of gestation before increasing again in late pregnancy [87]. Pregnant cows that will maintain their pregnancy to term have greater plasma PAG concentrations at day 30 than cows that will experience late embryonic mortality [87, 88]. However, cows with late fetal losses have relatively normal PAG concentrations at day 32 [87, 88].

High circulating PAG concentrations may also be indicative of impending pregnancy loss compared to average PAG concentrations [89]. These increased PAG concentrations could be due to the mother overcoming placental insufficiency, ultimately destructing the fetal-placental unit and releasing the placental products into circulation

[88]. Concentrations of PAG are associated with parity, AI service number, milk production, and metabolic diseases linked with pregnancy failure [88, 89]. Breed is also associated with PAG concentration. This concentration difference is noted in Brangus cattle, which have higher concentrations than Angus cattle throughout pregnancy [86].

Another way to quantify the normal or abnormal progress of pregnancy is to examine fetometric growth parameters, such as crown-rump length (CRL) or crown-nose length (CNL) [90]. Crown-rump length is the distance between the highest point of the head, or crown, and the most caudal extent of the buttocks at the tail head [91]. The entire bovine fetus can be scanned in month 2 of gestation, with accessibility of the thorax, abdomen, and pelvis declining to about 50% in months four and five [92]. Crown-rump length is limited approximately to day 70 of gestation as fetuses larger than 10 centimeters are difficult to project onto the screen [92]. The length of the head has linear correlation with CRL and therefore can be substituted for CRL if hard to obtain [90]. In Kahn's [92] study, the head was accessible 80% of the time and correlated with fetal age. Crown-rump length showed the least variation and highest correlation to age of pregnancy [92]. Thus, the size of the bovine fetus is directly proportional to fetal age and demonstrates linear growth throughout gestation.

Physiological and Environmental Contributors to Pregnancy Losses in Cattle

These common causes of pregnancy loss can manifest in several ways. The animal's metabolic status, especially the occurrence of a negative energy balance, can impact various aspects of the reproductive process. For example, the developing embryo is very sensitive to stress during early pregnancy. In addition to metabolic stress, heat stress and other environmental factors can negatively affect embryo

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competence. Clinical and subclinical periparturient diseases are also major issues in high producing dairy cows. The incidence of multiple diseases further reduces conception and pregnancy rates. This section will describe these and other major contributors to pregnancy loss in cattle.

<u>Metabolic Status</u>

Metabolic status of the cow, such as high nutrition, negative energy balance, and body condition score, greatly affects embryonic and fetal survival. Cattle grazing in a high-energy pasture prior to AI have more than a 30-percentage point reduction in embryo survival compared to cattle grazing a lower energy pasture [93]. By contrast, during periods of extreme negative energy balance, intake is insufficient for general body maintenance, therefore reproductive parameters and oocyte quality are greatly compromised [94]. Body condition loss is another aspect that significantly affects fertility. Underweight cows are subjected to longer periods of anestrous, lengthening the breeding interval [95]. Additionally, a one unit drop in body condition score from calving to 30 days post-partum increases the odds ratio for pregnancy loss by 2.41-fold [96].

Another important facet of metabolism is how it may control circulating steroid hormone concentrations. A high plane of nutrition (i.e. consumption of high quantities of feedstuff, and especially grains) will reduce circulating estradiol and P4 concentrations in cattle [97]. This occurs, at least in part, because the high metabolic activity of the liver inadvertently increases steroid hormone metabolism, and this reduces circulating concentrations of these hormones [93, 97].

This scenario of lowering steroid hormones is especially dramatic in lactating dairy cows. The increased feed intake needed to supply the energy requirements for lactation and resulting liver steroid metabolism are likely major contributors to infertility. Reductions in estradiol adversely affect ovarian dynamics, and specifically follicle development, creating a smaller follicle containing a less competent oocyte [97]. Reductions in P4 are thought to reduce uterine activity, and reductions in uterine histotroph production likely reduce conceptus development and hinder maternal recognition of pregnancy success [72, 74, 78].

<u>Environmental Factors</u>

One environmental factor that can have dramatic adverse effects on fertility is heat stress. To manage the effects of heat stress, cattle exert energy to maintain body homeostasis and dissipate heat, which negatively impacts reproductive parameters. The effects of heat stress can be attributed to decreased thermoregulatory ability, hyperthermia, or elevated uterine temperature [98, 99]. The temperature at which stress events begin, or the upper critical temperature, is 25-26°C in high-yielding dairy cows [100].

Several facets of reproduction are adversely affected by heat stress. Folliculogenesis, specifically follicular development and dominance, is altered during high heat exposure, and this decreases oocyte and subsequent embryo quality [101, 102]. Oocytes are extremely temperature sensitive, and any perturbation can inhibit their development [103]. Heat stressed oocytes have reduced competence to develop into blastocysts as the uterus is unable to support embryo metabolism [98, 104].

Additionally, uterine blood flow is decreased, diminishing the delivery of nutrients to support oocyte development while increasing uterine temperature [105].

Heat stress also contributes to reduced reproductive efficiency by limiting reproductive estrus expression/detection and decreasing conception rate [5]. There are decreases in estradiol-17β concentrations in heat stressed cows, and these reductions in estradiol limit estrus behavior [101, 102, 106, 107]. Mounting activities are especially reduced during the summer not only by reduced hormonal expression but physical lethargy [108].

There also are major problems with early embryo development during heat stress. Final oocyte maturation and ovulation are disrupted by elevated temperatures, inhibiting subsequent embryo development. Embryos retrieved from cattle heat stressed on day 1 post-estrus have decreased viability and development [109]. Additionally, there is a reduced percentage of embryos at the blastocyst stage of development when heat stress occurs on day 1 post-estrus. There is no effect of heat stress on embryonic development when stress occurs on day 3, 5, or 7 [109]. This is indicative of the embryo's developmental resistance and thermotolerance to maternal heat stress.

One way to overcome many of the detrimental effects of heat stress on the oocyte and early embryo is through ET. Transferring embryos from non-heat-stressed cows into heat stressed recipients increases pregnancy rates [110]. Prior to EGA, heat stress alters steroid synthesis, disrupts oocyte maturation, and blocks development at the 1-4 cell stage embryo. Once EGA occurs, the embryo becomes heat resistant and can defend itself from the detrimental effects heat stress imposes on subsequent

embryonic growth. Defense against heat stress improves as pregnancy progresses, therefore ET bypasses the most heat sensitive time and allows proper embryonic development [108, 109].

Fescue toxicosis is another environmental factor that causes infertility in cattle. Endophyte-infected fescue contains *Acremonium coenophialum*, a fungus that resides in fescue grass which produces toxins termed ergo alkaloids [15]. Cattle grazing infected fescue have reduced pregnancy rates [15, 111]. The mechanisms by which fescue toxicosis reduces reproductive performance are not fully understood, but at least two major causes for infertility exist. The first is a potential direct effect of alkaloid toxins on oocyte and embryo development [112]. Early stage embryos are extremely sensitive to ergot alkaloid and pregnancies are typically lost before day 30 of gestation [112]. A contributing, secondary factor to this infertility occurs because fescue toxicosis heightens thermal sensitivity. Cattle feeding on infected fescue spring and summer months are undoubtedly less fertile because of the damaging effects increased body temperature has on embryonic survival [15].

Clinical/Subclinical Diseases

Clinical and sub-clinical periparturient diseases are associated with reduced reproductive performance in cattle [113]. The prevalence of periparturient diseases in dairy cattle range from ~8-50%, depending on the disease. Inflammation, discomfort, and fever are characteristic for disease-ridden cows. Elevated body temperature and a suboptimal uterine environment increase the risk of pregnancy loss, as the cow is unable to achieve and maintain a normal pregnancy with these issues.

Mastitis is one of the most prominent diseases affecting lactating dairy cows [114]. Clinical mastitis is identified by alterations in milk composition and appearance, decreased milk production, swelling or heat in the infected quarters, or elevated body temperature [115]. There is a decreased conception rate both at first postpartum AI and in overall pregnancy rate in cows experiencing clinical mastitis [116]. Another study determined that cows with clinical mastitis between the time of AI to pregnancy confirmation were 2.8 times more likely to lose the pregnancy than cows without mastitis [117]. Also, conception rates were reduced in cows experiencing clinical or subclinical mastitis at insemination, and cows that did conceive experienced greater embryonic losses than non-infected cows [116, 117]. Subclinical mastitis, as defined as the presence of the pathogen but no visual abnormalities in the milk, has detrimental effects on reproductive performance. Days open, days to first service, and services per conception are increased in cows diagnosed with subclinical mastitis [115].

The mechanism by which clinical or subclinical mastitis affects reproductive performance is not known. Perhaps adverse consequences resulting from mammary gland inflammation changes endocrine profiles. Also, fever associated with some clinical mastitis events could compromise oocyte and embryo developmental potential.

Retained placenta, metritis, and ovarian cysts are other factors that contribute to lower conception rates in cows [113]. Additionally, milk fever is associated with reduced conception rate and endometritis results in delayed days to first service and greater services per conception [117, 118].

Pregnancy outcomes in dairy cattle are more profoundly reduced in cows experiencing two or more periparturient disorders [88, 119, 120]. Healthy dairy cows have a 51% pregnancy rate, whereas cows one case of a disease have a reduced pregnancy rate of 43% [119]. The incidence of more than one disease further reduces the pregnancy rate to 34% [119]. Also, there is a clear link between peri- and postpartum cow health on subsequent fertility, as seen with increased risk of pregnancy loss. These losses typically occur within the first 60 days in cattle with disorders [119]. There is a 40% prevalence rate across cows affected by multiple clinical and subclinical diseases [119, 120]. Consequently, a further reduction in reproductive efficiency is noted in cows affected by more than one disease.

The mechanisms that cause more profound infertility and pregnancy losses in these cattle are unclear. These diseases adversely affect milk production, body condition and overall animal health, therefore it is likely that the overall severity and duration of each disease state has indirect effects on reproductive processes. Long-term health effects can remain in cattle with disorders, which ultimately carry over and inhibit proper reproductive function. Resulting limitations in pregnancy status may occur because cattle are not at a physiological state required for optimal reproduction.

Using Bovine *In Vitro* Fertilization to Study Early and Late Embryonic Losses

The high incidence of embryonic losses has prompted the utilization of *in vitro* bovine embryo production to examine potential reasons for these losses. This laboratory uses an *in vitro* bovine embryo production system to study bovine embryo development. In vitro production (IVP) of embryos (fertilization and culture of embryos in a laboratory setting) is gaining popularity globally as a means for improving genetic

potential in beef and dairy cattle. Ovum Pickup (OPU) and IVF methods have been implemented in South America, more specifically Brazil and Argentina, where production and transfer of IVF embryos jumped from 2,858 in 2010 to over 400,000 in 2013. [2]. We are using IVF-ET as a model for investigating ways to improve embryo competency.

We have focused efforts on examining whether three uterine-derived growth factors can be used to encourage early trophectoderm development in bovine embryos. These factors are epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor 1 (IGF1).

Epidermal Growth Factor

Epidermal growth factor (EGF) is a uterine produced factor that stimulates cellular proliferation, differentiation, and migration in various cell types [121, 122]. EGF is expressed in the peri-implantation mouse uterus and enhances blastocyst formation, expansion, and zona hatching [123]. Receptors for EGF are present in the early mouse embryo between the 4- and 8-cell stage and the day 13-14 porcine conceptus trophectoderm [124, 125]. In vitro maturation of mouse, rat, human, porcine, and bovine embryos is encouraged with the addition of EGF to medium [126]. Specifically, addition of EGF in vitro increases protein synthesis and enhances differentiation in the mouse embryo [127]. In the bovine trophoblast cell line, EGF improves cell numbers and enhances cell proliferation, motility, and migration [128-130]. Furthermore, EGF stimulates proliferation and motility of bovine trophoblast cells during the post-implantation stage [131]. Also, as will be discussed later, early bovine embryonic and conceptus development is mediated by EGF [131].

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Fibroblast Growth Factor-2

Fibroblast growth factor 2 (FGF2), part of the FGF superfamily, is a locally produced hormone expressed by the uterine epithelium [131, 132]. The FGF family function as key regulators of cell proliferation, differentiation, and migration [133]. They also positively influence embryonic and trophectoderm development [133, 134]. Basic fibroblast growth factor (bFGF), FGF2, and FGF7 are detected in the uterine luminal fluid of mice, sheep/cattle, and pigs, respectively [132, 133, 135-137]. FGF2 and its receptor are present on bovine conceptuses during early development [132, 134]. The rise in FGF2 concentrations coincides with conceptus elongation and increased interferon-tau concentrations [136]. In ruminants, FGF2 stimulates IFNT mRNA expression and protein levels in trophectoderm [130, 132, 134, 136].

Insulin-like Growth Factor 1

Insulin-like growth factor I (IGF1) stimulates tissue growth, mitogenesis, proliferation, differentiation, and survival in various cell types [138, 139]. Both IGF1 and insulin like growth factor-II (IGF2) are produced by placental cells during pregnancy and levels of expression are maximal during the peri-implantation stage of embryo development [138, 140]. Reduction in IGF expression has been implicated in intrauterine growth restriction [138]. Moreover, IGFs regulate peri-conceptus and placental development in humans, rodents, and domestic animals [139, 141]. The addition of IGF1 to porcine trophectoderm cells (pTr) increases cell numbers and stimulates cell migration [139]. Furthermore, migration of ovine trophoblast cells (oTr) to the maternal epithelium is stimulated with IGF2 treatment [141]. Lastly, bovine

blastocyst and hatched blastocyst development is increased with the addition of IGF-I [126].

It also is noteworthy to mention that IGF1 improves post-transfer embryo survival in cattle [142]. Addition of IGF-1 advances development of bovine embryos to the blastocyst stage and improves blastocyst number. These advancements are especially noted in heat stress situations. The treatment of bovine preimplantation embryos improves resistance to heat shock by reducing effects of raised temperature on blastomere apoptosis and development to the blastocyst stage [142]. In bovine embryos, IGF1 regulates cell survival through the Akt-dependent pathway that prevents apoptosis [143]. The mechanism by which IGF1 works is not well understood, but the improvement in cell numbers by the addition of IGF1 may be able to block the increase of PGF2α through increased interferon-tau production. The improvements in cell number, pregnancy rates, and calving rates of IGF1 treated embryos are only significant during heat stress, demonstrating an interaction between season and culture with IGF1 [142].

Multiple Uterine Factor Supplementation Schemes

Although individual growth factor treatments have been successful in improving embryo competency, even greater improvements in developmental potential of bovine embryos may be possible by combining these uterine factors together during embryo culture. Xie et al. [131] reported that combining EGF, FGF2 and IGF1 treatments enhances the proliferation trophoblast CT1 cell line. This combination treatment also increases bovine IVP blastocyst rates, and increases trophoblast cell numbers in these blastocysts [131]. The percent of hatched blastocysts and blastocyst diameter are

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increasingly improved with the growth factor treatments, suggesting an additive effect of EGF, FGF2, and IGF1 [131]. Other examples of this phenomenon also exist. For example, combining IGF1, IGF2, FGF2, transforming growth factor-β (TGF-β), granulocyte-macrophage colony-stimulating factor (GM-CSF), and leukemia inhibitory factor (LIF) promote IVP bovine embryo development to the blastocyst stage [126]. Higher rates of blastocyst and hatched blastocyst development on day 7 and 8 and increases in overall cell numbers were also observed following this treatment [126].

To date, no information about post-transfer survival of embryos supplemented with multiple growth factors *in vitro* is available. Success of IVP bovine embryo development with the addition of various growth factors has been established, however these embryos have not been transferred to examine the effects on fetal/maternal fitness. Additionally, early indicators of pregnancy success such as PAGs and fetal CRL prove useful to predict success outcomes of IVP embryos without needing the hundreds of cows required to examine calving outcomes.

Summary and Concluding Remarks

Pregnancy loss has grave economic repercussions to producers, therefore it is imperative for cows to achieve and maintain pregnancy. The majority of losses occur during the early embryonic stage when the embryo fails to implant and signal its presence to the maternal system. Any perturbations throughout normal pregnancy progression can contribute to pregnancy loss. Metabolic status, environmental factors, and periparturient diseases are major factors impeding early embryonic development. Limiting various maternal stress effects is key to ensure successful embryonic development. Addition of a growth factor cocktail (EGF, FGF2, and IGF1) to IVP bovine

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embryos increases trophoblast cell number and blastocyst development. Placental development is mediated through trophoblast cells and is imperative for fetal growth and nourishment. Embryo competency is improved in vitro, however the effects of growth factor treatment on subsequent pregnancy establishment and maintenance and embryonic/fetal health remains to be explored.

Hypothesis and Objectives

The work completed in this thesis will expand upon previous observations made by this laboratory on combining uterine factors during IVP embryo production as a means for improving embryo competency. This research will test the hypothesis that promoting trophectoderm and thus placental development during early IVP embryo development will encourage positive interactions between the placenta and maternal system, and this will ultimately improve pregnancy retention in cattle. Animal number limitations prevent completion of an ET study where pregnancy rates throughout gestation can be calculated. Rather, alternative indicators of pregnancy success/failure will be examined in this work. The specific objectives of this study were to:

1. Determine if supplementing EGF, FGF2, and IGF1 to IVP bovine embryos improves the proportion of transfer-quality bovine embryos and the trophectoderm size of these embryos at day 7 of development, and

2. Determine if supplementing EGF, FGF2, and IGF1 to IVP bovine embryos influences fetal size and circulating PAG concentrations during early pregnancy.

Chapter 2 – Post-transfer Developmental Effects of Bovine Embryos Supplemented with Epidermal Growth Factor, Fibroblast Growth Factor 2, and Insulin-Like Growth Factor 1

Introduction

Pregnancy loss is one of the most predominant issues facing the dairy and beef cattle industries, causing reduced production efficiency and economic loss. Failure of lactating dairy cows to establish and carry a pregnancy to term results in an average loss of $555 per pregnancy [1]. Executing management strategies to minimize pregnancy losses is essential to reduce economic losses. Various environmental and physiological factors contribute to pregnancy loss, with uterine function being one of the major contributors to infertility. The use of various assisted reproductive techniques, namely ET, have been implemented to evade the damaging effects suboptimal uterine environments and other environmental and physiological factors have on embryonic development. *In vitro* production of bovine embryos and ET is gaining popularity globally as a means for improving genetic potential in beef and dairy cattle [2]. We are using IVF-ET as a model to further investigate ways to improve embryo competency and subsequent pregnancy maintenance.

There are three primary stages of pregnancy loss established by the Committee on Bovine Reproductive Nomenclature (1972). Losses are typically reflected in embryonic and fetal mortality. The first stage is early embryonic death, which encompasses the period of undetected losses that occur within the normal range of an estrous cycle (16-24 days) in cattle [3]. Losses during this period result from failures in embryonic genome activation, elongation, and maternal recognition of pregnancy. The second stage of pregnancy loss is termed late embryonic death which occurs between

25 and 42 days of gestation [3]. During this period, corpus luteum (CL) function is

maintained beyond a normal estrous cycle length. Failures in initiation of implantation,

placentome formation, or completion of embryonic differentiation can result in

pregnancy loss during this period [7]. The third period of pregnancy losses, which

occurs after day 42 when a fetus is distinguishable, are termed fetal losses [3]. The

causes for losses during this period generally go undetermined if not caused by known

infectious agents. Of the three stages, the majority of pregnancy losses occur during the

early embryonic stage [4]. Improvements in early embryonic development to reduce

these early pregnancy losses must, therefore, be explored.

This work focused on examining whether three uterine-derived growth factors

can be used to encourage early trophectoderm development in bovine embryos and

limit early embryonic loss. Epidermal growth factor (EGF), fibroblast growth factor 2

(FGF2), and insulin-like growth factor 1 (IGF1) are uterine produced growth factors

involved in cell proliferation, differentiation, and migration [121, 132, 138]. These

specific growth factors play a role in early bovine embryonic and conceptus

development. Increasing concentrations of EGF and FGF2 coincide with trophectoderm

development and conceptus elongation [130, 136]. Cell survival, peri-implantation

embryonic growth, and placental development is regulated by IGF1 [138].

Although individual growth factor treatments have been successful in improving

embryo competency, even greater improvements in developmental potential of bovine

embryos may be possible by combining these uterine factors together during embryo

culture. Combining EGF, FGF2, and IGF1 treatments enhances the proliferation of the

trophoblast CT1 cell line [131]. This combination treatment also increases bovine IVP blastocyst rates and increases trophoblast cell numbers in blastocysts [131].

This work will further pursue the addition of uterine-derived factors, which are known to improve embryonic development, to embryos prior to transfer. We hope to promote trophectoderm/placental development, encourage positive fetal and maternal interactions, and ultimately improve pregnancy rates and retention. Due to limited animal numbers, cows were aborted for reuse in future replicates. Markers were utilized to determine pregnancy success and fitness. These markers were circulating pregnancy associated glycoprotein (PAG) concentrations and crown-rump lengths (CRL), indicators of early placental and fetal fitness, respectively.

Methods

A timeline of events used for this study is provided in Figure 2-1.

Estrous Synchronization

A CIDR-Ovsynch protocol was used to synchronize beef cows [144]. The protocol was initiated by administering GnRH (100 μg; Cystorelin, Merial, Duluth, GA) and inserting an intravaginal progesterone device (Eazi-Breed CIDR containing 1.38 g P4, Zoetis Animal Health, New York). After 7 days, the insert was removed and PGF2α (25 mg; Lutalyse, Zoetis Animal Health) was administered intramuscularly. Approximately 55-60 hours after the Lutalyse injection, GnRH was administered. Cows were observed for estrus expression (day -1). Approximately one-third of the cows were artificially inseminated (AI) the morning following GnRH injection (day 0). The remaining cows were slated for ET. A multi-sire pool of Holstein semen was used for each AI. This same semen pool was used for in vitro fertilization (described below).

In vitro Bovine Embryo Production

The IVP of bovine embryos was completed as described previously [131, 145]. Bovine oocytes were purchased from DeSoto Biosciences (Seymour, TN). These oocytes were derived from Brown Packing (Gaffney, SC), and cumulus-oocyte complexes (COCs) were isolated and shipped overnight in a transport incubator at 38.5 °C. Individual tubes containing COCs were gassed with 5% CO_2 in air before sealing. Cumulus-oocyte complexes were transferred to fertilization medium, and exposed to BoviPure™ gradient-purified (Nidacon, Spectrum Technologies, Healdsburg, CA) bovine spermatozoa derived from the pool of frozen semen from 3 Holstein bulls described previously. After 18 h at 38.5°C in 5% CO_2 in humidified air, cumulus was removed by vortexing, and groups of 20-30 presumptive zygotes were placed in 50 µl drops of SOF containing 20 µg/ml essential amino acids (Sigma-Aldrich), 10 µg/ml nonessential amino acids (Life Technologies), 4 mg/ml fatty acid free bovine serum albumin (Sigma-Aldrich), and 25 µg/ml gentamicin sulfate (Life Technologies), and cultured at 38.5°C in 5% O_2, 5% CO_2, 90% N_2 [145]. At day 4 post-fertilization, ≥8-cell embryos were harvested, pooled and exposed either to the EFI treatment (10 ng/ml EGF, 10 ng/ml FGF2, 50 ng/ml IGF1) or carrier only (1% [w/v] bovine serum albumin). At day 7, embryo stage and quality were assessed. Only embryos that were categorized as compact morulae, blastocysts or expanded blastocysts and contained quality scores of 1 or 2 were used for ET [146].

Embryo Transfer

At day 7, individual transferable embryos exposed to either the EFI treatment or carrier only were placed into ET straws, maintained in a shipper incubator during

transport to the VA Tech Beef Center, and transferred to beef cattle at day 7 post-estrus. Transrectal ultrasonography was completed to determine the presence and location (left or right ovary) of a functional corpus luteum (CL). Those cows with no CL were excluded from the study. Information about the specific stage and number of embryos transferred within each treatment group is shown in Figure 2-2.

For ET, an epidural was administered using Lidocaine (5mL; VEDCO, St. Joseph, Missouri) injected into the spinal junction to aid manipulation of the cervix and uterine horns. The ET straw was loaded into an ET rod and prepared for transfer. The vulva was cleaned and spread for insertion of the rod by the technician. Embryos were placed in the upper one-third of the uterine horn ipsilateral to the CL.

Pregnancy Diagnosis

Pregnancy was diagnosed on day 28 post-ovulation using an Ibex portable transrectal ultrasound, equipped with a linear 8–5 MHz multifrequency transducer (E.I. Medical Imaging, Loveland, Colorado). A functional CL, presence of the amniotic sac (determined as fluid accumulation within the uterus) and presence of an embryo were features used to determine pregnancy.

Pregnancy Associated Glycoproteins

Blood samples from pregnant cows were collected via coccygeal vein on days 28, 42, and 56 of gestation. Presence of pregnancy associated glycoproteins (PAGs), and specifically pregnancy specific protein B (PSPB), was determined from blood plasma. The BioPRYN Quantitative PSPB ELISA (Enzyme-Linked Immunosorbent Assay) (BioTracking, Inc, Moscow, ID) was used for PSPB quantification. Standards of known PSPB concentrations were run with each assay to determine the concentration

of PSPB in each sample and all solutions needed for the antigen-capture ELISA were included in the kit. Study samples and kit standards were run in duplicate. Briefly, sample buffer, bovine plasma, and standards were added to antibody coated wells. The plates were incubated overnight (>5 hours) at room temperature (25°C). After the sample incubation, the plate was washed using a blot dry method. Next, buffer solution containing an antibody (Detector Solution) was added to the wells and incubated for one hour at room temperature. Following incubation, plates were washed and enhancer solution containing horseradish peroxidase enzyme (HRP) was added to wells and incubated for one hour. After the hour incubation, wells were washed and indicator solution containing TMB Substrate was added for 15-minutes. Then, stop solution was added and plates were read at a wavelength of 640nm using a Tecan Infinite Pro (Tecan Trading Ag, Switzerland) plate reader. The optical density plate reading was exported into the excel sheet provided by BioTracking for analysis.

Crown-Rump Length Determination

Transrectal ultrasonography was completed using an Ibex portable ultrasound, equipped with a linear 8–5 MHz multifrequency transducer (E.I. Medical Imaging, Loveland, Colorado) on all cows at gestational days 42 and 56. Fetal crown-rump length (CRL) was measured on day 42 and 56 and crown-nose length (CNL) was measured on day 56 [86]. Fetal CRL is classified as the distance from the top of the head (crown) to the base of the tail (rump). Fetal CNL is the distance from the crown to the tip of the nose. Additionally, fetal sex was determined on day 56-61 [8].

Replication of the Study

Three replicates of the study were completed. In the first replicate, 8 cows were AI and 14 cows received embryos (6 control and 8 EFI). For the second replicate, 9 cows were AI and 19 cows received embryos (10 control and 9 EFI). Finally, for the third replicate, 5 cows were AI and 19 cows received embryos (9 control and 10 EFI). Pregnancies were aborted by administering Lutalyse post-fetal sexing to permit the reuse of cows for subsequent study replicates. Termination of pregnancy was verified by ultrasound and cows were given a minimum of 4 weeks to allow sufficient uterine recovery. To avoid effects of heat stress, replicates were completed outside of the summer months.

Statistical Analyses

All data were analyzed using Analysis of Variance (ANOVA) with the General Linear Model (GLM) of the Statistical Analysis System (SAS). Main effects in the model included treatment group (AI vs. ET groups), replicate, and day (when appropriate). Pairwise comparisons were completed using the Probability of Difference (PDIFF) option in SAS. Data are presented as arithmetic means and standard errors of the mean (SEM).

Results

Stage of embryo development (morula, blastocyst, expanded blastocyst) was determined in control and EFI (10 ng/ml EGF, 10 ng/ml FGF2, 50 ng/ml IGF1) embryos on day 7 prior to ET (Fig. 2-2A). No differences were noted between ET groups at the morula or blastocyst stages of development. There was, however, a greater percentage of advanced, expanded blastocysts in the EFI group compared to the control group

(P<0.05). Embryo quality was also assessed on day 7 before transfer (Fig. 2-2B). The EFI group had a greater percentage of transferable embryos than the control group (P<0.05).

Pregnancy diagnosis was performed at day 28 (Fig. 2-3A). There were no differences in pregnancy rate at day 28 post-estrus among the AI and ET groups (EFI and control). Pregnancy rate at day 56 was not different, however 3 control ET and 2 EFI ET pregnancies were lost by day 56 (Fig. 2-3B).

The main effects of treatment and fetal sex on circulating concentrations of PSPB were examined on day 28, 42, and 56 (Fig. 2-4 and 2-5). There were no differences detected in concentrations at day 28 (Fig. 2-4A), day 42 (Fig. 2-4B), and 56 (Fig. 2-4C) among the AI and ET groups. There also were no main effects of fetal sex on circulating PSPB concentrations on day 28 (Fig. 2-5A), day 42 (Fig. 2-5B), and day 56 (Fig. 2-5C).

The interaction between fetal sex and treatment group on circulating PSPB concentrations at day 28, day 42, and day 56 was also examined (Fig. 2-6). No differences between treatment groups and fetal sex were evident at day 28, but there was a tendency for a sex by treatment interaction effect in cows with control embryos at day 28 (Fig. 2-6A). Heifer pregnancies tended to have lower circulating PSPB concentrations than bulls in the control embryos (P= 0.08). A treatment by fetal sex interaction was present at day 42 (Fig. 2-6B). This effect was observed in the control ET group, where cows with bull fetuses contained greater (P <0.05) PSPB concentrations than cows with heifer fetuses. Also, cows carrying heifers from control embryos contained less (P < 0.05) PSPB than cows with bull fetuses in the EFI ET group. Differences in circulating PSPB concentrations also existed at day 56 (Fig. 2-6C).

Again, this effect occurred because cows with bull fetuses from control embryos had greater (P < 0.05) circulating PSPB concentrations than pregnancies with heifer fetuses in the control group. No other differences among the groups were detected.

Crown-rump lengths (CRL) were measured via transrectal ultrasonography at day 42 and day 56 (Fig. 2-7). At day 42, there were no differences in CRL between treatment groups (Fig. 2-7A). There were differences in CRL at day 56 (Fig. 2-7B). The AI group had larger (P <0.05) CRL than both ET groups. There was no effect of fetal sex on CRL was noted at day 42 (Fig. 2-8A) or day 56 (Fig. 2-8B).

The interaction of treatment and fetal crown-rump length (CRL) on day 42 and day 56 also was assessed (Fig. 2-9). There were no effects of treatment group and fetal sex on CRL at day 42 (Fig. 2-9A). At day 56, there were CRL differences among treatment groups (Fig. 2-9B). Heifer fetuses from AI cows were larger (P < 0.05) than bull fetuses from cows receiving either a control or EFI embryo.

Discussion

This work established the beneficial effects of combinatorial growth factor supplementation in bovine embryo culture. The basis for this work stemmed from the increasing global use of IVF and OPU to improve genetic potential of IVP bovine embryos [2]. *In vitro* supplementation of a combination of growth factors (EGF, FGF2, and IGF1) has been previously studied in this lab [131]. However, embryonic development of IVP bovine embryos post-transfer has not been established. The focus of this research was to examine the embryonic and placental developmental of IVP-growth factor supplemented bovine embryos post-transfer.

The specific growth factors used in this study are involved in cell proliferation, differentiation and migration during early embryonic development. Supplementation of embryo culture media with EGF, FGF2, and IGF1 advanced embryo development and increased the number of transferable embryos. The *in vitro* embryonic development observed with growth factor supplementation was comparable to previously completed studies. Addition of IGF1, IGF2, FGF2, TGF-β, GM-CSF, and LIF promoted IVP bovine embryo development to the blastocyst stage and increased overall cell numbers [126]. These results suggest cooperative actions of multiple growth factors to improve embryo development *in vitro* and enhance the transferable quality.

There have been mixed findings on post-transfer consequences (maternal and fetal) of IVP bovine embryos. Pregnancy loss during the late embryonic and fetal period and abnormal placental development has been reported in cattle with IVP embryos [147, 148]. Heavier fetal weights, birth weights, and higher incidence of dystocia have also resulted from IVP embryos [149-151]. However, these developmental outcomes

have not been observed in IVP-derived offspring in more recent publications [152, 153]. Due to limitations in animal numbers, pregnancy associated glycoproteins (PAG) and crown-rump length (CRL) were used as alternative markers to examine placental fitness and fetal well-being.

Circulating concentrations of PAG of pregnant cows, specifically pregnancy specific protein B (PSPB), were analyzed at gestation days 28, 42, and 56. Concentrations of PAG typically increase around day 30 of gestation and then gradually decrease until late gestation [87]. In this study, concentrations of PSPB tended to follow a similar pattern, with the greatest average concentration across groups at day 28, and lowest concentrations at day 56. However, PSPB concentration levels were higher across days (~4-6 ng/ml) than previously reported concentrations (~2-3 ng/ml) analyzed by the same ELISA [154]. This difference could be attributed to the variation in cattle population. Non-lactating beef cows were used in this study, whereas lactating beef cattle were used in the previous study [154]. The reduced PAG concentrations in lactating cattle may reflect the elevated degradation of PAG in the liver or clearance by the kidney [155].

Lower PAG concentrations are typically associated with pregnancy loss [88]. Cattle experiencing embryonic losses (before day 42) tended to have lower circulating concentrations of PSPB compared to pregnant cows in the present study (data not shown). However, cattle losing pregnancy by day 56 had normal PSPB concentrations. This association could suggest PAG production is important for early placental development, but the circulating concentration is not indicative of fetal losses likely due to placental insufficiencies [3]. Additionally, PSPB concentrations did not differ between

the AI group and ET groups. These findings indicate sufficient placental support and development in cows receiving an IVP embryo, contrary to previous reports [148].

The effect of fetal sex by treatment on circulating PAG concentrations was also examined in this study. Interestingly, bull fetuses had higher circulating concentrations, but only within the control ET group. This could potentially be a carryover effect of *in vitro* embryo culture. Male embryos typically develop quicker *in vitro* and the advanced development could result in more active binucleate cells secreting PAG. Additionally, at day 42 the growth factor treated (EFI) heifer embryos had higher PAG concentrations than control heifer embryos. Again, this could be a result of culture conditions and improved development in the EFI embryos. Interestingly, higher PAG concentrations were not observed between male embryos of the ET groups. Taken together, these results suggest a potential improvement in placental fitness of EFI heifer embryos compared to control embryos. Because male embryos are more advanced *in vitro*, this effect may not be as noticeable.

Crown-rump length measurements are used to evaluate fetal age and developmental status [90]. Fetal size is not different during early development, however subspecies differences in fetal development are noted at day 54/55 of gestation [86]. Angus cows contain larger fetuses than Brangus cows at day 54/55 [86]. This deviation could be related the shorter gestation length in Angus cattle compared to Brangus cattle that have longer gestations and more time to develop. Due to these findings, we hypothesized any variations in fetal development between the AI and ET groups would be noted by day 56. Fetal CRL at day 42 was not different among groups, but at day 56 the AI fetuses were larger than both the EFI fetuses and control fetuses. Growth of ET

fetuses was not retarded or advanced which suggests addition of the growth factors improves early embryonic development, but these effects are nonexistent later in development.

There was no interaction of fetal sex and CRL at day 42, but CRL appeared to be influenced by fetal sex among treatment group at day 56. The AI cows carrying heifer fetuses had longer CRL than bull fetuses from both ET groups. In bovine embryo culture, male embryos are programmed during early development to be increasingly stress resistant and develop quicker than female embryos [156]. Although male embryos develop earlier *in vitro*, the culture conditions are suboptimal. After transfer, the embryo must adjust to the optimal *in vivo* environment and use more energy for survival rather than growth, which is likely why shorter CRL are seen in ET embryos. Compensatory growth could be attributed to the weight gained by the ET calves to reach normal birth weight. Bull calves tend to have longer gestation lengths and greater birth weights than heifer calves [157]. Additionally, calves produced by ET are similar in size at birth to conventional AI calves, but are more likely to experience alterations in birth weight [153]. Early embryo culture conditions and sensitivity could be to blame for the early lag in growth seen in the ET fetuses.

To conclude, growth factor supplementation improved embryo quality and advanced development in culture. Circulating PAG concentrations were not altered within the ET groups, however fetal sex effects were noted within the control group. Additionally, CRL did not differ between treatment groups at day 42, but at day 56 AI fetuses were larger than both ET groups. Our goal was to improve embryonic development without inducing large offspring syndrome and dystocia. Although

pregnancies were not carried to term, we can infer from the CRL measurements that EFI embryos were not advanced in growth. Future studies should investigate late gestation outcomes of growth factor treated embryos. We successfully improved the quality and development of embryos with growth factor supplementation, without affecting fetal and maternal interactions.

Chapter 3 – Interpretive Summary

Pregnancy loss is one of the major issues facing the beef and cattle industries, causing reduced production efficiency and economic loss. Various physiological and environmental factors contribute to losses throughout gestation. Any perturbations in the normal progression of pregnancy can result in pregnancy loss. The three stages of pregnancy loss include early embryonic loss, late embryonic loss, and fetal loss. Most losses occur during the early embryonic stage and are attributed to failures between the embryo and uterine environment. Use of bovine embryo transfer (ET) has been explored to evade the damaging effects of stress and suboptimal uterine environments on embryonic development. We implemented a bovine in vitro production (IVP)-ET scheme to further investigate ways to improve embryo competency and pregnancy retention. The goals of this work were to improve embryo quality with the addition of growth factors known to advance embryonic development (Epidermal growth factor, Fibroblast growth factor 1, and Insulin-like growth factor 1) and to encourage positive fetal/maternal interactions, ultimately improving pregnancy retention.

Supplementation with growth factors during in vitro culture prior to transfer improved embryo quality and development compared to control embryos. To adjust for limited cow numbers, pregnancies were aborted and cows were recycled for additional study replications. Alternative markers were used to determine fetal development and placental fitness, as pregnancies were not carried to term. Circulating concentrations of pregnancy associated glycoproteins (PAG) did not differ among treatment groups. There was a fetal sex by treatment effect within the control embryo group, where bull fetuses had higher PAG concentrations than heifer fetuses. This sex effect could be

attributed to the embryo culture conditions and advanced development of male embryos. Crown-rump length (CRL) measurements were used to determine fetal well-being. At day 56, fetuses (regardless of sex) in artificially inseminated cattle (AI) were larger than both ET groups.

Overall, with growth factor supplementation, we successfully produced higher quality embryos and improved the percentage of transferable embryos. This will prove helpful to improve efficiency of the bovine IVP industry. Alterations in PAG concentrations were not observed in the ET groups. Additionally, fetal CRL in the ET groups were shorter than AI fetuses. The objective was to produce a more developed embryo without creating damaging effects post-transfer. From these results, we can conclude addition of the growth factors did not harm embryonic/fetal growth or placental development. Altogether, this work provides new insight into the post-transfer effects of growth factor supplemented embryos on fetal and placental development. Further studies should examine the effects of growth factor supplementation on peri- and post-natal calf development and production.

Figure 2-1 Experimental Design. Cattle were synchronized using a CIDR-Ovsynch protocol initiated on day -10. Estrus was observed on day -1 and ~1/3 of the cows were artificially inseminated the following morning, day 0. The remaining cows were slated for embryo transfer, and *in vitro* produced bovine embryos were transferred on day 7. Blood samples were taken on day 21 and peripheral blood mononuclear cells (PBMC) were isolated to examine expression of interferon stimulating genes (ISG). Pregnancy was diagnosed on day 28 post-ovulation. Presence of pregnancy-associated glycoproteins (PAG) were determined from blood plasma on day 28, 42, and 56. Crown-rump lengths (CRL) were measured on day 42 and 56. Fetal sex was determined between day 56-60 and pregnancies were aborted to provide more animal numbers for future replicates.
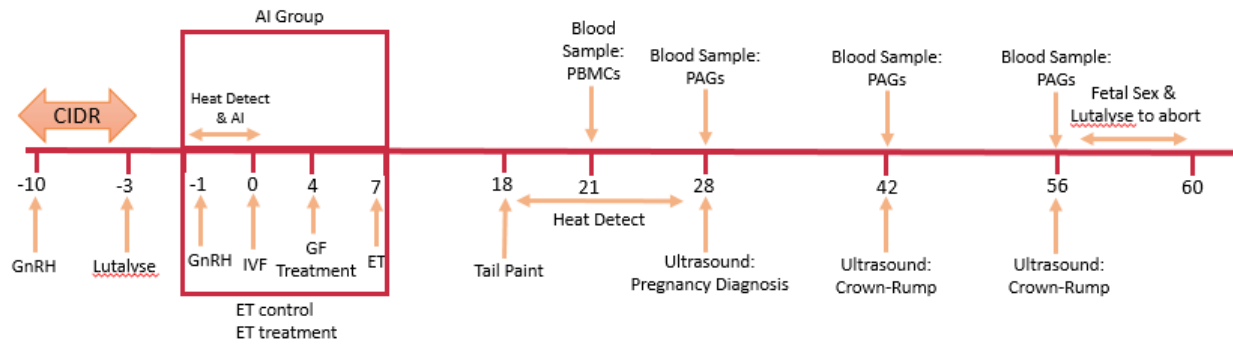
Figure 2-1

Figure 2-2 Information on number of cows synchronized, detected in estrus, and selected for AI or ET after completing a CIDR-Ovsynch protocol. Those cows not expressing estrus received a second administration of GnRH on day 0. Cows that were not AI and had no functioning CL were excluded from the replicate. Number of cows that were AI and number of cows that were used for ET (CL at day 7) are also included in the table.

Figure 2-2

	Replicate 1	Replicate 2	Replicate 3
CIDR-Ovsynch (#)	25	32	25
Detected in estrus day -1 (#)	9	22	14
Second GnRH day 0 (#)	16	10	11
Not used (#)	3	4	1
AI day 0 (#)	8	9	5
CL at Day 7 for ET (#)	14	19	19

Figure 2-3 Cow AI/ET information. For each replicate, the number of cows used for treatment groups and stage of embryo at transfer, when applicable, is shown.

*ML= morula, BL= blastocyst, EB= expanded blastocyst

Figure 2-3

	Replicate 1				Replicate 2				Replicate 3			
	# Cows Used	ML	BL	EB	# Cows Used	ML	BL	EB	# Cows Used	ML	BL	EB
AI	8	-	-	-	9	-	-	-	5	-	-	-
Control	6	6	-	-	10	2	5	3	9	-	4	5
EFI	8	3	1	4	9	-	3	6	10	-	5	5

Figure 2-4 **The influence of EFI treatment on the transferable quality of in vitro produced bovine embryos.** *In vitro*-produced bovine embryos were created, and at day 4 post-fertilization, embryos were cultured in medium containing a mix of three growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA). At day 7, embryo development (morula, regular blastocyst, expanded blastocyst) and percent transferable embryos were examined. Panel A: Percentage of embryos that achieved the compact morula, blastocyst and expanded blastocyst stages by day 7 post-fertilization. The asterisk indicates differences between treatment groups at the expanded blastocyst stage (P=0.02). Panel B: Percentage of embryos considered transferable. Embryos represent those that achieved compact morulae, blastocyst or expanded blastocyst stages and were given quality scores of 1 or 2. Different superscripts denote differences (P=0.05) (n=60 embryos/replicate).
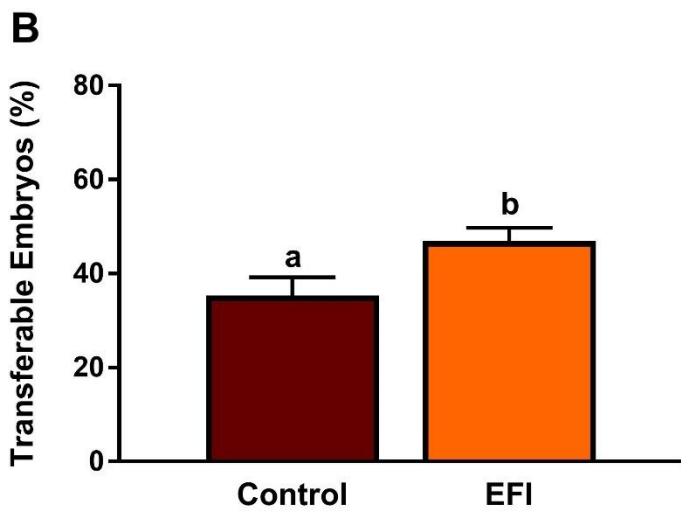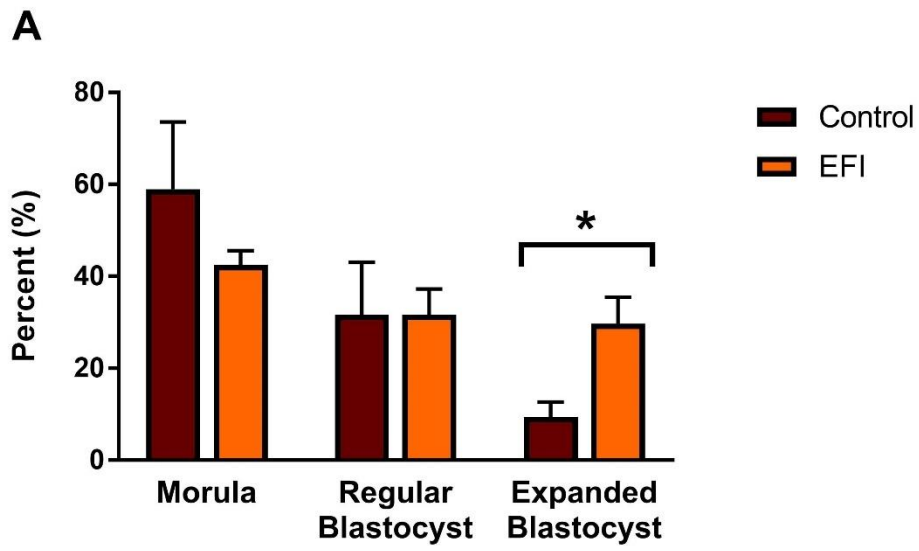
Figure 2-4

Figure 2-5 **Percentage of cows pregnant at day 28 and 56 of gestation. Cows either were artificially inseminated (AI) or received an** *in vitro*-**produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Panel A: At day 28, transrectal ultrasonography was performed to diagnose pregnancy. Panel B: Percent pregnant at day 56. No differences were detected among treatment groups (n=22-28).**

Figure 2-5

A

B
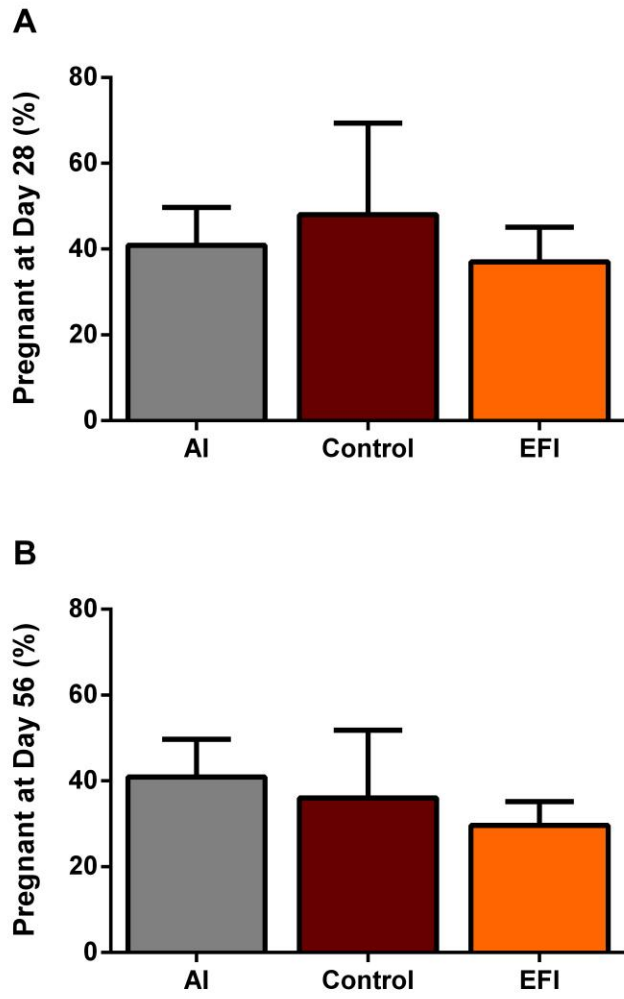
Figure 2-6 Circulating concentrations of pregnancy specific protein B (PSPB) at days 28, 42, and 56 of gestation. Cows either were artificially inseminated (AI) or received an *in vitro*-produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Pregnancy was diagnosed at day 28 by transrectal ultrasonography. Cows that aborted pregnancies between days 28 and 56 were not included in these analyses. At days 28, 42, and 56, blood was collected via coccygeal venipuncture. Circulating concentrations of PSPB were determined from harvested plasma. Panel A: PSPB concentrations at day 28. Panel B: PSPB concentration at day 42. Panel C: PSPB concentrations at day 56. Different superscripts within each panel denote differences ($P<0.05$) (n=8-9).
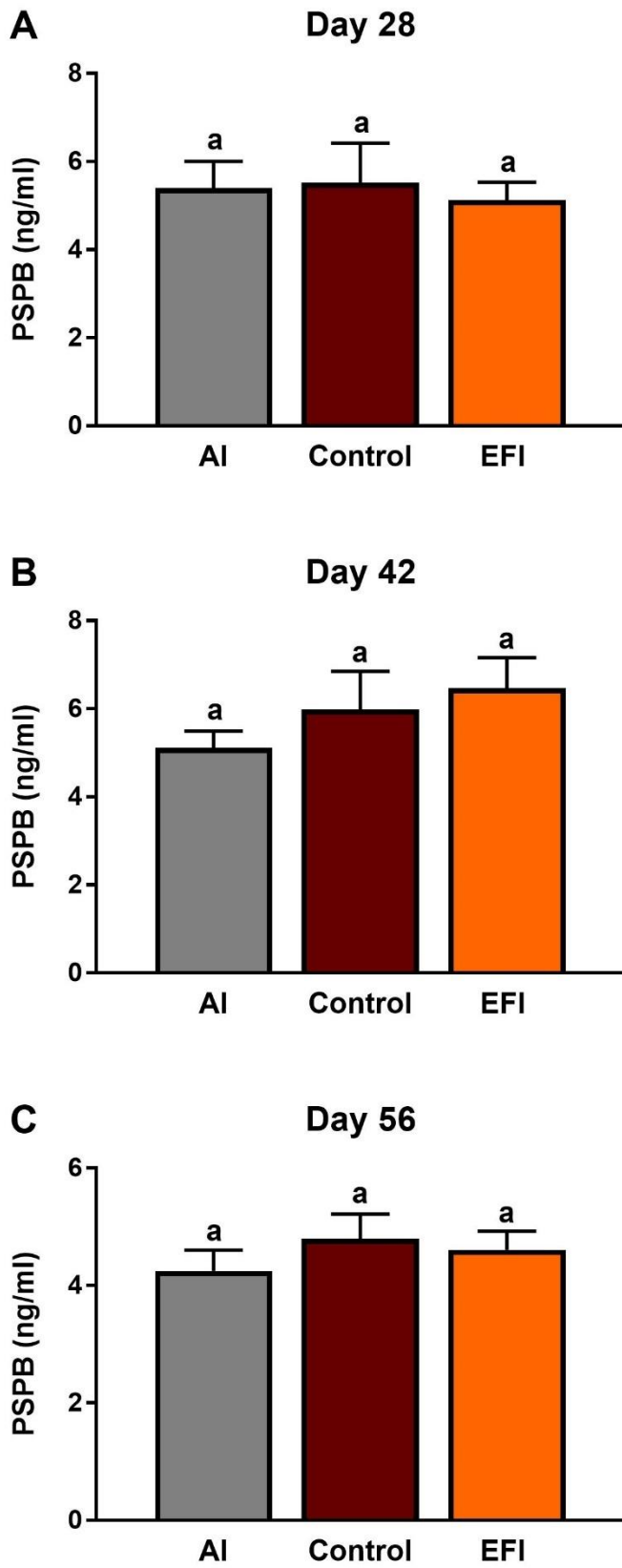
Figure 2-6

A   **Day 28**

B   **Day 42**

C   **Day 56**

Figure 2-7 **Effect of fetal sex on circulating pregnancy specific protein B (PSPB)**
concentrations in pregnant cows at days 28, 42, and 56. Cows either were artificially
inseminated (AI) or received an *in vitro*-produced embryo treated with growth factors
(EFI10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml
Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-
fertilization. Pregnancy was diagnosed at day 28. Cows that aborted pregnancies
between days 28 and 56 were not included in these analyses. Fetal sex was determined
by transrectal ultrasonography at day 56-60. At days 28, 42, and 56, blood was
collected via coccygeal venipuncture. Circulating concentrations of PSPB were
determined from harvested plasma. Panel A: PSPB concentrations for each fetal sex at
day 28. Panel B: PSPB concentrations for each fetal sex at day 42. Panel C: PSPB
concentrations for each fetal sex at day 56. Different superscripts within each panel
denote differences (P<0.05) (n=11-15).

Figure 2-7

A

Day 28
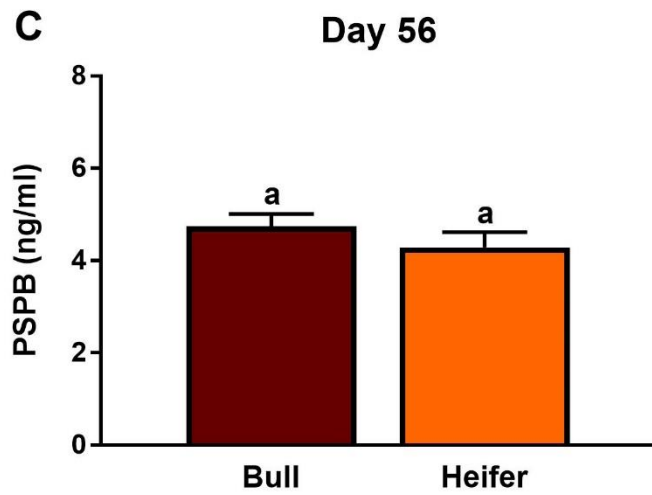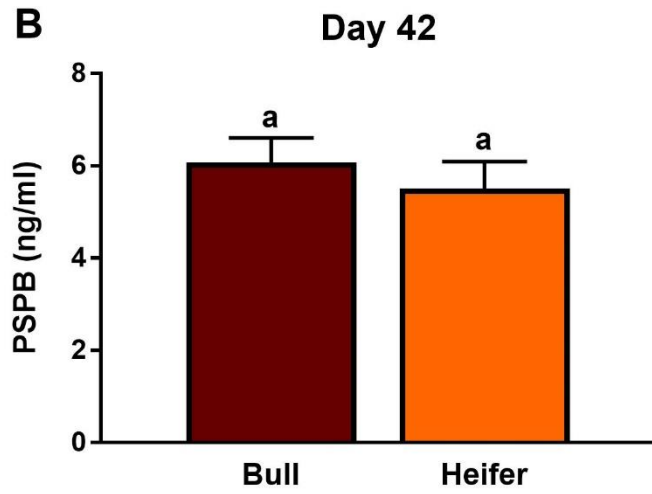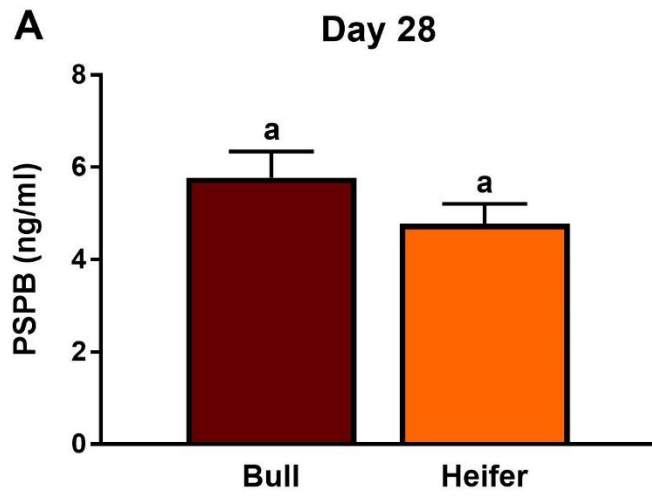

B

Day 42

C

Day 56

Figure 2-8 The interaction between fetal sex and treatment on circulating PSPB concentrations. Cows either were artificially inseminated (AI) or received an *in vitro*-produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Pregnancy was diagnosed at day 28. Cows that aborted pregnancies between days 28 and 56 were not included in these analyses. Fetal sex was determined by transrectal ultrasonography at day 56-60. Panel A: PSPB concentrations for each fetal sex in each treatment group at day 28. Panel B: PSPB concentrations for each fetal sex in each treatment group at day 42. Panel C: PSPB concentrations for each fetal sex in each treatment group at day 56. Different superscripts denote differences between groups (P<0.05) (n=3-6). The asterisk represents a sex-based tendency (*P*=0.08) within the control group.
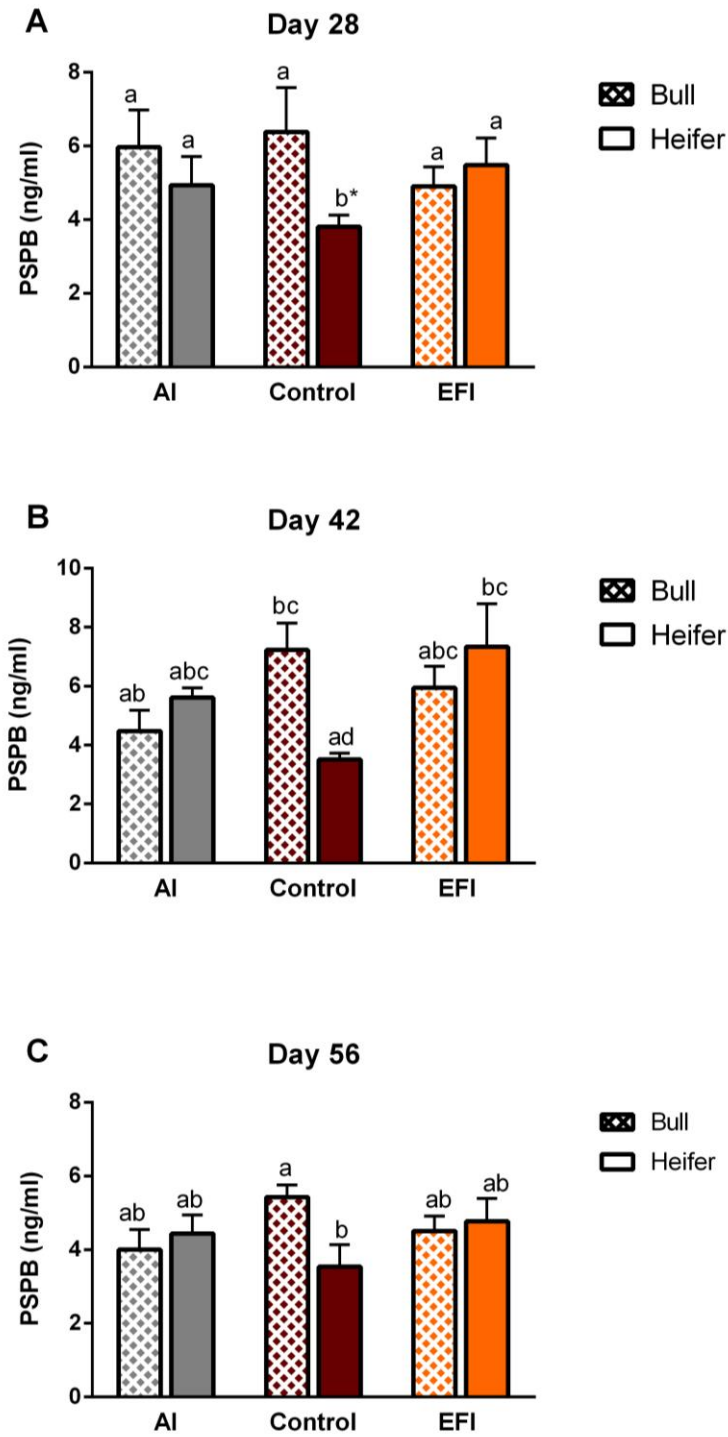
Figure 2-8

Figure 2-9 The effect of treatment on crown-rump lengths (CRL) in cattle at days 42 and 56 of gestation. Cows either were artificially inseminated (AI) or received an *in vitro*-produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Pregnancy was diagnosed at day 28. Cows that aborted pregnancies between days 28 and 56 were not included in these analyses. CRL was measured via transrectal ultrasonography at day 42 and 56 post-AI. Panel A: CRL at day 42. Panel B: CRL at day 56. Different superscripts within each panel denote differences ($P<0.05$) (n=7-9).

Figure 2-9

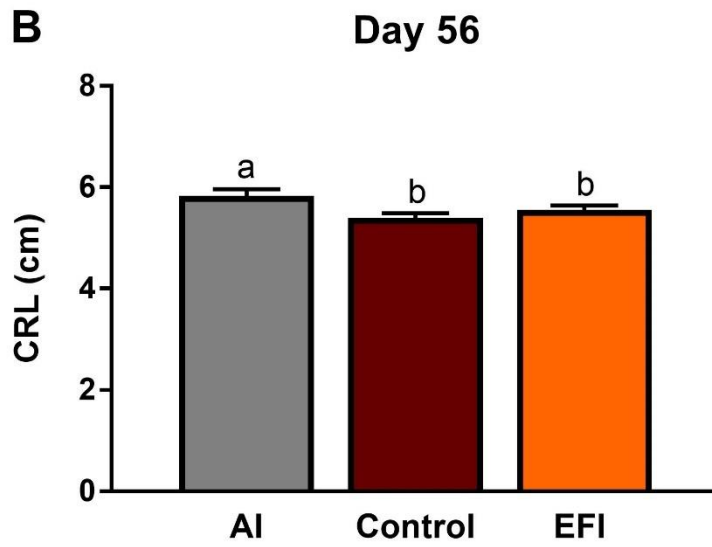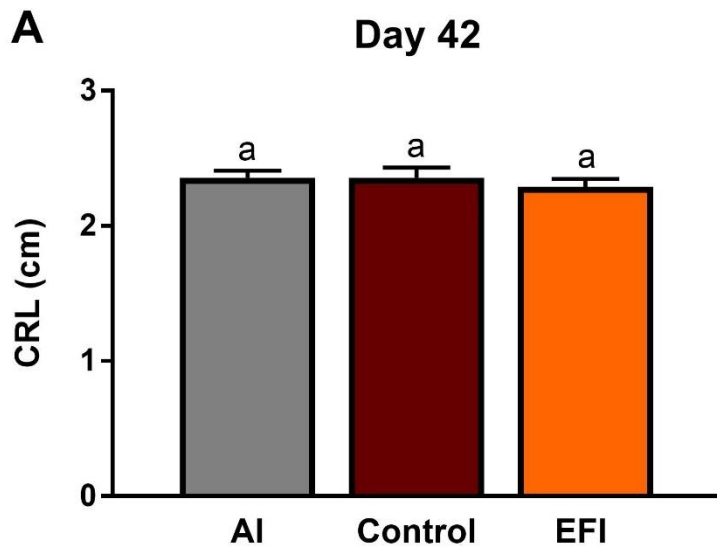**A**

Day 42

B

Day 56

Figure 2-10 The effect of fetal sex on CRL at day 42 and 56. Cows either were artificially inseminated (AI) or received an *in vitro*-produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Pregnancy was diagnosed at day 28. Cows that aborted pregnancies between days 28 and 56 were not included in these analyses. CRL was measured via transrectal ultrasonography on day 42 and 56 post-AI. Fetal sex was determined by transrectal ultrasonography at day 56-60. post-AI. Panel A: CRL measurements for each feta sex at day 42. Panel B: CRL measurements for each fetal sex at day 56. Different superscripts within each day denote differences (P<0.05) (n=10-14).
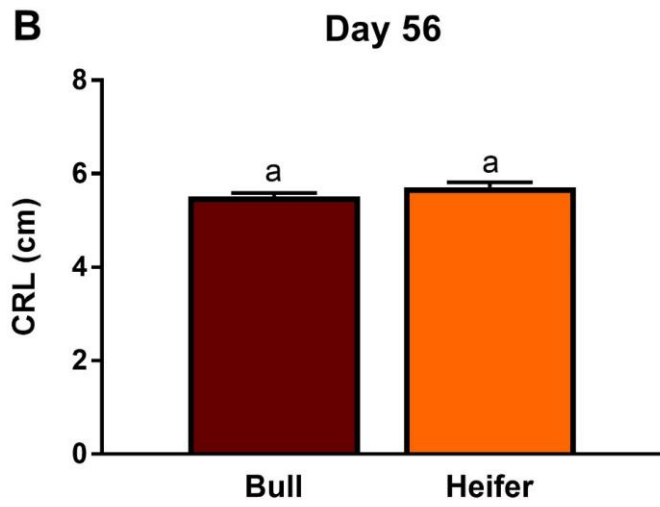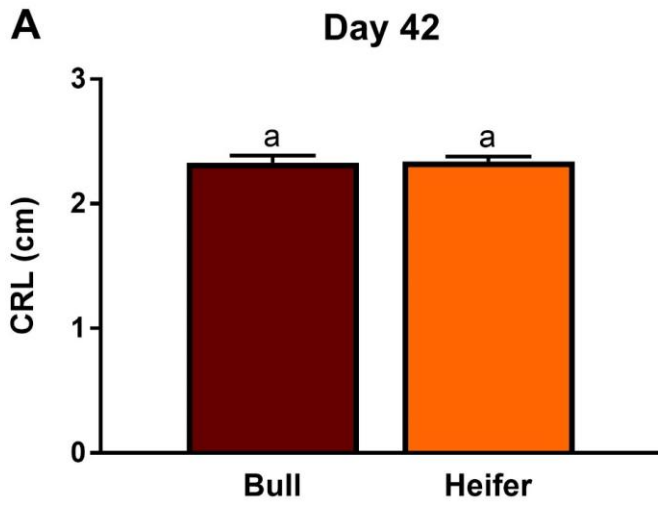
Figure 2-10

A

Day 42

B

Day 56

Figure 2-11 **Influence of treatments and fetal sex on CRL at day 42 and 56. Cows either were artificially inseminated (AI) or received an** *in vitro*-**produced embryo treated with growth factors (EFI; 10 ng/ml Epidermal Growth Factor, 10 ng/ml Fibroblast Growth Factor 2, 50 ng/ml Insulin-like Growth Factor 1) or carrier only (Control; 1% BSA) from day 4 to 7 post-fertilization. Pregnancy was diagnosed at day 28. Cows that aborted pregnancies between days 28 and 56 were not included in these analyses. CRL was measured via transrectal ultrasonography on day 42 and 56 post-AI. Fetal sex was determined by transrectal ultrasonography at day 56-60. post-AI. Panel A: CRL measurements for each fetal sex and treatment at day 42. Panel B: CRL measurements for each fetal sex and treatment at day 56 Different superscripts denote differences** (P<0.05) (n=3-6).

Figure 2-11

A Day 42

B Day 56
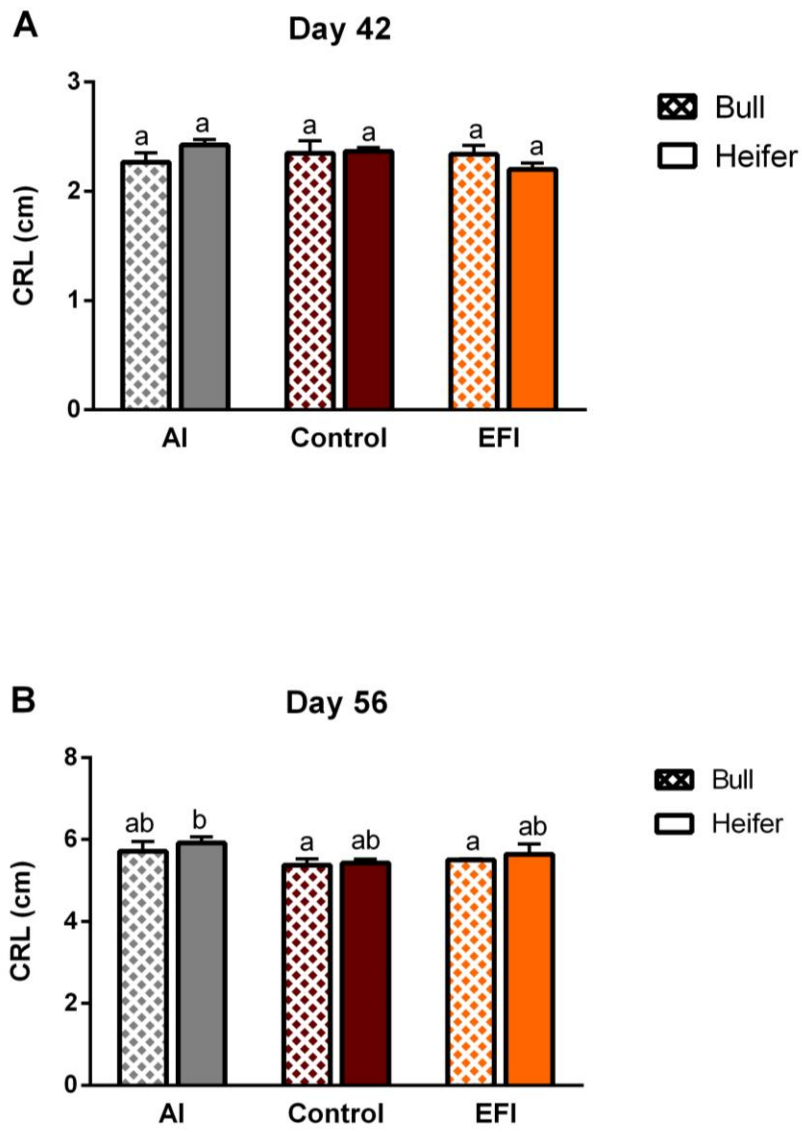
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