

DEVELOPMENT OF AN EXTENDED CULTURE SYSTEM FOR BOVINE
BLASTOCYSTS: EFFECTS OF OXYGEN TENSION, MEDIUM TYPE AND SERUM
SUPPLEMENTATION ON EMBRYO DEVELOPMENT AND INTERFERON-TAU
SECRETION

By

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To all of my friends and family who have helped me through this time in my life and help shape
the person I am today

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TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	4
LIST OF TABLES	7
LIST OF FIGURES	8
ABSTRACT	10
CHAPTER	
1 INTRODUCTION	12
2 LITERATURE REVIEW	15
The Estrous Cycle.....	15
Follicular Development and Ovulation	15
Luteal Development and Function	18
Luteolysis	19
Pregnancy	23
Fertilization.....	23
Early Embryonic and Trophectoderm Development.....	24
Implantation and Placental Development.....	26
Placental Hormones.....	28
Parturition.....	30
Maternal Recognition of Pregnancy	31
Discovery of the Maternal Recognition of Pregnancy Factor in Ruminants.....	31
The IFN τ Gene and its Expression.....	32
Antiluteolytic Action of IFN τ	33
Other Activities of IFN τ	35
Bovine Embryo Culture.....	37
Defined <i>In vitro</i> Culture Requirements	37
Differences between <i>In vivo</i> and <i>In vitro</i> -Derived Embryos.....	40
Extended Blastocyst Culture	42
Uterine Factors Affecting Embryo Development.....	44
Fibroblast Growth Factor-2 (FGF-2).....	46
Granulocyte-Macrophage Colony-Stimulating-Factor (GM-CSF)	47
Summary.....	48
3 MATERIALS AND METHODS	51
<i>In vitro</i> Embryo Production.....	51
Individual Embryo Culture from Day 8 to 11 Post-IVF.....	52
Evaluating Apoptosis and Total Cell Number.....	55
Interferon-tau Production.....	56

Statistical Analysis.....	56
4 RESULTS.....	59
Experiment 1: Effect of Medium Type and Oxygen Atmosphere on Blastocyst Survival, Development and IFN τ Production.....	59
Medium type did not affect the quantity of bioactive IFN τ , as assessed with an antiviral assay, in conditioned medium from blastocysts cultured individually from day 8 to 11 post-IVF (Fig 4-6). However, embryos incubated in 5% oxygen secreted more (P=0.002) IFN τ into medium than embryos incubated in 20% oxygen (Fig. 4-6). This effect is evident in both medium types both before and after normalizing data according to embryonic cell numbers on day 11 post-IVF (Fig. 4-7).....	60
Experiment 2: Serum Requirements for Extended Bovine Blastocyst Culture.....	60
Experiment 3: FGF-2-Mediated Induction of IFN τ Production in Bovine Blastocysts Depends on the Level of Atmospheric Oxygen.....	61
Experiment 4: The Combined Effects of FGF-2 and GM-CSF on Blastocyst Development and IFN- τ Production	62
5 DISCUSSION.....	81
LIST OF REFERENCES.....	88
BIOGRAPHICAL SKETCH	116

LIST OF TABLES

<u>Table</u>		<u>page</u>
2-1	A comparison of medium components for M-199 and KSOM.	57
2-2	Medium components that are only included in M-199.	58

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
4-1 Examples of degenerated embryos by day 11 post-IVF.	64
4-2 Examples of Hoescht 33342 and TUNEL staining for total cell counts and incidence of apoptosis.	65
4-3 Incubating blastocysts from day 8 to 11 post-IVF in M-199 and 5% O ₂ prevents degeneration.	66
4-4 Incubation in M-199 under 5% oxygen conditions yields greater total cell numbers on day 11 post-IVF.	67
4-5 Incidence of apoptosis is not affected by medium type or oxygen tension in bovine embryos from day 8 to 11 post-IVF.	68
4-6 Incubation in atmospheric oxygen increases IFN τ secretion from bovine blastocysts.	69
4-7 Interaction of medium type and oxygen concentration on IFN τ secretion on day 11 post-IVF.	70
4-8 Incubating embryos in M-199 containing 2.5 or 5% FBS with 5% oxygen limits degeneration from day 8 to 11 post-IVF.	71
4-9 Supplementation with FBS improves embryonic cell number after incubation in 5% oxygen.	72
4-10 Effect of serum supplementation on percentage of TUNEL positive cells during individual embryo culture from day 8 to 11 post-IVF.	73
4-11 Degree of serum supplementation and oxygen concentrations during culture impact IFN τ secretion.	74
4-12 Serum supplementation and atmospheric oxygen conditions act independently to mediate IFN τ production.	75
4-13 Effect of FGF-2 supplementation on cell numbers in embryos on day 11 post-IVF.	76
4-14 Main effects of FGF-2 supplementation and oxygen concentrations on IFN τ secretion from bovine blastocysts.	77
4-15 Treatment with FGF-2 and 20% oxygen increases IFN τ production from bovine blastocysts.	78
4-16 Effect of FGF-2 and GM-CSF supplementation on embryo cell number.	79

4-17 Supplementation with FGF-2 but not GM-CSF increases IFN τ production from bovine embryos80

Abstract of Thesis Presented to the Graduate School
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The bovine conceptus produces interferon-tau (IFN τ), and this factor acts on the endometrium to block the pulsatile release of prostaglandin F2 alpha (PGF $_{2\alpha}$) and prevent luteolysis. Factors secreted from the uterus control IFN τ gene expression in bovine and ovine conceptuses. The overall goal of this work was to develop a culture system that supports bovine embryonic development until day 11 post-*in vitro* fertilization (IVF) so it may be used as a model to investigate how uterine-derived factors regulate IFN τ synthesis and secretion. All embryos were produced via IVF and evaluated for overall quality, percent apoptotic nuclei (not study 3 and 4), total cell number and IFN τ content in the medium. In study 1, individual blastocysts were placed in one of two culture medium formulations, KSOM or M-199, containing 5% FBS and incubated in a 5 or 20% oxygen environment from day 8 to 11. Survival to day 11 post-IVF was greatest for blastocysts cultured in M-199/5% oxygen. None of the treatments affected percentage of apoptotic nuclei. IFN τ concentrations were greater for embryos cultured in 20% oxygen than for those cultured in 5% oxygen regardless of medium type. In study 2, individual blastocysts were cultured in M-199 supplemented with different protein

supplements at 5% or 20% oxygen from day 8 to 11 post-IVF. Blastocysts supplemented with serum substitute (ITS), 1% bovine serum albumin (BSA) or 1% fetal bovine serum (FBS) contained more degenerated embryos at day 11, fewer blastomeres, and increased percent apoptotic nuclei compared to blastocysts incubated with 2.5% and 5% FBS. IFN τ was detected only in embryos incubated in 2.5 or 5% FBS. In study 3, effects of FGF-2 treatment and oxygen concentration on blastocyst cell number and IFN τ secretion were evaluated. Individual blastocysts were cultured in M-199 supplemented with 2.5% FBS in 5% or 20% oxygen from day 8 to 11 and treated with or without 100 ng/mL FGF-2. Blastocysts in 5% oxygen, regardless of FGF-2 treatment, yielded embryos with greater cell numbers. Blastocysts incubated in 20% oxygen had greater IFN τ secreted in culture medium than those incubated in 5% oxygen. FGF-2 treatment increased IFN τ secretion in blastocysts incubated in 5% oxygen but not in blastocysts cultured in 20% oxygen. In study 4, blastocysts individually cultured in M-199 supplemented with 2.5% FBS at 5% oxygen and treated with 100 ng/mL FGF-2, 100 ng/mL GM-CSF, 100 ng/mL of both FGF-2 and GM-CSF, or no growth factor treatment (control). There were no effects of FGF-2 and GM-CSF treatment on IFN τ secretion, although individual treatment comparisons showed greater IFN τ secretion in FGF-2 treated blastocysts when compared to controls. GM-CSF treatment did not affect IFN τ secretion in blastocysts compared to controls; however mean IFN τ concentrations were slightly increased and not different from FGF-2 treatment. Co-supplementation did not increase IFN τ secretion in blastocysts when compared with controls. In summary, a culture system for maintaining individual blastocyst development until day 11 post-IVF has been identified. These outcomes indicate that this culture system can be used to better understand how IFN τ expression and embryo development is regulated by growth factors present in the uterine secretory milieu, such as such as FGF-2 and GM-CSF.

CHAPTER 1 INTRODUCTION

The sustainability and profitability of livestock production systems relies on suitable reproductive capacities. Reproductive problems preside in nearly all domesticated animals used for food production, and numerous components of the reproductive process usually are involved with reproductive insufficiencies. Problems associated with detecting estrus for artificial mating and failure to maintain pregnancies to term are the predominant problems associated with reproduction in dairy and beef cattle [1]. These issues will typically result in extended calving intervals, decreased lifetime milk production, and an increase in the number of artificial inseminations required to achieve a pregnancy [2]. Dairy producers stand to lose an average of \$550 in lifetime milk potential with each reproductive cycle that fails to produce a calf [3]. The use of estrous synchronization and timed artificial insemination protocols has diminished some of these issues related to fertility in cattle; however no solutions for embryonic mortality have remained elusive to date.

It is estimated that 57% of all failed pregnancies occur between days 3 and 21 of pregnancy when the newly formed embryo must develop, announce its presence to the maternal system, and begin attaching to the uterine lining [1]. Several crucial developmental events occur during this period of embryonic growth and proliferation. Newly formed embryonic cells divide and begin differentiating into a variety of embryonic and extra-embryonic tissues that will give rise to the fetus and placenta. The developing conceptus, a term used to describe the embryonic mass and its associated placental tissues, must also signal its presence within the maternal system to sustain a progesterone-dominated environment conducive for pregnancy. In ruminants, this crucial event occurs by placental production of a Type I interferon, termed interferon-tau (IFN τ).

Adequate development of the initial placental tissue lineages and ample production of IFN τ are vital for pregnancy success in cattle and other ruminant species.

The predominate method of studying early bovine embryonic development is through the utilization of *in vitro* production and *in vitro* culture of embryos. The production of bovine embryos using *in vitro* maturation, fertilization, and culture technologies provides scientists with a powerful tool for examining the initial events of embryogenesis in cattle and related species. Establishing culture conditions that support bovine embryo development during the blastocyst stage would offer considerable benefits. For example, extended culture could provide additional end-point measurements that can be used to assess how media, supplements, and atmospheric conditions impact the production of embryos suitable for recipient transfer. Also, a blastocyst-based culture system could assist in describing how uterine-derived factors influence embryo development. Uterine secretions are essential for proper conceptus elongation. Ewes that fail to develop uterine glandular epithelium, a model created by exposing neonatal ewes to progestins, are unable to nourish a pregnancy past the hatched blastocyst stage [4, 5].

Extending bovine embryo culture beyond the blastocyst stage has been completed by several groups. Culturing groups of embryos in microdrops of medium sustains blastocyst viability to day 10 or 11 post-*in vitro* fertilization (IVF) [6-8]. Bovine blastocyst development after hatching (i.e. protrusion from the zona pellucida) is completed, at least to some extent, either by creating trophectoderm outgrowths on extracellular matrices [7, 9-11] or by provoking elongation by placing bovine blastocysts in agarose tunnels [12-14]. Unfortunately, the embryonic disk, or epiblast, fails to develop properly in most cases, and the resulting tissue mass is comprised primarily of trophectoderm and primitive endoderm [9, 12-14].

Insufficient quantities of the maternal recognition of pregnancy factor, interferon-tau (IFN τ), also are observed in ewes containing little to no glandular epithelium [4, 5]. Several intrinsic and extrinsic factors affect IFN τ expression during early pregnancy [15, 16], but length of the developing conceptus is the predominant determinant of overall IFN τ production [17-19]. Therefore, it is imperative that the physiological events associated with early conceptus development and IFN τ production be fully understood so that resolutions for pregnancy failures can be conceived.

We examine several of the basic reproductive processes surrounding early embryonic and conceptus development in cattle and provide an update on our current knowledge of key biological processes that influence fecundity. The literature review will begin with a brief overview of the estrous cycle in cattle, followed by an overview of pregnancy where a description of embryonic and conceptus development is presented. Also, an overview of IFN τ expression and action will be provided. A brief review of past and present bovine embryo culture systems and their similarity with *in vivo* derived embryos will be reviewed. The literature review will end by discussing some uterine-derived factors that impact embryonic development. The research focus of this thesis then will be presented. The overall goal of the research was to develop a culture system that can be used to examine bovine blastocyst development. Through a series of studies, a suitable system for maintaining blastocyst viability was created, and its efficacy for use in examining how uterine-derived factors affect embryo development and IFN τ production was tested.

CHAPTER 2 LITERATURE REVIEW

The Estrous Cycle

The estrous cycle consists of a series of predictable physiological events that occur between successive periods of sexual receptivity, also known as estrus. These series of reproductive events occur throughout the female's adult life until it is interrupted by pregnancy and, in some species, season of the year. The estrous cycle provides the female with repeated opportunities to copulate and become pregnant. Estrus is preceded by the development and ovulation of a single follicle in cattle, and hormones secreted by this follicle drive female receptivity to males and ovulation. Immediately following ovulation, the tissue mass that made up the follicle differentiates into a body of tissue termed the corpus luteum (CL). As the CL ages, copious amounts of progesterone are produced to support uterine secretory activities and diminish uterine contractility, both of which sustain pregnancies in cattle. If there is no pregnancy, the corpus luteum will undergo regression, or luteolysis, and the female will return to estrus. In cattle, the interval between estrus activity, or the length of the estrous cycle, averages from 20 to 23 days.

Follicular Development and Ovulation

Folliculogenesis is the process by which follicles capable of being ovulated are formed on the ovary in the female from a pool of naïve, or primordial follicles [20]. Folliculogenesis is an ongoing process in cattle that begins prior to puberty, and cycles of follicle development and death (or atresia) are seen repeatedly throughout estrous cycles and pregnancy. Follicles are present on the ovary in a hierarchy according to their stage of development and functional status [21]. These "waves" of follicle growth occur by the developmental progression of primordial follicles through three primary processes: 1) recruitment, 2) selection, and 3) dominance [22].

During follicle recruitment, a group, or cohort, of follicles begins developing from the primordial pool and mature over a 45 to 60 day period. A selection process occurs as cohorts of follicles continue to increase in size and eventually only a single follicle will continue to develop. The smaller, subordinate follicles will undergo a degenerative process, known as atresia, and fail to ovulate. Greater than 99% of ovarian follicles will undergo atresia throughout the lifetime of the cow [23]. The remaining follicle, which now is termed the dominant follicle, limits the ability of other follicles to develop and is capable of ovulating if endocrine events associated with hypothalamic-pituitary-ovarian axis permit it. During the first follicular wave of an estrous cycle, luteal-derived progesterone levels do not permit ovulation. As a result, these dominant follicles will also undergo atresia, allowing a new follicular wave to begin.

Folliculogenesis is controlled by hormones produced by the anterior pituitary, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH). As follicles develop, they become responsive to gonadotropins, and primarily FSH, which mediates further development. When the dominant follicle reaches a particular differentiated state it is hypothesized that its growth can be sustained by lower levels of FSH which, in conjunction with inhibin, do not facilitate the further recruitment and development of subordinate follicles. Inhibin is a 32-kDa dimeric protein composed of a disulfide bonded α - and β -subunit that is produced by granulosa cells under the control of FSH [24, 25]. Within this dominant follicle, theca cells produce androgens in response to LH. The granulosa cells within the follicle then aromatize these androgens to estradiol under the control of FSH [26]. As the dominant follicle emerges, increased levels of estrogen and inhibin will feed back onto the pituitary and decrease FSH secretion. The reduction in circulating FSH prevents most large follicles from continuing to develop. The production of both LH and FSH is controlled by numerous systemic and

neuronal signals. One of the primary mediators of both hormones is a hypothalamic peptide known as Gonadotropin Releasing Hormone (GnRH). This factor is released from hypothalamic nuclei in specific regions of the hypothalamus and acts on LH and FSH-producing cells in the anterior pituitary via transport through a closed portal hypothalamic-pituitary system to stimulate hormone production and release [27, 28].

Ovulation of a dominant follicle depends on whether luteal-derived progesterone is present. When circulating progesterone concentrations are high, which is the case throughout 80% of the estrous cycle, the dominant follicle will undergo atresia after 3-4 days of dominance. The regression of the dominant follicle lifts the inhibin-mediated block in folliculogenesis and permits a successive wave of follicle selection and dominance. Typically, two to three waves of follicle growth/atresia exist during an estrous cycle, but as little as one follicular wave and as many as four have been reported [29, 30].

Ovulation of the dominant follicle will occur in the absence of elevated luteal progesterone. This process is driven by changes in the hypothalamic and pituitary responsiveness to ovarian hormones. Notably, the loss of progesterone permits follicle-derived estradiol to interact with the hypothalamic surge center, which generates the preovulatory surge of LH [31, 32]. Between 24-36 hours after the LH surge, the dominant follicle will rupture, releasing the oocyte. This rupturing is mediated by histamine and prostaglandin E₂ (PGE₂) production, which increases blood flow to the ovary after the LH surge, resulting in an increase in hydrostatic pressure within the follicle [33]. Theca cells of the follicle begin producing progesterone, which stimulates collagenase synthesis by the theca interna cells to breakdown collagen in the follicle. Also, granulosa cells increase follicular fluid secretion, causing intrafollicular pressure build up

[33]. Finally, $\text{PGF}_{2\alpha}$ causes smooth muscle contraction within the ovary. Collectively, these coordinated events result in follicle rupture [33].

Luteal Development and Function

Progesterone is the hormone responsible for preventing ovulation, preparing the uterus for pregnancy, and maintaining a pregnant state in cattle. Progesterone is produced by the CL, which is a mass of tissue created from differentiated follicle cells around the time of ovulation. During ovulation, the follicle wall collapses and the basement membrane between the theca interna and granulosa cells breaks down, giving way to the development of an extensive vascular network where blood vessels invade the antral space of the follicle forming the corpus hemorrhagicum (bloody body) and eventually the corpus luteum (CL; yellow body). In domestic animals the major luteotropin is LH, which stimulates development of the CL and production of progesterone. The CL is comprised of two types of luteal cells: large and small. Unlike the small luteal cells, large luteal cells do not respond to LH stimulation, however, they are responsible for a majority of the basal secretion of progesterone [34]. In cattle, LH pulses are required for the development of a fully functional CL [35-39].

One of the important actions of progesterone is to prevent ovulation. Progesterone acts on the hypothalamus to reduce GnRH pulse frequency and prevent the hypothalamic surge center from generating an LH surge. These activities reduce LH and FSH pulse frequency but increase pulse amplitude of LH and FSH; conditions that favor luteal function. Gonadotropin production is sufficient to allow follicular development to occur, however ovulation is prevented by the negative feedback actions of progesterone on the hypothalamus [40].

Copious progesterone secretion by the CL is vital for the maintenance of pregnancy. Progesterone stimulates the endometrium to produce nutrients, growth factors,

immunosuppressive agents, enzymes, and ions needed for conceptus development [41]. Among inseminated cows, lower systemic concentrations of progesterone are associated with lower pregnancy rates [42, 43]. Progesterone actions on the uterus are mediated by its interaction with progesterone receptors present in luminal and glandular epithelium, the primary site of uterine histotroph production. In several species, like the ewe, luminal and glandular epithelia show a loss of progesterone receptors just before conceptus implantation. This decrease in progesterone receptors seems to be needed for onset of differentiated functions of progesterone, in terms of secretory protein production, such as uterine milk proteins and osteopontin. The actions of progesterone on the endometrium throughout the majority of gestation are mediated by the endometrial stroma, which remains progesterone receptor positive. Stromal cells are capable of producing several types of growth factors, including hepatocyte growth factor (HGF) and two forms of fibroblast growth factors, FGF-7 and -10. These factors are responsive to progesterone and mediate the interactions that are crucial for pregnancy support [44].

Luteolysis

Luteolysis is the termed used to describe the structural and functional demise of the CL. This process is essential for the initiation of a new reproductive cycle and another mating opportunity if cows are not pregnant. Two main phases of luteolysis include 1) a loss of ability to synthesize and secrete progesterone and 2) the structural demise of the CL itself [38, 45]. During most of the estrous cycle, high levels of progesterone create a block on the uterus to prevent it from generating the luteolytic signal that prompts CL regression. After a set period of time (12 days in the ewe; 16 days in the cow) this block is diminished by the estrous cycle-dependent loss of progesterone receptors in luminal and glandular epithelium [39], which enables the endometrium to become able to produce hormones that induce luteolysis. Prostaglandin $F_{2\alpha}$

(PGF_{2α}) is a prostaglandin produced by the uterus which is capable of terminating a pregnancy at any time in the cow.

Throughout the estrous cycle, progesterone stimulates the accumulation of phospholipids in the luminal and glandular epithelia of the endometrium, and these substrates are used to generate arachidonic acid, which is required for synthesis and secretion of PGF_{2α} [38, 46]. As progesterone receptor (PR) abundance decreases in luminal and glandular epithelium, estrogens acting through their receptors, termed estrogen receptors (ERs), stimulate the expression of oxytocin receptors (OTR) [46, 47]. In sheep, OTRs first appear on day 14 and reach a peak at estrus [48, 49], while in the cow OTRs appear between days 15 and 17, just prior to luteolysis [50-52]. There is some debate as to how this up-regulation of OTRs occurs. It has been hypothesized using ewes as the model species that estradiol is acting through its receptor to cause the up-regulation on OTR. However if this were an estradiol-dependent event, then it would be plausible to assume then that ER receptors would need to be up-regulated first. Two studies conducted by Robinson and colleagues would argue otherwise in the cow. In one experiment, luminal epithelium ER did not increase until between day 16 and 18 in the cow, slightly after, approximately 1-2 days later, than the initial up-regulation of OTRs. Additionally, OTR mRNA was detectable on day 14, while ER mRNA was not detectable until day 16 [53]. In ewes, there is conflicting evidence as to which receptor is up-regulated first. Spencer and colleagues found that ER concentrations increase before OTR [54] while Wathes and Hamilton observed the exact opposite [49]. It remains plausible that an up-regulation of ER is not required for estradiol to stimulate OTR expression in endometrium, or perhaps the OTR gene up-regulation is mediated via estradiol acting in a paracrine manner in other cells, such as the deep

gland ERs [53]. Results from several studies indicate that estradiol is not required for the up-regulation of OTRs, although it may enhance the process [55-58].

In the cow and other ruminant species, oxytocin is released by both the posterior pituitary and the CL and can act on the endometrial epithelium to produce $\text{PGF}_{2\alpha}$. The synthesis and release of $\text{PGF}_{2\alpha}$ from the uterus acts back on the CL to, amongst other things, induce further oxytocin release, and this oxytocin stimulates further production of uterine $\text{PGF}_{2\alpha}$ [59, 60]. This positive feedback loop that is created continues until the CL is fully degraded and luteolysis is complete [61].

$\text{PGF}_{2\alpha}$ is considered a luteolysin and causes luteolysis by both abating progesterone production and inducing the structural demise of the CL. While the exact mechanism remains unclear, it is hypothesized that functional luteolysis by $\text{PGF}_{2\alpha}$ is mediated by the endothelial cell-derived vasoconstrictive peptide known as endothelin-1 (ET-1), which can alter the normal pattern of progesterone synthesis [62]. $\text{PGF}_{2\alpha}$ is thought to activate the ET-1 gene in luteal endothelial cells, thus stimulating ET-1 production. ET-1 secretion then further stimulates luteal $\text{PGF}_{2\alpha}$ production, which will act in a paracrine manner on the CL to further enhance ET-1 synthesis and secretion [37, 62, 63]. ET-1 binds to a specific receptors found on both small and large luteal cells. This activation then decreases both basal and LH-induced production of progesterone, possibly by interrupting the cAMP mediated pathway leading to progesterone production [62]. This mechanism of action has been elucidated in the rat [64, 65] and pig [66], although it still remains unclear if this same pathway interruption occurs in the cow and ewe. At the time of structural luteolysis on the cow and ewe, it was shown that both ET-1 concentration and mRNA encoding for ET-1 were greatest in luteal tissue at this time [62]. Further evidence

shows that ET-1 blocks progesterone production in bovine and ovine luteal cell culture and PGF_{2α} treatment stimulates ET-1 expression and secretion in these luteal cells [67-69].

A variety of agents have been implicated as mediators in the PGF_{2α}-induced structural demise of the CL in the bovine and ovine, but one of the major players is tumor necrosis factor-α (TNF-α). Several groups have shown that PGF_{2α} elevates TNF-α production by macrophages in both the bovine and ovine CL [70, 71]. There is evidence to suggest that TNF-α induced apoptosis plays a role in structural luteolysis. Several reports indicate that apoptosis occurs during luteolysis in ruminants [72-74], while in rodents there is a strong correlation between apoptosis and maximal expression for PGF_{2α} receptor mRNA [75]. PGF_{2α} elevates TNF-α levels in both the bovine and ovine CL and demonstrated that endothelial cells express high levels of TNFR1, a TNF-α receptor type, and that they are sensitive to TNF-α-induced apoptosis *in vitro* [70, 71].

One group positively linked both PGF_{2α} induced ET-1 and TNF-α in the resulting functional and structural demise of the corpus luteum. As previously discussed, ET-1 plays a key role in functional luteolysis through the inhibition of progesterone production by luteal cells and TNF-α has been linked to apoptosis in luteal endothelial cells. It has been hypothesized that during early and mid luteal phases, the high levels of progesterone that still persist, in conjunction with low levels of TNF-α and a TNFR1, prevent apoptosis in endothelial cells. It seems the progesterone producing cells are resistant to apoptosis and serves to protect luteal cells [76]. However, at the time of luteolysis, PGF_{2α} stimulates ET-1 secretion by luteal cells and TNF-α production by local macrophages up-regulate one another's production via a positive feedback loop, which synergize to inhibit progesterone production. These low progesterone levels in conjunction with increased TNFR1 expression [70, 71] facilitate TNF-α apoptosis of

the endothelial cells in the CL. The end result leads to both functional and structural luteolysis [76].

Pregnancy

If copulation takes place during estrus, there is an opportunity for the sperm and egg to unite and form a new organism, which initially is termed a zygote or embryo. This embryo will develop first in the oviduct and then in the progesterone-dominated uterine environment. By a particular period in development, the developing placenta must attach to the uterine wall to create a direct line of communication between mother and fetus and allow for nutrient, gas and waste exchange. The placenta will serve as this essential connection between the mother and fetus throughout the duration of gestation until a critical series of signals are initiated to induce parturition, or birth of the fetus.

Fertilization

Fertilization is the process by which sperm and egg unite and their haploid chromosomes form a new, genetically distinct diploid organism [77]. Sperm travels through the female reproductive tract in a regulated process that ensures those spermatozoa with normal morphology and vigorous motility will have the greatest chance of fertilization [78]. Sperm that remain viable through the arduous journey through the cervix and vagina enter the oviduct and travel to the uterotubal junction where a single sperm will fuse with the oocyte and contribute its genetic material to create a genetically distinct organism.

Upon insemination, spermatozoa in the female reproductive tract are not capable of fertilizing an oocyte. Before fertilization can occur, three major phases of final spermatozoa maturation and development must take place. The first phase involves an alteration of the sperm membrane, an event termed capacitation. In the bovine, capacitation occurs in the oviduct of the female reproductive tract by interactions with glucosaminoglycans [79-82]. This interaction

leads to altered flagellar motility and the development of the capacity for the sperm to fuse with the oocyte [77]. The sperm then penetrates the cumulus oophorus, which consists of cumulus cells and extracellular matrix. Once through the cumulus oophorus, the sperm comes in contact with and binds to the zona pellucida, at which point the acrosome reaction is initiated by ZP3. This constituent glycoprotein of the zone pellucida, called ZP3, binds to the receptors on the anterior head of the acrosome-intact sperm [83, 84]. Once ZP3 binds, a chain of events occurs through the activation of phospholipase C (PLC) and elevated cytosolic calcium levels. This directly drives the acrosome reaction by continuously pushing calcium into the plasma membrane. Acrosome reacted sperm penetrate the zona pellucida where fusion of the outer acrosome membrane to the plasma membrane takes place, resulting in sperm-egg adhesion and a release of acrosomal contents [77]. Finally, an increase in cytosolic calcium instigated by sperm egg fusion induces the oocyte to proceed through its final phases of meiosis. This activity also induces cortical granules to expel their contents. This event alters the zona pellucida to limit polyspermy [85].

Early Embryonic and Trophectoderm Development

Once fertilization has occurred, the male and female pronuclei of the zygote will undergo syngamy within the cytoplasm. The newly formed zygote begins mitotic cellular divisions to generate multiple embryonic cells, or blastomeres. This newly formed entity generally is referred to as an embryo, but in reality this new entity is best referred to as a conceptus since it also contains the ability to form extraembryonic tissues (placenta). Both terms will be used in this narrative. During the first few mitotic divisions, each blastomere has the capability to develop into a completely formed individual [86], an event known as totipotency. As development progresses, totipotency is lost in some blastomeres but not others. The initial segmentation of

embryonic cells is the first in many differentiation events that the embryo proper and extraembryonic membranes go through to generate a viable fetus and placenta.

It takes approximately 96 hours for the bovine embryo to travel through the oviduct and into the uterine body. Normally, bovine embryos have reached the 16-cell stage in development, which equates to developing through four cell divisions. After this point, subsequent mitosis generates a mass of cells, termed a morula. Blastomeres will continue to undergo division and will begin to compact. During compaction, tight junctions form between blastomeres lining the outside of the embryo. Soon thereafter, the first signs of blastocoele formation become evident as fluid begins to build up in the center of the morula [87, 88]. The timing of blastocoele formation varies among individual embryos and is dependent on the rate of fluid entry into the newly formed cavity and the extent of tight junction formation between blastomeres [87, 89, 90]. During the compaction phase of morula development, two cell types may be distinguished from one another [88]. A population of flattened, outer cells will form the trophoctoderm. This tissue will give rise to outer layer of the placenta. The remaining inner cells will gather at one pole of the embryo and form the inner cell mass, which also is termed the embryonic disk, or epiblast [87]. These cells eventually will differentiate into various fetal and extraembryonic tissues.

By approximately day 9-10 of development, a small slit forms in the zona pellucida, allowing the blastocyst to separate from the zona pellucida in a process termed “hatching” [91, 92]. Once hatched, the inner cell mass will bulge to the outside of the sphere-shaped blastocyst and is clearly discernible as the embryonic disk, which is still covered by trophoctoderm cells, termed the Rauber’s layer. As hatched blastocyst development progresses, trophoctoderm cells within the Rauber’s layer will be shed.

Over the next several days, the bovine conceptus remains free floating in the uterine lumen as embryonic and extraembryonic development continues [87]. Between days 8 and 10 of development, endoderm emerges from the inner cell mass and lines blastocoel cavity underneath the trophoctoderm. This tissue eventually will form the inner border of the yolk sac. Between days 12 to 14 of development, the outer cells of the inner cell mass begin to polarize and differentiate into embryonic ectoderm. At this point the inner cell mass appears to be composed of multiple cell layers [87]. Between days 14 to 16 of development, the mesoderm layer migrates from the inner cell mass between the trophoctoderm and endoderm to separate the yolk and amniotic sacs, as well as forming the inner border of the chorion and eventually the umbilical cord [93].

Approximately the same time that embryonic cell lineages are emerging (days 12-16), the conceptus begins to elongate first into an ovoid shape and soon thereafter into tubular and finally a filamentous form [93]. After 17-18 days of development, the embryo may occupy two-thirds of the uterine horn ipsilateral to the ovary bearing the ovulation and may be greater than 160 mm in length [94]. Within 1-3 days later (day 18-20), the embryo usually occupies the entire gravid uterine horn and encroaches into the contralateral horn. At this stage of the development the amnion forms through encroachment of mesoderm and inversion of the trophoctoderm. Finally, a heartbeat is detectable between 20-22 days of development [95].

Implantation and Placental Development

Implantation is best described as a transitional stage during pregnancy where the conceptus assumes a fixed position on the endometrium and begins its intimate physiological relationship with the uterus [96]. The extent to which placental invasion into the uterus is dictated by the type of placenta utilized by that particular species. In the bovine and other ruminants, a synepitheliochorial placenta forms between mother and fetus. The placental cells

responsible for uterine invasion in cattle and other ruminants are termed binucleate cells or multinucleated giant cells [97-100]. These cells are first detected around day 16-17 of pregnancy and compose approximately 20% of the trophoblast by day 25 of pregnancy in cows. They remain abundant until just a few days before parturition, at which time there is a significant decline in binucleate cell number [99, 101, 102]. The binucleate cells originate from mononucleated trophoblast and undergo a process known as cytokinesis, where nuclear division occurs but there is no cytoplasmic division. These binucleate cells contain large secretory granules which occupy about 50% of their volume [99, 100]. Once these cells have transformed to binucleate cells they migrate into the maternal epithelial layer and fuse with the maternal epithelial cells to form trinucleated hybrid cells. The lysis of these giant placental-uterine cells creates the syncytium during early pregnancy, and their presence throughout pregnancy is thought to facilitate uterine-placental communications and potentially promote nutrient and gas exchange [97-99].

The trophoblast of the bovine conceptus possesses areas of numerous microvilli that form into structures known as the cotyledons, which attach to specific areas on the endometrium known as caruncles. The finger-like projections on the fetal cotyledon fit into crypts of the maternal caruncle and together they make up the unit known as a placentome [93, 99]. The placentomes serve as the sites of nutrient, gas and waste exchange between the mother and fetus. Additionally, they act as a barrier by preventing the migration of non-placental cells across the two entities [103]. These placentomes can grow to a size of 10-12 cm long and 2-3 cm thick towards the end of gestation in cattle. They are found throughout both uterine horns but are larger in the horn containing the pregnancy. The contact surface area between the fetus and

mother can reach as much as 130 m² due to this extensive interdigitation between the highly villus cotyledon and plentiful crypts of the caruncle [93, 99].

Grosser originally defined the ruminant placenta as syndesmochorial because the maternal epithelial layer degraded during early pregnancy [104]. His initial conclusions were that the fetal epithelial layer lysed uterine epithelial cells so the placenta could be in direct contact with the maternal connective tissue. Future work questioned this contention, notably because during mid- and late-gestation a majority of the uterine epithelium is reformed in the intercaruncular areas in cattle, sheep, deer and goats. In sheep and deer, caruncular areas remain free of uterine epithelium throughout pregnancy, but epithelial re-growth is observed in caruncular regions of the cow and doe (female goat) [99, 105, 106]. In all ruminant species, limited placental cell migration and fusion persists in the intercaruncular zones throughout pregnancy. Today, some researchers classify the ruminant placenta as epitheliochorial (or desmochorial) under the misconception that the syncytial state is transient [107, 108]. However, the synepitheliochorial or syndesmochorial classification remains the more precise terminology for placentation in ruminants.

Placental Hormones

The placenta acts as an endocrine, paracrine, and autocrine organ and produces a wide range of steroid and peptide hormones that facilitate fetal development and aid in altering the maternal physiology to support pregnancy [103]. Several hormones are commonly produced by mammalian placentae, including estrogens, progesterone, and placental lactogen. However, ruminants also produce some unique secretory factors, including IFN τ , the maternal recognition of pregnancy factor in ruminants. This unique hormone will be discussed later in the review.

In ruminants and many other mammalian species, the placenta produces estrogens and progesterone. The main placenta-derived estrogen product is estrone-3-sulfate. Production of this

variant may be detected locally as early as 33 days of gestation in cattle and is first detected in peripheral circulation around day 70-100 of gestation. Its peak concentrations reach 5-30 nmol/L from about day 265 of gestation through parturition [109-111]. The biological role of this estrogen variant has yet to be discovered, however it is implicated in regulating myometrial activity, placental maturation and softening of the birth canal [109-112].

Placentae from various species produce copious quantities of progesterone. However, unlike the sheep and horse, where the placenta will take over as the dominant source of progesterone at a particular stage of gestation, progesterone production by the bovine placenta is not sufficient to maintain pregnancy. Instead, luteal-derived progesterone must also be available throughout gestation to sustain a pregnant state [110].

One of the main protein hormones produced by the ruminant placenta is placental lactogen (PL). PLs are produced by the binucleate cells [113-115]. This non-glycosylated, single-chain, 23-kDa protein is structurally similar to prolactin and growth hormone. It can bind and activate prolactin and growth hormone receptors [103, 113-120]. The onset of PL production in the ewe occurs on day 16 of pregnancy [121, 122]. In the cow, there is a slow rise of PL throughout pregnancy corresponding with a continuous fall in the fetal serum beginning at about day 75 of pregnancy [123]. Throughout gestation in the cow and greater than two thirds of gestation in the ewe, fetal PL concentrations remain consistently higher than those in maternal blood [123, 124].

The precise functions of PL in the ruminant remains speculative, but recent work implicates this hormone in regulating endometrial gland development. In utero biological effects of ovine PL on endometrial differentiation and function has been demonstrated in ewes receiving ovarian steroid replacement therapy, where intrauterine injections affected endometrial function

via uterine milk protein mRNA and osteopontin mRNA levels and uterine gland density were increased [125]. In addition to stimulating endometrial gland morphogenesis, placental lactogen has also been implicated in regulating water and electrolyte balance, mediating fetal growth and development, and potentially serving to regulate metabolism and behavior [126, 127].

Parturition

Parturition is a hormonal process where the fetus signals to the mother that it is ready to be expelled from the uterus. Stress signals initiated by the fetus must lead to enormous changes within the maternal environment to transition from a state conducive to maintaining a pregnancy to one that facilitates removal of the fetus from the uterus. Progesterone produced by the CL maintains its dominance as the main hormone of pregnancy in cattle, with a gestation of approximately 280 days. By approximately 250 days of gestation, plasma progesterone concentrations reach approximately 7-8 ng/mL and drop steadily afterwards to about 3-4 ng/mL. The day before parturition progesterone drops to a nearly undetectable level [128]. Conversely, estradiol concentrations remain fairly low throughout most of gestation (approximately 300 pg/mL by mid-gestation) but increase just before parturition (4000-6000 pg/mL) [129].

Current dogma implicates fetal stress signals as the initiator of parturition in many mammals. In sheep and presumably cattle, fetal cortisol appears to be the initial parturition signal. Cortisol, stimulated by corticotrophin releasing hormone (CRH) and produced by the adrenal cortex, will act on the placenta to use progesterone as a substrate for estrogen production, thereby reducing progesterone secretion by the placenta. This shift in steroid production increases myometrial contractions [130]. During the cortisol surge at the onset of labor, a feed-forward endocrine loop between cortisol, CRH, and ACTH is established in the fetus, and this drives the endocrine events that produce active labor [131, 132]. It is hypothesized that increased uterine activity is, in part, due to exogenous oxytocin production by the posterior pituitary of the

mother, which is released in a pulsatile manner during pregnancy. Myometrial OTR concentrations in the cow will increase gradually throughout pregnancy, reaching its peak weeks before the onset of labor. Within caruncular endometrium, OTR levels remain low until just prior to parturition. A sudden surge of oxytocin into the maternal blood system at the onset of labor along with the elevated concentration of myometrial OTRs is the likely cause of myometrial contractions during parturition [133].

Maternal Recognition of Pregnancy

Once the newly created conceptus has begun to develop, an essential line of communication between the embryo and uterus must be established to maintain a pregnant state. It is particularly crucial that the embryo signal its presence to the mother to ensure continued CL function. This process is one component of what is commonly known as maternal recognition of pregnancy. In ruminant species, this key signaling process is directed by a novel protein and member of the Type I interferon family, termed IFN τ .

Discovery of the Maternal Recognition of Pregnancy Factor in Ruminants

Maternal recognition of pregnancy in cattle and sheep was first investigated in the 1960s by Drs. Moor and Rowson. In a series of studies they determined that the presence of a viable embryo by day 12 and 16 post-estrus in ewes and cows, respectively, was required for the continuation of CL function beyond the time of a normal estrous cycle [134-138]. The nature of this factor remained unknown until Martal, et al. (1979) discovered the embryo-derived factor, initially termed trophoblastin, was proteinaceous in nature [139]. Intrauterine injections of homogenates from day 14-16 ovine embryos successfully maintained the CL lifespan by one month, while intrauterine injections of homogenates from day 21-23 ovine embryos did not extend the CL lifespan [139, 140]. Shortly thereafter, researchers at the University of Florida discovered that low molecular weight proteins isolated from ovine and bovine conceptus

contained this antiluteolytic activity. When injected into uterine lumen of non-pregnant ewes or cows during diestrus, conceptus secretory proteins from day 15-16 ovine conceptuses and 16-18 bovine conceptuses successfully extended luteal function in cyclic ewes and cows [140, 141]. These proteins were termed trophoblast protein-1, or oTP-1 and bTP-1 for the ovine and bovine counterparts, respectively. Sequencing of oTP-1 cDNA isolated from a day 13 ovine conceptus cDNA expression library determined that the protein product was structurally similar to Type I interferons (IFN) [139, 142]. This newly discovered trophoblast-derived interferon was then named interferon-tau (IFN τ).

The IFN τ Gene and its Expression

Expression of IFN τ genes is distinct from many other Type I IFNs. Of particular note is the lack of IFN τ expression in response to a virus or pathogen challenge, which is a hallmark feature of most other Type I and Type II (IFN γ) IFNs [143, 144]. Also, IFN τ is not expressed by a variety of cell types, including immune cells, as are most other IFNs. Instead, IFN τ genes are transcribed solely in the trophoblast for a specific period during early pregnancy [145, 146].

These unique features of IFN τ expression likely were conceived as the ancestral IFN τ gene was created in peromyscid ruminant ancestors soon after their divergence from other Artiodactyls, such as camels, llamas and pigs, approximately 36 millions years ago. It is proposed that the ancestral IFN τ gene was created by duplication of the IFN ω gene. Coincident or soon after this duplication event, the promoter region of the new IFN gene was replaced by sequences that permitted placental-specific gene expression [16, 146, 147]. IFN τ genes have been identified in the Bovidae, Cervidae and Giraffidae families by Southern Blot analysis, but comparable genes could not be found in other mammalian species [16, 148].

IFN τ mRNA is expressed for a brief period of time during maternal recognition, beginning early in bovine embryo development and reaching peak gene expression at day 14 coincident with the onset of conceptus elongation in cattle. [145, 149-152]. IFN τ mRNA can be detected until approximately day 25 of development but is not expressed thereafter [145, 150, 151]. IFN τ protein can be detected in conditioned medium as early as the late morula or early blastocyst stage of development, approximately day 6-7 post-fertilization [153, 154]. Although IFN τ mRNA abundance reaches its peak at day 13-14 of development, the overall production of IFN τ protein continues to increase between days 13 and 19 of pregnancy due to the considerable growth of trophoctoderm over this period of development [149, 155-157].

Antiluteolytic Action of IFN τ

The extension in CL function is considered as the primary activity of IFN τ during early pregnancy. IFN τ accomplishes this task by acting on the uterus to limit the pulsatile secretion of uterine-derived PGF $_{2\alpha}$ normally occurring during late diestrus. Recombinant forms of both ovine and bovine IFN τ are able to mimic the biological activities of native IFN τ . Intrauterine injections of recombinant bovine and ovine IFN τ in cattle extend luteal function and repress the pulsatile release of uterine PGF $_{2\alpha}$ in response to oxytocin challenge [150, 158-160].

In a pregnant ewe and cow, IFN τ limits PGF $_{2\alpha}$ pulsatility by limiting expression of oxytocin receptors within the endometrial epithelium. Flint and Sheldrick (1986) demonstrated that oxytocin receptor concentrations are decreased in early pregnancy compared to the concentrations of a normal estrous cycle in ewes [161]. This effect of pregnancy could be mimicked by administering IFN τ or ovine conceptus secretory proteins to cyclic ewes [139]. Studies in ewes also revealed that a decrease in estrogen receptor abundance corresponded with the decreased number and binding affinity of oxytocin receptors on the endometrium [162, 163].

This activity is mediated within the endometrium by two IFN-regulated transcription factors, Interferon Regulatory Factor-1 (IRF-1) and Interferon Regulatory Factor-2 (IRF-2). IRF-1 is a transcriptional activator of IRF-2, which is best known as a transcriptional repressor [164]. Luminal and glandular epithelial expression of both IRF-1 and IRF-2 increases in non-pregnant ewes supplemented with IFN τ [54]. The current working model of the antiluteolytic actions of IFN τ to limit oxytocin receptor expression, and thus luteolysis, is that IFN τ stimulates IRF-1 gene transcription in endometrial epithelium, which in turn increases IRF-2 gene transcription. IRF-2 represses ER gene expression by binding to an interferon regulatory factor element, and this loss in ER expression prevents the up-regulation of OTR expression. Collectively, this loss in OTR prevents oxytocin from inducing PGF $_{2\alpha}$ synthesis [54, 164, 165].

The estrogen receptor-mediated theory of oxytocin receptor gene expression regulation appears to be well suited for the physiological events observed in ewes, but cattle are slightly different in their mechanism of action. Most notably, in a non-pregnant state, increased abundance of OTRs precedes that of increased ER expression in bovine endometrium [52, 53]. Based on this physiological difference between ewes and cows, it is possible that cows and ewes differ in the mechanisms used to prevent oxytocin receptor gene expression. Perhaps IFN τ acts directly on oxytocin receptor expression in cattle and also acts indirectly by regulating estrogen receptor expression during early pregnancy in both species. As mentioned earlier, there is conflicting evidence in sheep as to which receptor is up-regulated first. Spencer and colleagues found that ER concentrations increase before OTR [54] while observations by Wathes and Hamilton contradicted these findings [49].

Interferon-tau also modifies prostaglandin metabolism in endometrium during early pregnancy. The production of endometrial-derived prostaglandins is controlled by a series of

enzymes. Phospholipase A₂ (PLA₂) is an OTR-responsive enzyme that cleaves membrane-bound phospholipids to generate arachidonic acid [166]. Arachidonic acid is then converted to PGH₂ by cyclooxygenase -1 and -2 (COX-1, COX-2), which are also known as prostaglandin endoperoxide H synthases 1 and 2 (PGHS-1 and PGHS-2). PGH₂ is then converted to various other PGs by specific enzymes, including PGE and PGF synthases, which generate PGE₂ and PGF_{2α}, respectively. In the presence of progesterone, recombinant bovine IFNτ can enhance the secretion of PGE₂, thereby increasing the PGE₂/PGF_{2α} ratio [167]. A biphasic PG response to IFNτ treatment has been observed by several laboratories. Lower levels of IFNτ similar to levels found during early conceptus develop at the time of pregnancy recognition suppress COX-2 expression in primary bovine endometrial cells or a bovine endometrial cell line (BEND cells) [160, 168, 169]. Conversely, exposure to high concentrations of IFNτ in these cell types, reminiscent of levels in the uterus after maternal recognition of pregnancy, stimulates COX-2 expression [160, 169]. Collectively, IFNτ appears to shift the predominate prostaglandin production away from PGF_{2α} and toward PGE₂, thus permitting the putative luteotrophic actions of this prostaglandin to favor CL maintenance [59, 170-172].

Other Activities of IFNτ

Interferon-tau possesses the classical activities of Type I IFNs (antiviral, immunomodulatory), and it is possible that these activities facilitate pregnancy in cattle, sheep and other ruminants. IFNτ inhibits the proliferation of cultured lymphocytes [173, 174] and inhibits the proliferation of mitogen-stimulated or mixed population lymphocytes [173, 175-178]. IFNτ also activates natural killer cells indirectly by increasing granulocyte chemotactic protein-2 production in the endometrium [132, 179]. Moreover, IFNτ limits the expression of major histocompatibility complex (MHC) class I molecules in the luminal epithelium during

placentation [180]. The physiological importance of IFN τ 's antiviral activity remains uncertain, but it seems intuitive to contend that IFN τ may act in a prophylactic capacity to limit viral infections early in pregnancy.

IFN τ also is implicated in orchestrating events to create a favorable uterine environment for the maintenance of pregnancy. The expression of several uterine proteins is regulated by IFN τ . One major IFN τ -induced uterine protein is ubiquitin cross reactive protein (UCRP), which also is known as IFN stimulated gene-15 or -17 (ISG15 or ISG17) [181-183]. UCRP conjugates with cytosolic proteins and prompts their degradation in cells. This activity of UCRP may be integral to establishing and maintaining pregnancy, although this precise function of UCRP remains uncertain. Granulocyte chemotactic protein-2 (GCP-2), which was discussed earlier for its role in immunomodulatory activities, is induced by IFN τ . Other IFN τ -induced uterine proteins include Mx proteins [184, 185], 2'5'-oligoadenylate synthetase [186], and a member of the 1-8 family, termed Leu13 [187]. One well described function of Mx protein is in the antiviral response, and it is unclear if it has a critical activity during early pregnancy or merely is facilitative during early pregnancy in ruminants [185, 188, 189]. 2'5'-oligoadenylate synthetase activates a constitutively expressed latent endonuclease, ribonuclease L (RNase L). This enzyme cleaves viral and cellular RNA and blocks viral replication and initiation of apoptosis in some cell types [186, 190, 191]. Its importance during maternal recognition of pregnancy remains uncertain. Leu13 was first described as a T-cell surface antigen implicated in inhibiting cell growth [192, 193]. Leu13 is localized primarily in the glandular epithelium, and it was hypothesized that this member of the 1-8 protein family, which is known for its involvement in adhesion, wound healing, and inflammatory responses, plays a role in adhesion of the conceptus to the endometrium and in the development of the placentome [187, 192-194].

Bovine Embryo Culture

Today's bovine embryo culture systems are capable of generating blastocyst stage embryos that can be transferred to recipients with fair pregnancy outcomes [195-199]. However, *in vitro* culture systems continue to remain inferior to the maternal environment, and identifying components provided by the maternal uterine environment that are not currently present in these culture systems is a major focus surrounding research regarding *in vitro* embryo culture. As the identification of more uterine-derived factors affecting embryonic development arise, our understanding of embryo development continues to become more complete.

Early attempts of bovine embryo culture met with little success as embryos would arrest at the 8- to 16-cell stage due to a lack of adequate support embryonic genome activation [200-202]. Several culture systems have been devised to overcome this obstacle. Traditionally, two types of culture systems were used: those utilizing somatic cells in the culture medium, or co-culture systems, and those that do not depend on somatic cells or serum supplementation, or "defined" culture systems [203]. Co-culturing embryos with somatic cells was once the preferred method of bovine and ovine embryo culture [204]. However, the disadvantage of co-culturing with somatic cells is that the identification of true embryotrophic compounds or other components of the physical environment are masked by the interaction that exists between the culture medium and somatic cells [205, 206]. This led to the development of several defined culture systems.

Defined *In vitro* Culture Requirements

There is much debate over an accurate definition of "defined" embryo culture system. A definition given by J.G. Thompson (1996) is that a defined culture system is one implementing a culture medium and physical environment using identifiable components prior to embryo culture. One of the primary reasons for such debate over the definition of a "defined" culture system is

due to the common usage of serum, which contains uncharacterized compounds as well as defined compounds that vary in their concentration among preparations [205]. Serum is a complex combination of both small and large molecules with growth-promoting and growth-inhibiting activities, depending on the source of serum. The addition of serum is commonplace during final oocyte maturation *in vitro*, and unknown agents in serum stimulate differentiation of cumulus cells [207-209]. Bovine serum albumin (BSA) is a common medium supplement and is the predominant protein in reproductive tract. Some of the other potential benefits of BSA in culture systems include its potential role in chelation and blocking non-specific binding sites on surfaces [203]. There are reports of serum-free bovine oocyte maturation protocols available, but others contend that these systems are not sufficient to mature oocytes to the point where they can be fertilized *in vitro* and develop to the blastocyst stage [210-212].

Presently, a variety of defined media exist for culturing bovine embryos after fertilization. Types of culture medium can be broken down into two main subtypes: complex and simple. Complex culture medium, such as Tissue Culture Medium-199 (with Earle's salts) and Menezo's B2 medium, contain various components including amino acids, vitamins, salts, nucleotides and purines. These types of components typically reflect the needs of somatic cell growth rather than for early embryo development, although the bovine embryo will develop in these media [205, 213]. Simple medium formulations, which include most of the so-called culture media designed specifically for embryos, typically are salt solutions supplemented with some specific substrates and a supplemental protein source, usually BSA. Examples include synthetic oviductal fluid (SOF; plus amino acids and BSA), which was formulated based on the make-up of luminal oviduct components, potassium simplex optimized medium (KSOM),

chemically defined medium (CDM), Charles Rosenkrans 1 (CR-1), Chatot/Ziomek/Bavister medium (CZB), and Hamster embryo culture medium (HECM) [203, 214].

Carbohydrates are an important source of energy for the developing embryo. Prior to compaction and morula development, the early bovine embryo does not readily utilize glucose as its primary energy source. In fact, glucose can be toxic to early developing embryos [215, 216]. Supplementation with pyruvate and/or lactate are excellent sources of energy for pre-compacted bovine embryos [203, 205, 217]. Work from Bavister and colleagues found that supplementing culture medium with both essential and non-essential amino acids had an overall stimulatory effect on embryo development, although some specific amino acids (phenylalanine, valine, isoleucine, tyrosine, tryptophan, and arginine) inhibited development [218, 219]. Glutamine, in particular, is a vital component of embryo culture media based on work in mice where glutamine supports embryo development in mouse strains prone to arresting at the 2-cell stage [220]. Bovine embryos undergo a change in their requirements for essential and non-essential amino acids as development progresses [221], although most culture procedures do not alter medium formulations between the 1-cell and blastocyst stages.

Optimal embryo development *in vitro* also relies on other important considerations. The optimal temperature for bovine *in vitro* maturation, fertilization, and development is between 38.5 and 39 °C [222, 223]. Maintenance of intracellular pH is critical for numerous cellular mechanisms [224]. In the oviduct and uterine fluid, regulation of pH occurs through HCO_3^- and its equilibration with CO_2 , which varies throughout the estrous cycle [225, 226]. Traditionally, embryo culture was conducted in an atmospheric oxygen level (approximately 20%), which is considerably greater than that found in the maternal environment [227]. Recent work in bovine embryos note that reducing the oxygen levels during early development to between 5 and 7%

improves proportion of embryos developing to the blastocyst stage [215, 228-231]. Presumably, the results from these studies indicate that lowering the oxygen tension limits the production of reactive oxygen species, thereby providing embryos with an environment that does not compromise membrane integrity and basic intracellular functions.

Culture medium for early *in vitro* blastocyst development should be tailored to the stage of development of the embryo. Earlier embryo development, up to day 5 post-fertilization, requires a simple base medium containing either no serum or a low concentration of serum, essential and non-essential amino acids (sometimes already in the base medium), pyruvate, lactate, glutamine, and preferably no glucose. Embryo development after the late morula/early blastocyst stage can be supported similarly by either a simple or complex medium type with supplemented glucose. Throughout culture, it is preferable to utilize a 5% oxygen environment, as this more closely replicates the oxygen environment an *in vivo* embryo is exposed to in utero.

Differences between *In vivo* and *In vitro*-Derived Embryos

Although healthy looking blastocysts are routinely obtained from *in vitro* maturation, fertilization, and culture methods, these embryos remain somewhat inferior to their *in vivo* derived counterparts. Most notably, pregnancy rates following transfer of *in vitro*-derived embryos is lower than transfer of *in vivo*-derived embryos to recipients [232-235].

Morphologically, *in vitro*-derived bovine embryos contain a darker cytoplasm, greater lipid content, and specifically more triglycerides, swollen blastomeres, and a more fragile zona pellucidae [236-239]. Also, *in vitro*-derived bovine embryos have a greater incidence of chromosomal abnormalities [236-242].

Blastocyst yield from *in vitro* produced embryos is affected by oocyte quality. While in the dominant follicle, the oocyte undergoes maturation events that lead to developmental competence for fertilization and subsequent embryo development. Several studies indicate that

oocytes matured *in vivo* are more competent to become fertilized and develop to the blastocyst stage than those matured *in vitro* [243-248]. Interestingly, work by Lonergan and colleagues showed no differences in cleavage rate between *in vivo* and *in vitro* fertilized bovine oocytes. This suggests that although fertilization is required to create embryos, it probably is not a limiting factor in producing embryos *in vitro*. The *in vivo* fertilized group had a greater proportion of oocytes reaching the blastocyst stage than *in vitro* fertilized oocytes, although there was no difference in quality, as determined by vitrification and thawing. However, a higher proportion of matured oocytes that developed to blastocysts after fertilization *in vivo* compared with *in vitro* fertilization. This result indicates that events surrounding the time of fertilization may play a role in developmental competence of oocytes, while the actual location of fertilization has no effect on blastocyst quality [249].

Two related experiments conducted by Lonergan and colleagues exemplify how *in vitro* development is suboptimal [249]. In their studies, *in vivo* and *in vitro* matured and fertilized zygotes were placed in either an *in vitro* culture system or in the oviduct of ewes until day 7 post-fertilization. No differences in cleavage rates or the percentage of oocytes that developed to blastocysts were observed, but blastocyst development and hatching rates increased in embryos incubated in ovine oviducts regardless of whether oocytes were matured and fertilized *in vivo* or *in vitro* [249]. Similarly, other work indicates that viability of *in vitro* produced embryos is 15-25% less than their *in vivo* produced counterparts [250-252]. The addition of serum to *in vitro* culture systems also is deleterious to post transfer development and promotes early blastulation, decreases cell number, increases apoptosis, and reduces rates of glycolysis [253-259].

A subset of genes also is expressed differentially between *in vitro* and *in vivo* derived bovine embryos. For example, mRNA for connexin 43, a gap junction protein, is lower in

abundance and Bax, a cell death factor, is elevated within *in vitro*-derived bovine embryos compared with *in vivo* derived embryos [260, 261]. A study using cDNA microarrays evaluated the differential expression of 384 genes or expressed sequence tags between *in vitro* and *in vivo* derived blastocysts. The results indicated that approximately 85% of these genes had reduced expression for *in vitro*-derived blastocysts compared to their *in vivo* counterparts [262].

In summary, the *in vitro* production of bovine embryos is a powerful tool to describe the events associated with normal embryo development in cattle. However, current *in vitro* systems are inadequate in several regards and improvements in these systems or more stringent selection and/or grading of embryos produced by these systems is required to obtain higher quality embryos.

Extended Blastocyst Culture

The continuation of embryo culture during and beyond the blastocyst stage can be accomplished, at least to a certain degree, but most experiments conducted with *in vitro*-derived bovine embryos end on day 7 or 8 post-fertilization when they reach the blastocyst stage. One method of extending the traditional culture systems is by simply placing the embryo into a new medium drop. Using this type of conventional culture procedure, hatched blastocysts are inclined to attach to the bottom of the culture dish and form trophectoderm outgrowths. Under these culture conditions, trophectoderm cultures can be maintained for extended periods but the remainder of the embryo (i.e. the embryonic disk) will degenerate on or after day 13-15 post-IVF [263, 264]. More recently, Alexopoulos and colleagues successfully extended embryo culture to day 27 post-fertilization when supplementing medium with Fetal Bovine Serum (FBS). Embryo development reached a spherical stage and seemed to match up with typical *in vivo* embryonic development. This was determined by evaluation of trophectoderm proliferation and whether or not a hypoblast was established (Day 11). More frequently, however, there was a divergence

from normal *in vivo* embryo development when embryos attached down to collagen gel matrix and initiated (spread) putative trophectoderm cell growth [9].

Using agarose tunnels to promote elongation is an intriguing concept for extended blastocyst culture. This method was first reported by Stringfellow and Thompson [265] and has been investigated more recently by others [13, 266]. These agar gel tunnels are created with 3% (w/v) agarose suspended in Dulbecco PBS with Ca^{2+} and Mg^{2+} , poured into a frame for vertical gels with a comb containing glass capillaries. Once the agarose is set, the comb of capillaries is removed and cut into 3 pieces, each containing four or five tunnels approximately 12-14 mm in length and 1 mm in diameter. Using this agarose scaffold, 52% of blastocysts elongated to a minimum of 1.6 mm by day 12 post-IVF. These embryos completely filled the space between the tunnel walls, while blastocysts cultured without an agarose scaffold averaged 1.3mm in diameter and possessed no signs of elongation. By day 14 post-IVF, 24% of the blastocysts remained viable and continued to grow within the tunnels, reaching an average of 4.3 mm in length. However, by day 16 post-IVF, only 2 embryos continued to grow in the tunnels, one reached 12 mm in length and the other 8 mm in length. In summary, this system successfully induced elongation but few embryos remained viable by days 14 and 16.

Another extended culture system utilizing agarose gel tunnels that achieved success was developed by Brandão and colleagues [12]. This group developed the Post Hatching Development (PHD) system. In this system, a Petri dish was set up with two glass capillary combs were placed in opposite directions from one another with the attached ends resting on the top edge of the dish and the closed ends on the bottom of the dish. The Petri dish was filled with 2.4% agarose in PBS, supplemented with either 4.5% or 9% FBS. When the gel was set, 2 mL of PHD medium (SOFaaci with 0.5% glucose and 10% FBS) was poured over the surface and the

combs were removed. Diagonal tunnels 20 mm in length and 1.2 mm wide were formed, filled with medium, and each tunnel contained one blastocyst. With this system, 56% of embryos initiated elongation and reached average length of 2.9-4.4 mm, depending on the quality score. Some embryos were not subjected to analysis and remained in the PHD system, reaching lengths of 10.1 mm by day 17 and 12.5 mm by day 19. However, only 23% showed continuous elongation by day 15 post-IVF. The development of this system allowed for a successful definition of a stable culture system for evaluating rapid growth, elongation, and initial differentiation of bovine post-hatching embryos that were produced exclusively by *in vitro* production [12].

In summary, while *in vitro* bovine embryo culture has met with some success in developing culture systems up to the hatching phase of development, there is still the issue of our inability to fully replicate the maternal uterine environment. Uterine endometrial glands secrete factors that are vital for proper conceptus development, elongation and placentation [4, 5, 267]. These more advanced stages of embryonic development create an even more problematic situation from what already exists in our culture systems. As the embryo grows and differentiates it requires a more complex array of energy, proteins, growth factors and many other undefined components than our systems currently provide. Nonetheless, attaining such a system will prove invaluable both commercially and experimentally.

Uterine Factors Affecting Embryo Development

This literature review has provided evidence arguing that the natural maternal environment provides factors that are vital to the production of healthy embryos, and scientists have been unable to precisely replicate embryo development using *in vitro* culture systems. Several uterine secretions are implicated as embryotrophic factors, and an overview of some of these factors is provided below.

One particularly well studied growth factor that plays a role in embryonic development is insulin-like growth factor-I (IGF-I). Uterine and placental production of IGF-I occurs in several species, including the rat [268], human [268, 269], pig [270] and sheep [271, 272]. Several studies implicate IGF-I as an embryotrophic factor in porcine, murine, human and bovine embryo development [273-276]. More recently IGF-I has been proposed to function primarily as a survival factor, and decreases the rate of apoptosis during *in vitro* culture [277, 278]. Also, IGF-I limits the deleterious effects of heat shock during bovine embryo culture [279, 280], and supplementation with IGF-I increases pregnancy rates of *in vitro* produced bovine embryos following transfer to recipients subjected to environmental conditions that could induce heat stress [281, 282].

Several other uterine-derived factors play vital roles in conceptus development. In the mouse, several factors, including transforming growth factor (TGF- α or TGF β 1), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-II), and epithelial growth factor (EGF) promote early embryo development *in vitro* [283-285]. Transcripts for TGF- β 2 and insulin-like growth factor (IGF-II) and the receptors for PDGF and IGF-II are detectable throughout preimplantation development in the bovine, indicating that these mRNAs are products of both the maternal and the embryonic genome origin in the cow. Platelet derived growth factor (PDGF) is produced in myometrial cells and promotes bovine embryo development beyond the 16-cell stage when treated for 9 days after 24 hours post-fertilization, although it does not increase blastocyst rate [286]. In that same study, TGF α was, in fact, able to increase the percentage of embryos developing to blastocysts where PDGF could not.

A few uterine-derived factors also are implicated in regulating IFN τ production during early pregnancy. The two that are particularly interesting to this laboratory include fibroblast growth factor-2 (FGF-2) and granulocyte-macrophage colony-stimulating-factor (GM-CSF).

Fibroblast Growth Factor-2 (FGF-2)

Fibroblast growth factors (FGF) represent a large family of paracrine and autocrine modulators consisting of 23 distinct polypeptides that contain mitogenic, chemotactic, and angiogenic activities responsible for various physiological processes, such as cell migration, cell differentiation, cell survival, and tumorigenesis [287-289]. These FGFs associate with a family of plasma membrane-spanning receptors containing intrinsic tyrosine kinase activity termed FGFRs. Four distinct genes encode these receptors and alternatively spliced products generate receptor sub-types that contain two or three immunoglobulin-like extracellular domains [287, 289-291]. Alternative splicing occurs primarily within the third immunoglobulin-like domain. This alternative splicing creates receptor subtypes possessing distinct ligand-binding specificities [289]. Each of the four FGFRs are activated by a specific FGF subset, although FGF-1 and FGF-2 have the ability to bind to numerous receptor subtypes and exert varying actions on target cells [289, 292].

Several members of the FGF family, such as FGF-4, and -7, have been implicated in influencing early embryo development. FGF-4 determines the developmental fate of murine trophectoderm cells [293, 294]. FGF-7, typically known to stimulate proliferation and migration of different epithelial cell types, was shown to increase the surface area of the trophoblast in the mouse [295]. Additionally, FGF-7 stimulates the proliferation and differentiation of porcine trophectoderm [296].

The first FGF to be identified was FGF-2, or basic FGF. FGF-2 was first described as a mitogen for mouse 3T3 fibroblasts [290] and is now best known as a mitogen and angiogenic factor [297-300]. FGF-2 is produced by the uteri of several species during early pregnancy and has been implicated in regulating placental development in several species [297, 299]. In pigs, rodents and rabbits, FGF-2 is primarily localized to the luminal epithelial layer of the endometrium during diestrus and early pregnancy [8, 301-303]. The loss of FGF-2 function in mice does not have major impacts on embryonic and placental development although defects in wound healing and heart contractility have been observed [304, 305]. Supplementation of FGF-2 in mouse embryo culture medium increased trophoblast outgrowths [295]. FGF-2 also promotes gastrulation in rabbits, limits differentiation in human embryonic stem cells in culture, and improves bovine blastocyst development when used in conjunction with transforming growth factor- β (TGF- β) [306-310]. Additionally, FGF-2 has been implicated in the regulation of placental attachment and formation of the syncytium in sheep [311].

Most recently, FGF-2 has been implicated in influencing IFN τ production in the bovine blastocyst. In ruminants, FGF-2 is produced by both the endometrium and the conceptus and in the endometrium is localized primarily in the luminal and glandular epithelium throughout the estrous cycle and early pregnancy [8, 312]. FGF-2 supplementation increased IFN τ mRNA concentrations in a bovine trophoblast cell line (CT-1 cells) and increased IFN τ protein secretion from both CT-1 cells and bovine blastocysts [8].

Granulocyte-Macrophage Colony-Stimulating-Factor (GM-CSF)

This cytokine is best known for its ability to mediate inflammation and other immune responses in macrophages, granulocytes and eosinophils. GM-CSF was first identified in activated T cells for its role in proliferation and differentiation of myeloid haematopoietic cells

[313, 314]. GM-CSF has been implicated in playing a role in embryo and placental development in several species. Mice exhibiting a disrupted GM-CSF gene produce smaller litter sizes and a reduction in offspring survival. Additionally, mice deficient in GM-CSF have exhibited reduced placental capacity, likely due to a reduction in the proliferation and differentiation of mononucleated trophoblast cells [313, 315]. GM-CSF injections in mice prone to abortion reversed their high absorption rates and increased fetal and placental weights [316]. Furthermore, human and mouse embryos possess GM-CSF receptors beginning at the first cleavage stage and treatment with GM-CSF improves blastocyst development [317, 318]. GM-CSF mRNA is localized within the placenta of the mouse [317, 319-321].

GM-CSF is implicated in mediating embryo development and IFN τ production in sheep and cattle. GM-CSF mRNA and protein abundance is greatest in the luminal and glandular epithelium of the bovine and ovine, while protein is detected in the oviduct and uterine flushes [322, 323]. GM-CSF treatment improves blastocyst development in cattle [317, 324, 325]. Several reports suggest that GM-CSF supplementation can increase IFN τ mRNA and/or protein production in bovine and ovine trophoctoderm [314, 326-328], however others were not able to detect such affects [329]. There also is evidence that the conceptus, and more precisely IFN τ , regulate uterine production of GM-CSF. Based on several findings, the ability of IFN τ to stimulate uterine GM-CSF is indirect, and is caused by the IFN τ stimulating PGE₂ production in uterine endometrium and PGE₂ stimulating GM-CSF expression in uterine lymphocytes and endometrium [330-332].

Summary

The estrous cycle consists of a series of predictable physiological events which occur throughout the female's adult life and provides her with repeated opportunities to copulate and

become pregnant. Follicles grow and develop in waves, eventually allowing one to become dominant and ovulate, forming a corpus luteum (CL). If the female is pregnant, the conceptus will signal its presence, via the maternal recognition of pregnancy signal Interferon-tau (IFN τ), maintaining the pregnant state. Otherwise, a complex series of events will occur, leading to luteolysis and return to estrus for another breeding opportunity.

Once the zygote has become fertilized, it develops first in the oviduct and later in the uterine lumen. At the blastocyst stage, trophoctoderm is distinguishable from non-differentiated embryonic cells in the inner cell mass, and proliferation of trophoctoderm changes the size and shape of the developing conceptus into a filamentous structure that will begin attaching to the uterine lining and forming the outer components of the placenta. Eventually, placentomes will be created by the coupling of placental-derived cotyledons with uterine-derived caruncles.

Throughout the lifetime of the placenta, several placental hormones are produced and function in various capacities to drive placental and fetal development and maintain the pregnant state until parturition. One vital product of the early placenta is IFN τ , which is a type I interferon expressed solely by the trophoctoderm. It is responsible for maintaining the uterus in a pregnancy-receptive state during the second and third weeks of pregnancy.

Current bovine embryo culture systems are capable of producing transferable embryos that result in fair pregnancy outcomes; however, they remain inferior to those created in the maternal environment. The maternal uterine environment contains a complex array of components which have yet to be fully characterized, and current culture systems cannot completely replicate that environment. Extension of embryo culture beyond the hatched blastocyst stage of development is particularly challenging, yet a few systems have met with success in inducing elongation. Developing such extended embryo culture systems will certainly be a valuable tool for better

understanding the physiological events associated with bovine embryo development and IFN τ production.

The following research project was conceived to develop an *in vitro* culture system that extended bovine embryo culture past the initial formation of blastocysts on days 6 to 8 post-IVF so that it could be used to improve our understanding of embryo development during this crucial period of development. To follow is a culture scheme that was developed to sustain bovine blastocysts in culture up to at least day 11 post-IVF. Several experiments were completed to determine how medium formulation, atmospheric conditions, and putative embryotrophic factors affect embryo quality, development, and IFN τ production in bovine blastocysts. This work is expected to be valuable for future research describing how blastocyst development progresses and understanding how uterine-derived factors influence bovine conceptus development.

CHAPTER 3 MATERIALS AND METHODS

***In vitro* Embryo Production**

In vitro maturation, fertilization and culture procedures [333-335] were used to generate the bovine embryos used in these studies. Bovine ovaries from dairy and beef breeds were collected from Central Beef Packing Co. (Center Hill, FL) and stored in sterile saline (or 0.9% [w/v] NaCl) with 100 units/mL penicillin and 100 µg/mL streptomycin (Chemicon International) at room temperature during transport. Ovaries were rinsed in warmed (38°C) sterile saline containing 100 units/mL penicillin and 100 µg/mL streptomycin to remove blood and debris. Follicles (2-10 mm) were slashed with a scalpel blade and rinsed in a beaker filled with 75 mL of Oocyte Collection Medium [Tissue Culture Medium-199 (M-199) with Hank's salts, without phenol red; (Hyclone), 2% (v/v) bovine steer serum (BSS; Pel-Freez) containing 2 units/mL heparin, 100 units/mL penicillin, 0.1 µg/mL streptomycin, 1 mM glutamine] to liberate and collect Cumulus Oocyte Complexes (COCs) containing one or more complete layers of compact cumulus cells. COCs were transferred in groups of 10 to 50 µL drops of Oocyte Maturation Medium [OMM; M-199 with Earle's salts, 10% (v/v) BSS, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Bioniche), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate, 1 mM glutamine] equilibrated to 38.5°C in 5% CO₂ and 95% humidified air and covered with mineral oil. COCs were incubated for 22-24 hours at 38.5°C in 5% CO₂ and 95% humidified air.

Following maturation, COCs were washed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid -Tyrode's Albumin Lactate Pyruvate solution (HEPES-TALP) and placed into 600 µL of In vitro Fertilization-Tyrode's Albumin Lactate Pyruvate solution (IVF-TALP) (Cell and Molecular Technologies). Frozen and thawed sperm from three different Holstein bulls (Genex Cooperative, Inc) were pooled and viable sperm were separated with a

Percoll gradient [333]. COCs were fertilized with approximately 1×10^6 spermatozoa/well. Fertilization wells were spiked with 25 μ L of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μ M epinephrine) to promote fertilization and incubated at 38.5°C in 5% CO₂/95% humidified air for 18-20 hours. Within each replicate, a different combination of three bulls was used for fertilization.

After fertilization, prospective zygotes were vortexed in HEPES-TALP containing 1000 units/ml hyaluronidase (Sigma-Aldrich Co.) to remove cumulus layers. Denuded oocytes were cultured in 50 μ L drops (n=25-35 zygotes/drop) of modified Potassium-Simplex-Optimized Medium (KSOM; Caisson Laboratories) at 38.5°C in 5% CO₂/5% O₂/90% N₂ [333]. This low oxygen environment was provided by a tri-gas chamber. The proportion of cleaved embryos assessed on day 3 post-*in vitro* fertilization (IVF) averaged $73.0 \pm 1.7\%$ among studies and the percentage of oocytes that developed to blastocysts on day 8 post-IVF was $16.1 \pm 1.2\%$.

Individual Embryo Culture from Day 8 to 11 Post-IVF

On day 8 post-IVF, all blastocysts were removed from culture in KSOM and washed in 0.01M Phosphate buffered saline containing 1 mg/mL of Polyvinyl Pyrrolidone (PBS/PVP; Fisher Scientific). The stage of blastocyst development was recorded (early, expanded, hatched) and embryos were evenly distributed across treatment groups according to the stage of blastocyst development and placed in medium drops (30 μ l) covered in mineral oil.

Experiment 1: Determination of Whether Medium Type and Atmospheric Oxygen Concentration Affects Embryo Development to Day 11 Post-IVF

Individual embryos were placed in 30 μ L drops of either M-199 containing 5% [v/v] fetal bovine serum (FBS; Invitrogen Corp.), 0.011 mM gentamicin (Sigma-Aldrich Co.), non-essential amino acids (Sigma-Aldrich Co.; 0.0001 mM of each L-alanine, L-asparagine•H₂O, L-aspartic

acid, L-glutamic acid, glycine, L-proline, and L-serine), and 0.019 mM sodium pyruvate (Sigma-Aldrich Co.) or KSOM containing 5% [v/v] FBS, 0.011 mM gentamicin, and non-essential amino acids. A comparison of M-199 and KSOM medium components is provided in Table 2-1 and 2-2. Embryos were incubated at 38.5°C in either a 5%CO₂/5%O₂/90% N₂ (5% oxygen group) or a 5% CO₂/95% air atmosphere (20% Oxygen group). On day 11 post-IVF the stage and quality of embryo development was recorded. Embryo quality was determined by visual observation for expansion and/or hatching and whether the embryo had degenerated.

Degenerated embryos were classified as having partial to complete collapse or deterioration of cell membranes, more specifically the trophectoderm, and/or rupturing of the zona pellucida. Embryos were washed once in PBS/PVP, fixed in 4% [w/v] paraformaldehyde (Polysciences Inc.) for 10 minutes and stored in 500 µL PBS/PVP with 0.02% [w/v] sodium azide (NaN₃) until further use. Spent medium (25 µL) was collected from each drop and stored at -20°C. Four independent replicate studies were completed with 5 to 12 blastocysts per treatment in each replicate (total n=20 to 46 blastocysts/treatment).

Experiment 2: Determination of Whether Serum Type or Concentration Affects Embryo Development to Day 11 Post-IVF

Blastocysts collected on day 8 post-IVF were cultured individually in 30 µL drops of M-199 with 0.011 mM Gentamicin, Non-Essential Amino Acids (0.0001 mM of each L-alanine, L-asparagine•H₂O, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine), and 0.019 mM sodium pyruvate was supplemented with either insulin-transferrin-selenium (670 µg/L sodium selenite (anhydrous), 1.00 g/L insulin, and 0.55 g/L transferrin) (ITS; Invitrogen Corp.), 1% [w/v] bovine serum albumin (BSA; Sigma-Aldrich Inc.), or 1%, 2.5%, or 5% [w/v] FBS at 38.5°C in either a 5% or 20% oxygen atmosphere. On day 11 post-IVF, blastocyst development

and quality was assessed and embryos and medium samples were processed as described previously. Two independent replicate studies were completed with 3 to 6 blastocysts per treatment in each replicate (total n=6 to 16 blastocysts/treatment).

Experiment 3: Determination of Whether FGF-2 Supplementation and Oxygen

Concentration Affects Embryo Development and IFN τ Concentration to Day 11 Post-IVF

Day 8 blastocysts were cultured individually in 30 μ L drops of M-199 supplemented with 2.5% FBS, 0.011 mM gentamicin, non-essential amino acids (0.0001 mM of each L-alanine, L-asparagine•H₂O, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine), 0.019 mM sodium pyruvate and either 0 or 100 ng/mL of recombinant bovine fibroblast growth factor-2 (boFGF-2; R&D Systems, Inc.) at 38.5°C in either a 5% or 20% oxygen atmosphere. BoFGF-2 was purchased as a lyophilized powder and was reconstituted in M-199 containing 0.1% [w/v] BSA. Aliquots were stored at -20°C. All treatments, including controls, were provided to cells with identical amounts of BSA carrier (20 μ g BSA/ml medium). On day 11 post-IVF, blastocyst development and quality was assessed and embryos and medium samples were processed as described previously. Three independent replicate studies were completed with 8 to 10 blastocysts per treatment in each replicate (total n=25 to 30 blastocysts/treatment).

Experiment 4: Determination of Whether FGF-2 and GM-CSF Co-supplementation

Affects Embryo Development and IFN τ Concentration to Day 11 Post-IVF

Day 8 blastocysts were cultured individually in 30 μ L drops of M-199 supplemented with 2.5% FBS, 0.011 mM Gentamicin, Non-Essential Amino Acids (0.0001 mM of each L-alanine, L-asparagine•H₂O, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine), 0.019 mM sodium pyruvate and either 100 ng/mL boFGF-2, 100 ng/mL of recombinant porcine GM-CSF (poGM-CSF; R&D Systems, Inc.), both FGF-2 and GM-CSF (100 ng/ml of each), or no

cytokine/growth factor treatment (control) at 38.5°C in a 5% oxygen atmosphere. Both boFGF-2 and poGM-CSF were purchased as a lyophilized powder and were reconstituted in M-199 containing 0.1 [w/v] BSA. Aliquots were stored at -20°C. All treatments, including controls, were provided to cells with identical amounts of BSA carrier (20 µg BSA/ml medium). On day 11 post-IVF, blastocyst development and quality was assessed and embryos and medium samples were processed as described previously. Three independent replicate studies were completed with 8-10 blastocysts per treatment in each replicate (total n=24-28 blastocysts/treatment).

Evaluating Apoptosis and Total Cell Number

The Terminal dUTP Nick-End Labeling (TUNEL) procedure was performed on individual embryos fixed in 4% [w/v] paraformaldehyde using the *In Situ* Cell Death Detection Kit with a Fluorescein dye (Roche Diagnostics) [334]. Individual embryos were washed in 50 µL drops of PBS/PVP and permeabilized in 50 µL drops composed of PBS with 0.5% [v/v] Triton X-100 and 0.1% [w/v] sodium citrate for 15 minutes at room temperature. Positive controls were incubated in a 50 µL drop of RQ1 RNase-free DNase (50 U/ml; New England BioLabs) diluted in PBS/PVP at 37 °C for 1 h. Embryos were washed in PBS/PVP and incubated with 25 µL drop of TUNEL reaction mixture according to manufacturer instructions for 1 hour at 37°C in a dark and humidified environment. Negative controls were incubated in the absence of terminal deoxynucleotidyltransferase. All embryos were then placed in 50 µL drops of a 0.5 mg/mL Hoescht 33342 (Molecular Probes). Embryos were washed 4 times in 500 µL of PBS/PVP, placed on microscope slides, and mounted with 10-12 µL of Prolong Gold Antifade mounting medium (Invitrogen Corp.). Epifluorescence microscopy was used to determine the total number

of nuclei (blue fluorescent stain) and number of TUNEL-positive nuclei (green fluorescent stain) in each blastocyst.

Interferon-tau Production

The amount of biologically active IFN τ present in conditioned medium from each bovine blastocyst culture was determined by using antiviral assays [178]. Briefly, 20 μ l of conditioned medium collected at day 11 post-IVF was diluted serially to determine when each sample was able to prevent vesicular stomatitis virus-induced cell lysis by 50%. The activity of each sample was compared with a bovine recombinant IFN τ developed in the laboratory [151] that had been standardized with recombinant human IFN α (EMD Biosciences Inc.; 3.84×10^8 IU/mg). The average activity of the bovine recombinant IFN τ standard was 8.03×10^8 IU/mg.

Statistical Analysis

All analyses were conducted using the general linear model of the Statistical Analysis System (SAS Institute Inc.; Cary, NC). Differences in percentage of degenerated embryos among treatments, total cell number per embryo, percentage of apoptotic cells per embryo, and antiviral activity of spent medium were determined using least-squares analysis of variance (LS-ANOVA). In most studies, the antiviral activity of IFN τ in medium samples was normalized for total cell number. Pair-wise comparisons were completed to further partition effects among treatment groups. In all experiments, the results are presented as arithmetic means \pm SEM.

Table 2-1. A comparison of medium components for M-199 and KSOM.

COMPONENTS	M-199 MOLARITY (mM)	KSOM MOLARITY (mM)
AMINO ACIDS		
Glycine	0.667	0.050
L-Alanine	0.281	0.050
L-Arginine hydrochloride	0.332	0.299
L-Asparagine H ₂ O		0.050
L-Aspartic acid	0.226	0.051
L-Cystine		0.050
L-Glutamic acid	0.455	0.045
L-Glutamine	0.685	1.001
L-Histidine hydrochloride-H ₂ O	0.104	0.099
L-Isoleucine	0.305	0.200
L-Leucine	0.458	0.200
L-Lysine hydrochloride	0.383	0.199
L-Methionine	0.101	0.050
L-Phenylalanine	0.152	0.100
L-Proline	0.348	0.050
L-Serine	0.238	0.050
L-Threonine	0.252	0.200
L-Tryptophan	0.049	0.025
L-Tyrosine		0.100
L-Valine	0.214	0.200
INORGANIC SALTS		
Calcium chloride (CaCl ₂) (dihyd.)		1.701
Magnesium sulfate (MgSO ₄ *7H ₂ O)		0.200
Potassium chloride (KCl)	5.330	2.485
Monopotassium phosphate (KH ₂ PO ₄)		0.353
Sodium bicarbonate (NaHCO ₃)	26.190	25.003
Sodium chloride (NaCl)	117.240	95.721
OTHER COMPONENTS		
D-Glucose (Dextrose)	5.560	0.200
EDTA		0.013
Sodium lactate syrup		10.003
Sodium pyruvate		0.200

Table 2-2. Medium components that are only included in M-199.

AMINO ACIDS	MOLARITY (mM)	OTHER COMPONENTS	MOLARITY (mM)
L-Cysteine hydrochloride-H ₂ O	0.000568	2-deoxy-D-ribose	0.00373
L-Cystine 2HCl	0.108	Adenine sulfate	0.0248
L-Hydroxyproline	0.0763	Adenosine 5'-phosphate	0.000576
L-Tyrosine disodium salt dihydrate	0.153	Adenosine 5'-triphosphate	0.00165
VITAMINS		Cholesterol	0.000517
Alpha-tocopherol phosphate	0.0000142	Glutathione (reduced)	0.000163
Ascorbic Acid	0.000284	Guanine hydrochloride	0.0016
Biotin	0.000041	Hypoxanthine Na	0.00294
Choline chloride	0.00357	Phenol red	0.0531
D-Calcium pantothenate	0.000021	Ribose	0.00333
Folic Acid	0.0000227	Sodium acetate	0.61
i-Inositol	0.000278	Thymine	0.00238
Menadione (Vitamin K ₃)	0.0000581	Tween 80®	20 mg/ml
Niacinamide	0.000205	Uracil	0.00268
Nicotinic acid (Niacin)	0.000203	Xanthine-Na	0.00224
Para-Aminobenzoic Acid	0.000365		
Pyridoxal hydrochloride	0.000123		
Pyridoxine hydrochloride	0.000121		
Riboflavin	0.0000266		
Thiamine hydrochloride	0.0000297		
Vitamin A (acetate)	0.000305		
Vitamin D ₂ (Calciferol)	0.000252		
INORGANIC SALTS			
Calcium chloride (CaCl ₂) (anhyd.)	1.8		
Ferric nitrate (Fe(NO ₃)-9H ₂ O)	0.00173		
Magnesium sulfate (MgSO ₄) (anhyd.)	0.397		
Sodium phosphate monobasic (NaH ₂ PO ₄ -H ₂ O)	1.01		

CHAPTER 4 RESULTS

Experiment 1: Effect of Medium Type and Oxygen Atmosphere on Blastocyst Survival, Development and IFN τ Production

Bovine blastocysts were cultured individually in two medium types and under different oxygen tensions to assess if embryo development could be extended to day 11 post-IVF in culture. On day 11 post-IVF, a portion of blastocysts were considered degenerated. These embryos possessed a partial to complete collapse or deterioration of cell membranes, more specifically the trophectoderm, and/or rupturing of the zona pellucida. Those embryos which appeared extremely unhealthy were small in size and did not seem to show any signs of having an expanded trophectoderm. Embryos which appeared healthy upon visual observation were large, rotund, and expanded, some extensively so, and most were hatched from their zona pellucida by day 11 post-IVF ($67.5 \pm 2.8\%$ overall mean and SEM). For a comparison of typical degenerated and healthy embryos under nuclear fluorescent staining (Hoescht 33342), refer to Fig. 4-1 and 4-2.

No main effects of medium type or oxygen concentration on rate of degeneration were detected but a medium type by oxygen interaction was evident ($P=0.05$), where incubation in M-199 in 5% oxygen prevented degeneration whereas from 23 to 26.5% of embryos incubated in other medium and oxygen atmospheric conditions were degenerated at day 11 post-IVF (Fig. 4-1, 4-3). Accurate cell counts could not be retrieved from these embryos and conditioned medium from these embryos did not contain measurable antiviral activity. Therefore, only data collected from non-degenerated embryos were used for subsequent analyses.

Total embryonic cell numbers on day 11 post-IVF were affected by the main effects of medium ($P=0.04$) and oxygen concentration ($P=0.03$) with M-199 and 5% oxygen treatments providing greater day 11 cell numbers (data not shown). A medium by oxygen interaction was

detected ($P=0.006$), and embryos cultured in TCM-199 in 5% oxygen contained greater ($P<0.01$) cell numbers at day 11 post-IVF than other culture treatments (Fig. 4-2, 4-4). No medium type or oxygen concentration effects, including interactions, were noted on the percentage of apoptotic nuclei on day 11 post-IVF (Fig. 4-5). In each group, percent TUNEL positive nuclei ranged from 1.5-3%.

Medium type did not affect the quantity of bioactive IFN τ , as assessed with an antiviral assay, in conditioned medium from blastocysts cultured individually from day 8 to 11 post-IVF (Fig 4-6). However, embryos incubated in 5% oxygen secreted more ($P=0.002$) IFN τ into medium than embryos incubated in 20% oxygen (Fig. 4-6). This effect is evident in both medium types both before and after normalizing data according to embryonic cell numbers on day 11 post-IVF (Fig. 4-7).

Experiment 2: Serum Requirements for Extended Bovine Blastocyst Culture

In the previous study embryos were maintained in medium containing 5% [v/v] FBS based on the laboratory's previous observations for group-cultured bovine blastocysts (Michael, 2006). The objective of this study was to define the minimum serum requirements for blastocyst survival *in vitro* to day 11 post-IVF. Treatments included M-199 containing previously described additives and 1% BSA (Negative control), Insulin-transferrin-selenium (ITS) containing 1% BSA, or 1%, 2.5% and 5 % FBS. Embryos were incubated in either 5% or 20% oxygen. During the experiment it became evident that embryos cultured with 1% BSA and ITS + 1% BSA had a high incidence of degeneration (approximately 75%) regardless of oxygen exposure and these treatments were not replicated. Rather, the remainder of the experiment was completed only with 1%, 2.5% and 5% FBS treatment groups. The incidence of degeneration was greater ($P=0.04$) for embryos cultured in 20% oxygen than those cultured in 5% oxygen (Data not shown). Moreover,

serum concentration affected degeneration rate ($P=0.04$). This effect was most evident for embryos cultured in 1% FBS versus those cultured in 2.5 and 5% FBS in 5% oxygen (Fig. 4-8).

Number of cells on day 11 post-IVF also was impacted by oxygen atmosphere ($P=0.001$) and serum supplementation to a lesser degree ($P=0.09$). As shown in Figure 4-9, cell numbers at day 11 post-IVF were increased ($P<0.05$) as serum concentrations in medium increased in embryos incubated in 5% oxygen. However, this effect was not observed in embryos incubated in 20% oxygen. There were no differences among serum and oxygen treatments in the proportion of apoptotic nuclei in blastocysts on day 11 post-IVF (Fig. 4-10).

Serum concentration and oxygen concentration influenced the quantity of bioactive IFN τ produced by the individual embryos (Fig. 4-11). Embryos cultured in 1% FBS produced less ($P<0.05$) IFN τ per cell at day 11 post-IVF than embryos either 2.5% or 5% FBS treatments, regardless of oxygen level. Moreover, IFN τ levels were below the detection limit for the assay (0.1 ng/ml) in a majority of the medium samples derived from embryos cultured in medium containing 1 % FBS. A clear increase ($P=0.02$) in IFN τ concentrations was detected in embryos cultured in 20% oxygen than those cultured in 5% oxygen. The effects of serum concentrations in medium and oxygen atmosphere remained evident when individual treatment comparisons were made (Fig. 4-12).

Experiment 3: FGF-2-Mediated Induction of IFN τ Production in Bovine Blastocysts Depends on the Level of Atmospheric Oxygen

Based on the work completed in experiments 1 and 2, incubating embryos individually in drops of M-199 containing several additives, including 2.5% FBS, permits continued blastocyst development and measurable IFN τ production from day 8 to 11 post-IVF. This extended culture system was used herein to determine if the level of atmospheric oxygen affects the ability of FGF-2 to influence IFN τ production. Bovine blastocysts were cultured individually from day 8

to day 11 post-IVF with or without 100 ng/mL FGF-2 in either 5% or 20% oxygen. No clearly degenerate embryos were observed in this study. Also, TUNEL analysis was not completed since previous studies did not detect any significant changes in percentage of apoptotic nuclei based on oxygen atmosphere (see experiments 1 and 2).

As observed in previous work, embryos cultured in M-199 with 2.5% FBS in a 5% oxygen atmosphere contained greater cell numbers ($P < 0.001$) on day 11 post-IVF than embryos cultured in 20% oxygen (Fig. 4-13). Supplementation with 100 ng/mL FGF-2 did not affect cell numbers for embryos regardless of the oxygen environment during culture. IFN τ concentration in conditioned medium at day 11 post-IVF was not affected by FGF-2 supplementation ($P = 0.1$) and clearly increased by incubating embryos in 20% oxygen ($P = 0.01$) (Fig. 4-14). When the effects of FGF-2 supplementation and oxygen levels were partitioned further, it became evident that FGF-2 supplementation was able to increase ($P = 0.06$) IFN τ concentrations in conditioned medium when embryos were incubated in a 5% oxygen atmosphere but not when they were incubated in 20% oxygen (Fig. 4-15).

Experiment 4: The Combined Effects of FGF-2 and GM-CSF on Blastocyst Development and IFN- τ Production

Several reports implicate GM-CSF as a mediator of IFN τ production in bovine embryos and trophectoderm cell lines [314, 323, 328], although this observation could not be observed in some studies [329]. A final study was completed to determine if GM-CSF affects blastocyst development and/or IFN τ secretion in this newly developed embryo culture system and determine if GM-CSF and FGF-2 act cooperatively to mediate embryo development and/or IFN τ secretion. All embryos were cultured individually in 30 μ l drops of M-199 containing 2.5% FBS under a 5% oxygen atmosphere. Either boFGF-2 (100 ng/mL), poGM-CSF (100 ng/mL), both FGF-2 and GM-CSF (100 ng/mL of each), or no growth factor treatment (Control) was added to

medium immediately prior to incubation at day 8 post-IVF. On day 11, embryos receiving GM-CSF treatment had fewer ($P=0.03$) cells per embryo regardless of whether FGF-2 was co-administered (data not shown). FGF-2 supplementation did not influence resulting cell numbers when compared with controls, but cell numbers were greater ($P<0.04$) in FGF-2-supplemented embryos on day 11 post-IVF than embryos supplemented with poGM-CSF (Fig. 4-16). The incidence of apoptosis was not measured in this study.

There were no main effects of FGF-2 or GM-CSF supplementation on IFN τ concentrations in conditioned medium at day 11 post-IVF when data were normalized to account for differences in day 11 cell counts. However, individual treatment comparisons determined that IFN τ concentrations in conditioned medium were greater ($P=0.02$) in boFGF-2-supplemented cultures versus non-treated controls (Fig. 4-17). Interestingly, poGM-CSF supplementation did not significantly affect IFN τ concentrations when compared to control values, but the mean concentrations of IFN τ in medium were increased slightly and were not significantly different from boFGF-2-supplemented samples. Co-supplementation with boFGF-2 and poGM-CSF did not increase IFN τ concentrations in conditioned medium when compared with controls.

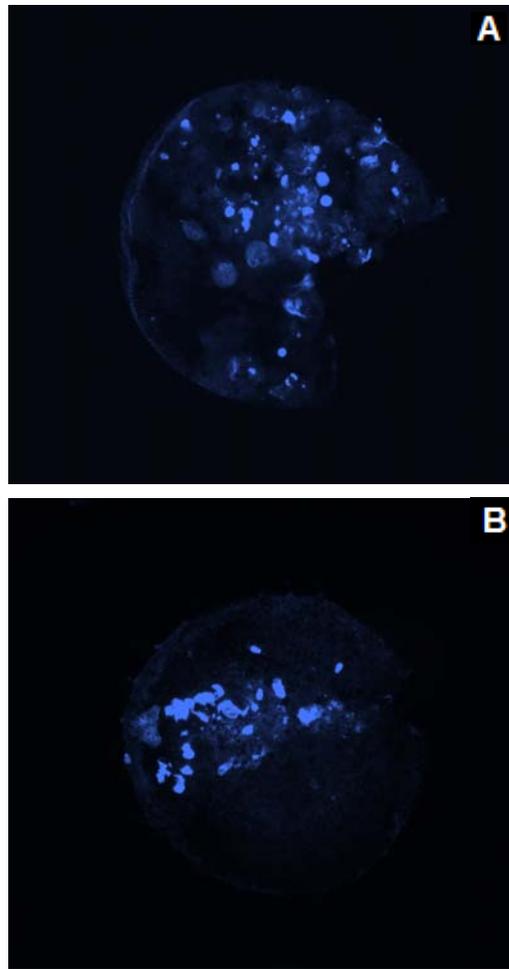


Figure 4-1. Examples of degenerated embryos by day 11 post-IVF. Degeneration on day 11 of development was determined by visual assessment of embryo integrity (see methods section for detail). Panel A and B depict degenerated embryos stained with Hoescht 33342 nuclear stain. Overall visual appearance is poor with inner cellular contents collapsed or absent, with presence of degenerated cellular debris and in some cases the zona pellucida has ruptured.

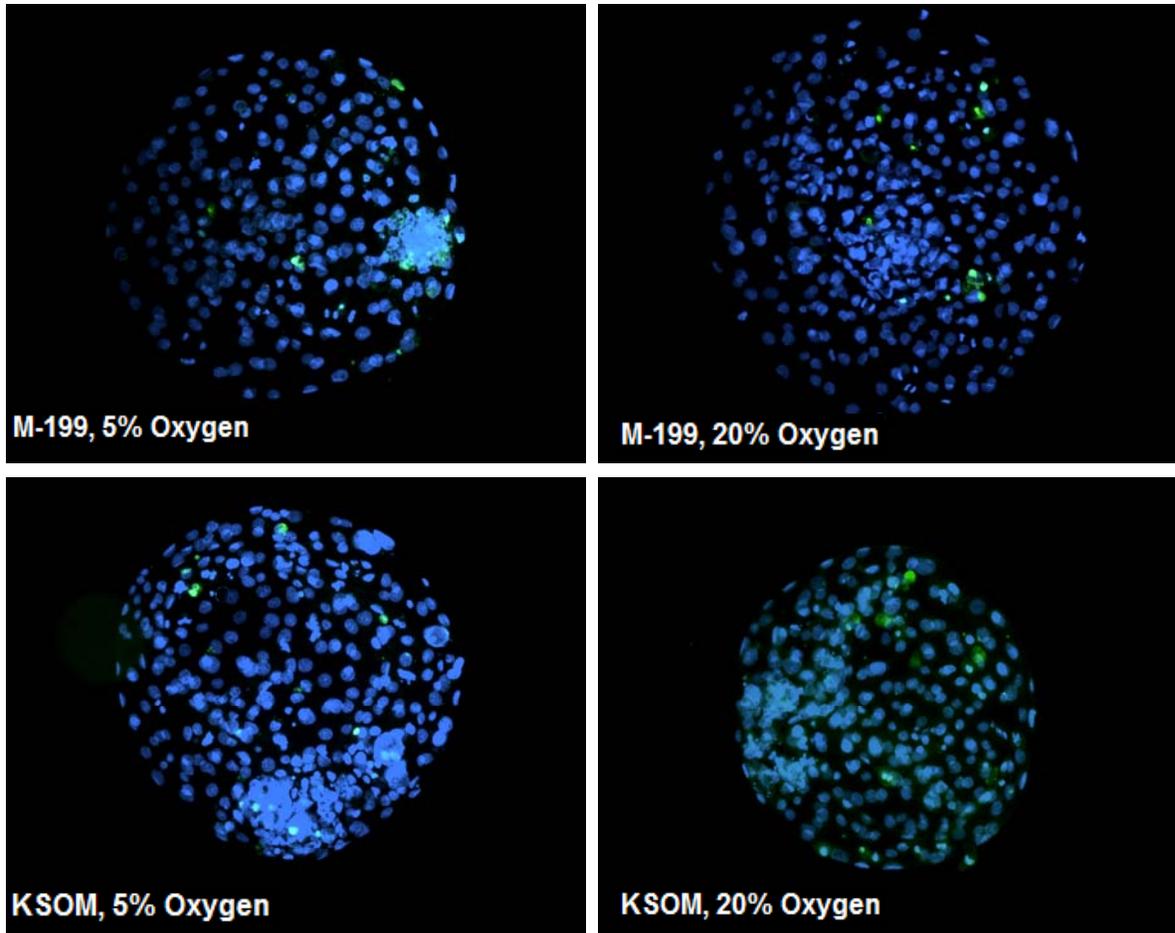


Figure 4-2. Examples of Hoescht 33342 and TUNEL staining for total cell counts and incidence of apoptosis. The total number of embryonic cells and the percentage of embryonic cells that were TUNEL positive at day 11 post-IVF was determined for embryos cultured individually in KSOM or M-199 and 5% or 20% oxygen were recorded using epifluorescent microscopy.

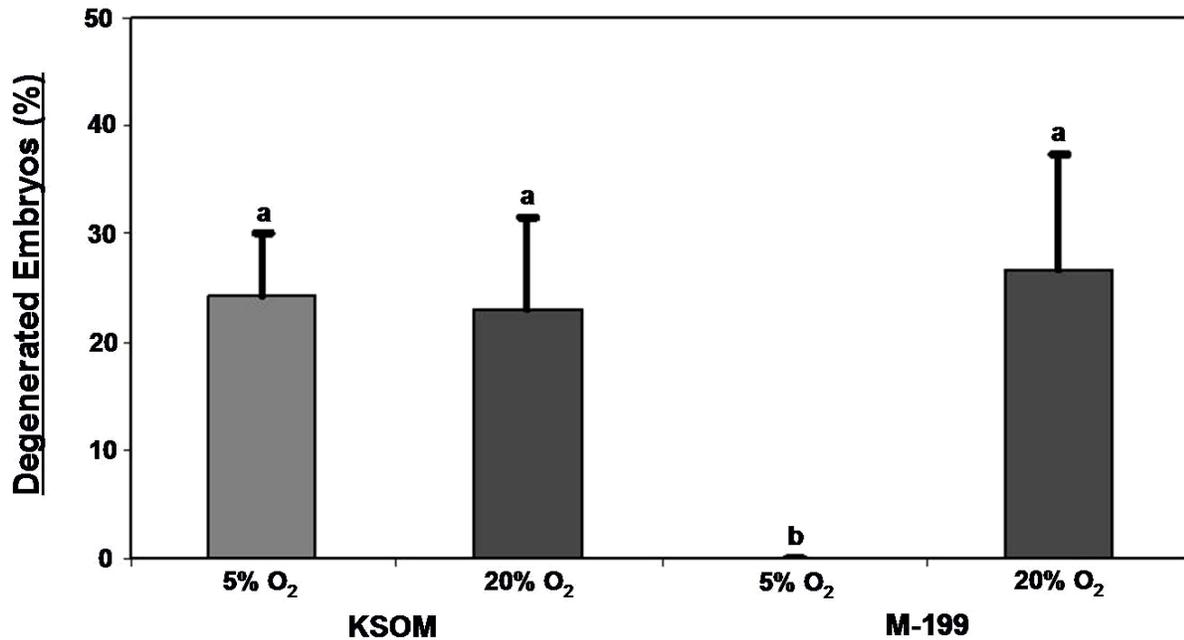


Figure 4-3. Incubating blastocysts from day 8 to 11 post-IVF in M-199 and 5% O₂ prevents degeneration. The incidence of embryo degeneration, as characterized by partial to complete collapse or deterioration of cell membranes, more specifically the trophoctoderm, and/or rupturing of the zona pellucida, was determined for embryos cultured from day 8 to 11 post-IVF in KSOM or M-199 and 5% or 20% oxygen. Degeneration on day 11 was determined by visual assessment of embryo integrity (see methods section for detail). Four independent replicate studies were completed with a total n=20 to 46 blastocysts/treatment. Different superscripts above bars represent differences (P<0.05). Error bars indicate mean standard error.

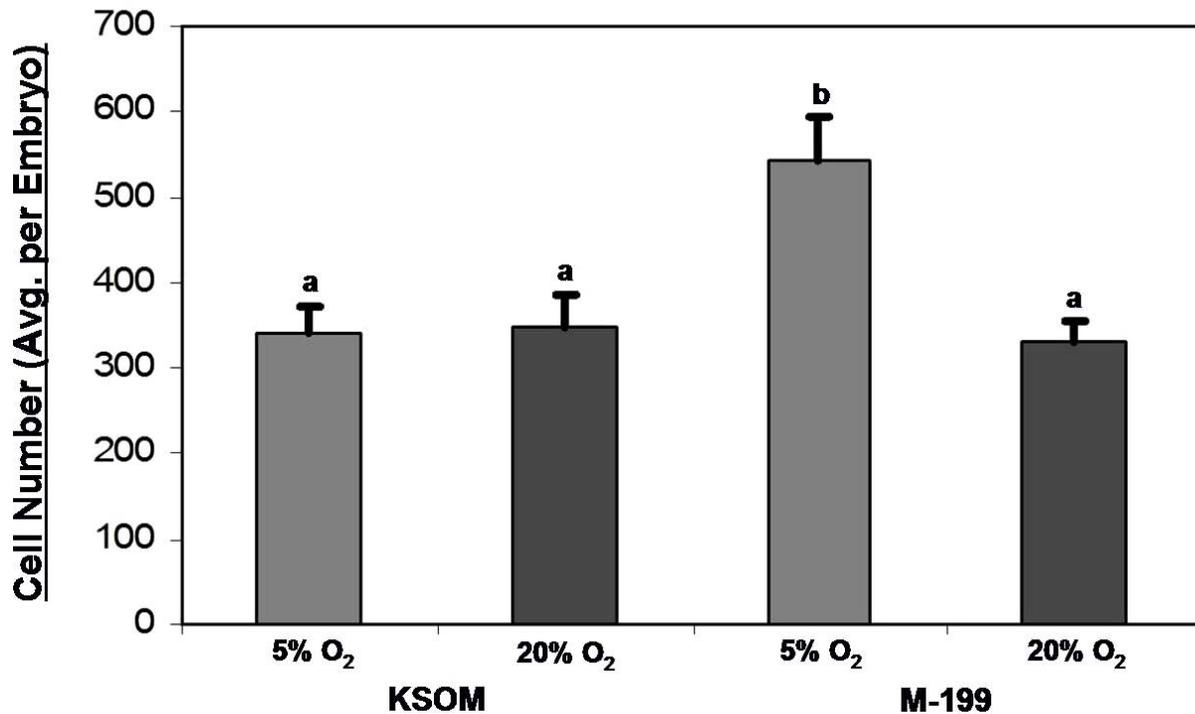


Figure 4-4. Incubation in M-199 under 5% oxygen conditions yields greater total cell numbers on day 11 post-IVF. Embryos were cultured individually in drops of KSOM or M-199 and 5% or 20% oxygen from day 8 to 11 post-IVF. Number of embryonic cells was determined at day 11 post-IVF was determined by staining nuclear DNA (Hoescht 33342) and counting nuclei using epifluorescent microscopy. Four independent replicate studies were completed with a total n=20 to 37 blastocysts/treatment. Different superscripts above bars represent differences (P<0.05). Error bars indicate mean standard error.

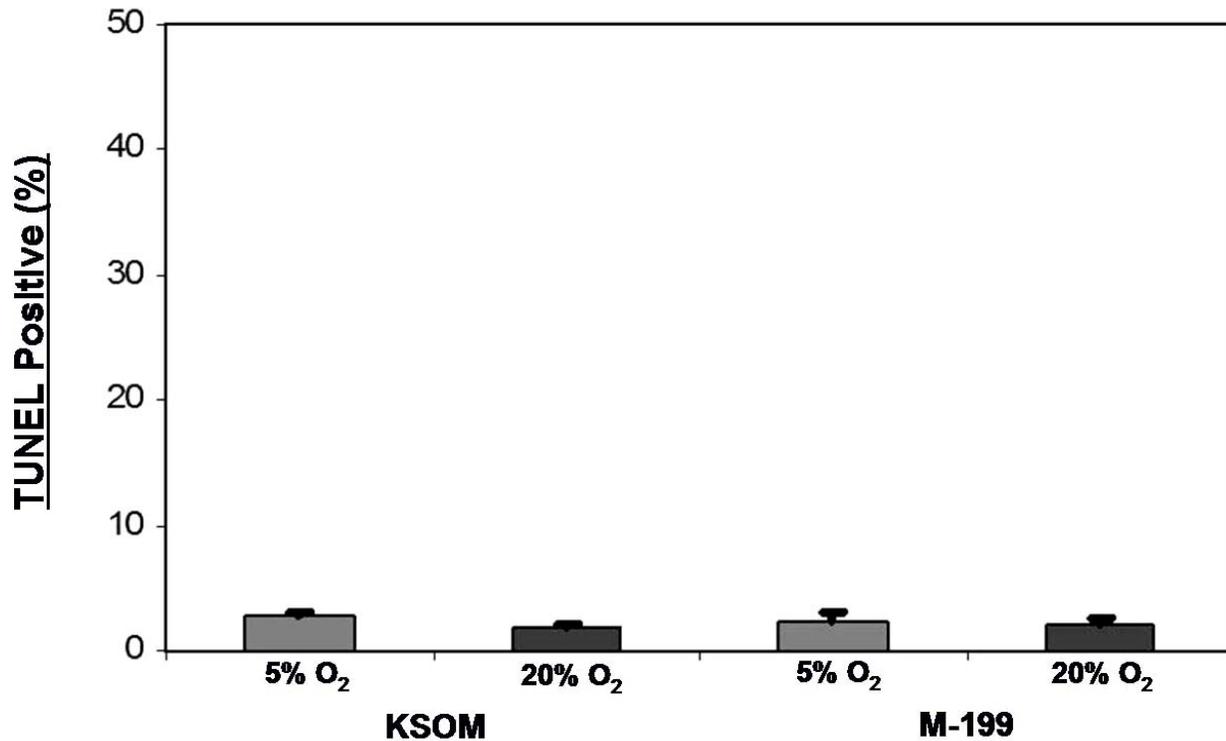
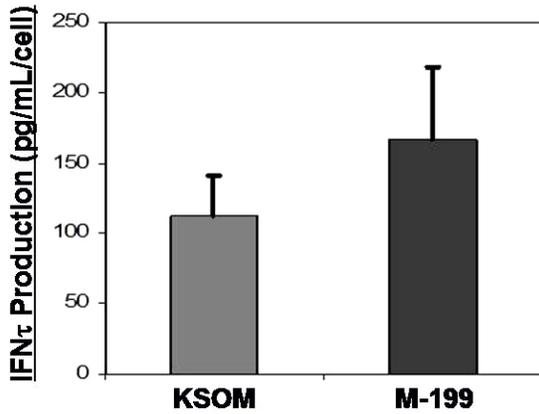


Figure 4-5. Incidence of apoptosis is not affected by medium type or oxygen tension in bovine embryos from day 8 to 11 post-IVF. The percentage of embryonic cells that were TUNEL positive at day 11 post-IVF was determined for embryos cultured individually in KSOM or M-199 and 5% or 20% oxygen were recorded using epifluorescent microscopy. Four independent replicate studies were completed with a total n=20 to 37 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

A.



B.

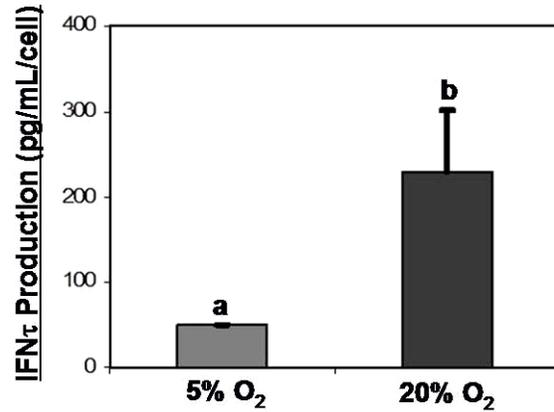


Figure 4-6. Incubation in atmospheric oxygen increases IFN τ secretion from bovine blastocysts.

The quantity of bioactive IFN τ present in conditioned medium on day 11 post-IVF was determined for embryos cultured from day 8 to 11 post-IVF in KSOM or M-199 and 5% or 20% oxygen. Results depict the main effects of medium type (A.) and oxygen concentration (B.) on IFN τ concentrations in conditioned medium. Four independent replicate studies were completed with a total n=20 to 37 blastocysts/treatment. Different superscripts above bars represent differences (P=0.02). Error bars indicate mean standard error.

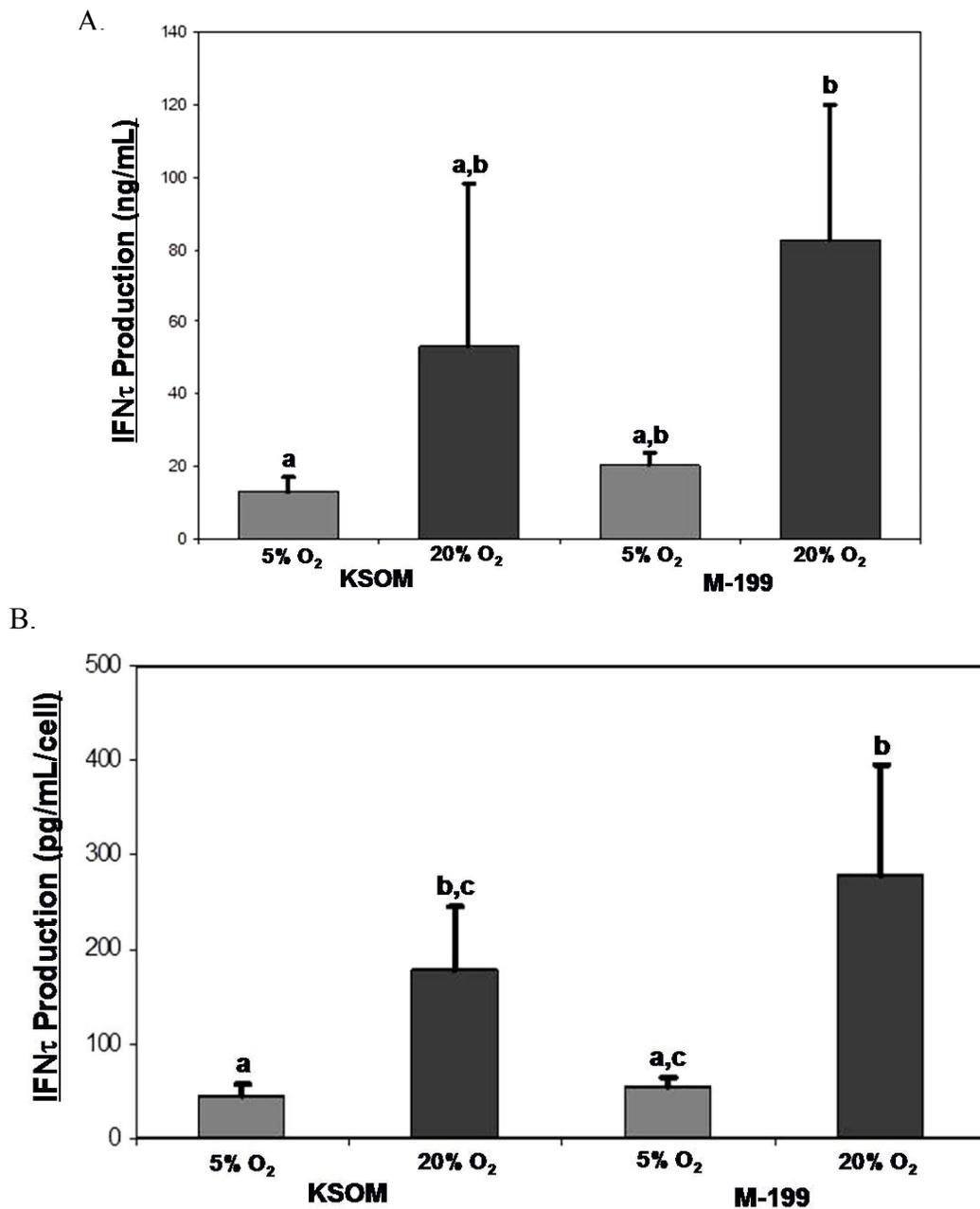


Figure 4-7. Interaction of medium type and oxygen concentration on IFN τ secretion on day 11 post-IVF. Concentrations of IFN τ in conditioned medium are depicted for embryos cultured in KSOM or M-199 and 5% or 20% oxygen from day 8 to 11 post-IVF. Data presented include IFN τ concentrations in medium prior to (A.) or after (B.) data were normalized to account for variation in embryo cell number. Four independent replicate studies were completed with a total n=20 to 37 blastocysts/treatment. Different superscripts above bars represent differences (P<0.05). Error bars indicate mean standard error.

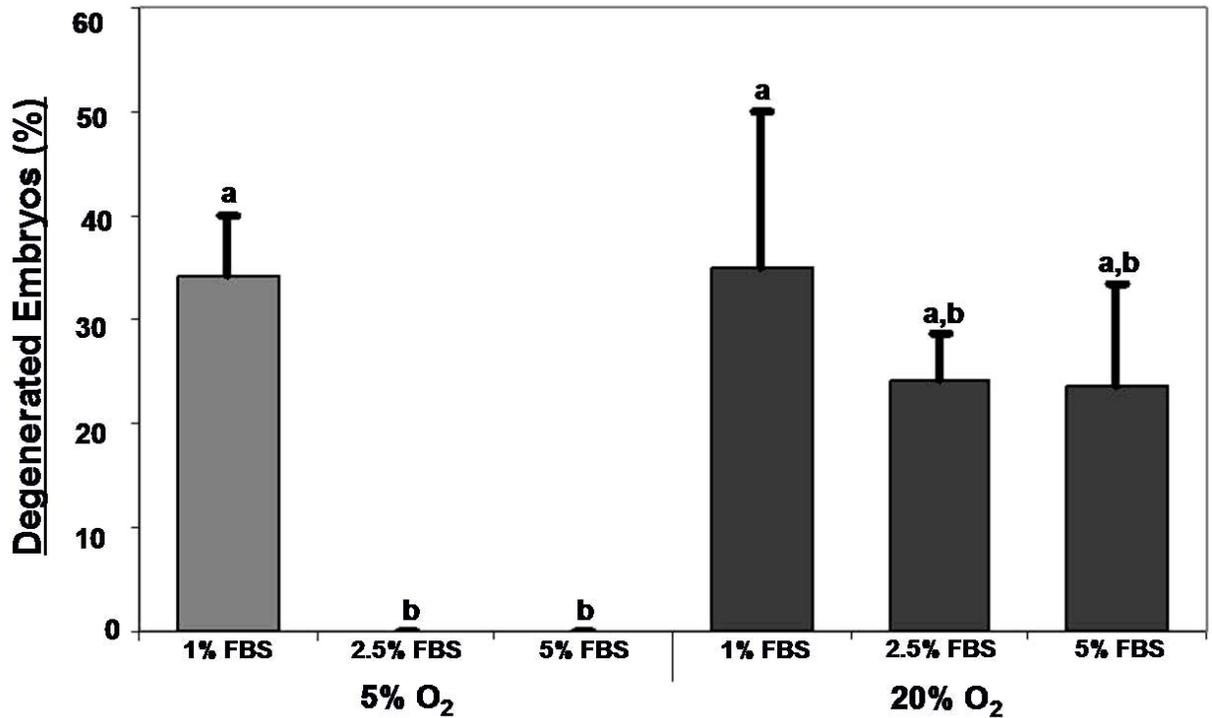


Figure 4-8. Incubating embryos in M-199 containing 2.5 or 5% FBS with 5% oxygen limits degeneration from day 8 to 11 post-IVF. The incidence of embryo degeneration was determined for embryos cultured individually from day 8 to 11 post-IVF in M-199 containing 1%, 2.5% or 5% FBS. Degeneration on day 11 was determined by visual assessment of embryo integrity (see methods section for detail). Two independent replicate studies were completed with 6 to 12 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

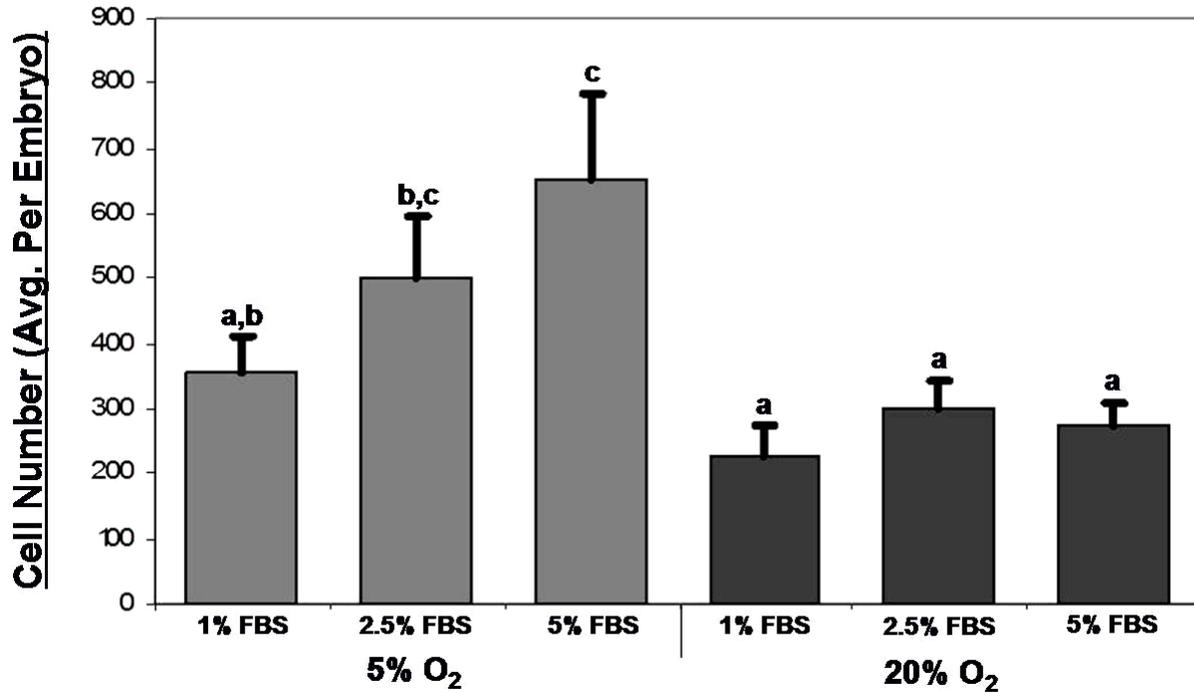


Figure 4-9. Supplementation with FBS improves embryonic cell number after incubation in 5% oxygen. Embryos were cultured in M-199 supplemented with 1%, 2.5% or 5% FBS and incubated in 5% or 20% oxygen from day 8 to 11 post-IVF. Number of cells at day 11 post-IVF was counted by epifluorescent microscopy after staining nuclear DNA (Hoescht 33342 staining). Two independent replicate studies were completed with a total of 6 to 12 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

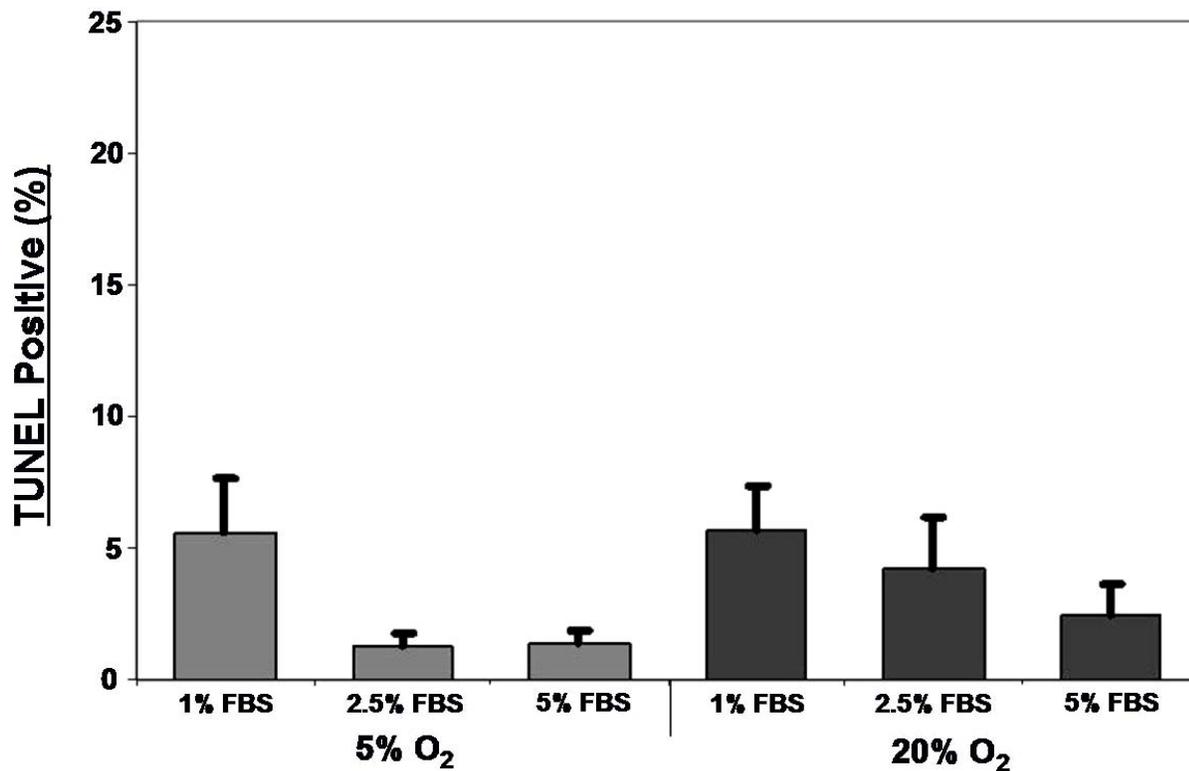


Figure 4-10. Effect of serum supplementation on percentage of TUNEL positive cells during individual embryo culture from day 8 to 11 post-IVF. Embryos were cultured individually from day 8 to 11 post-IVF in M-199 containing 1%, 2.5% or 5% FBS. The percentage of TUNEL positive cells in each embryo was determined using a fluorescent stain for DNA nicking followed by nuclear DNA counter-staining. TUNEL positive and total nuclei were counted by epifluorescent microscopy. The number of TUNEL positive cells was compared to the total cell numbers. Two independent replicate studies were completed with a total of 6 to 12 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

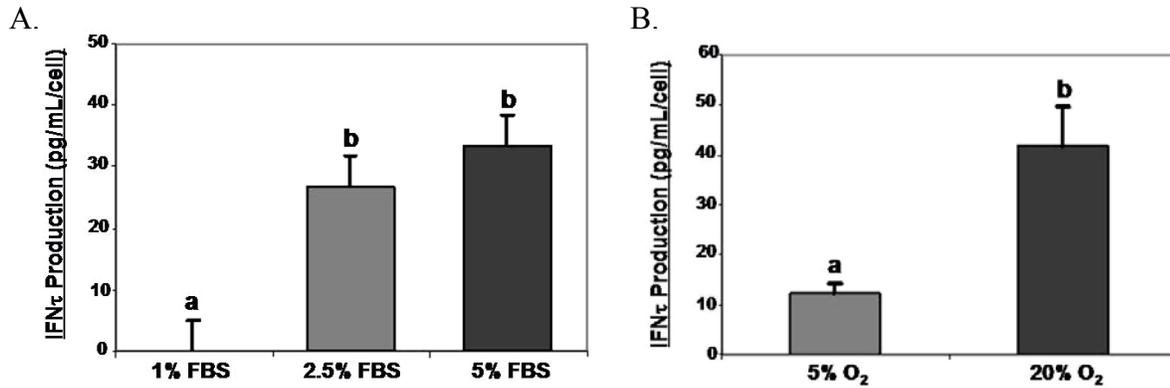


Figure 4-11. Degree of serum supplementation and oxygen concentrations during culture impact IFN τ secretion. Blastocysts were cultured individually in M-199 supplemented with 1%, 2.5% or 5% FBS in either 5% or 20% oxygen from day 8 to 11 post-IVF. Concentration of bioactive IFN τ in conditioned medium at day 11 post-IVF was normalized by cell number. Data represent the main effects of level of serum supplementation (A.) and atmospheric oxygen concentration (B.) on IFN τ secretion. Two independent replicate studies were completed with a total of 6 to 12 blastocysts/treatment. Different superscripts above bars represent differences (P<0.05). Error bars indicate mean standard error.

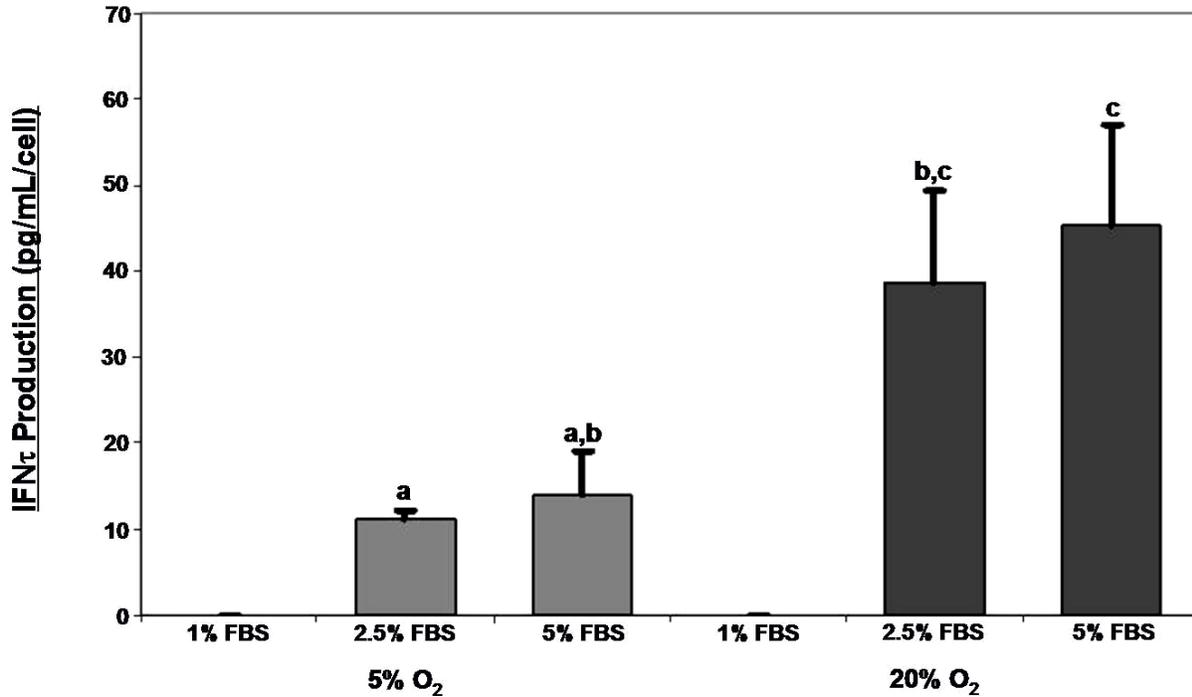


Figure 4-12. Serum supplementation and atmospheric oxygen conditions act independently to mediate IFN τ production. Blastocysts were cultured individually in M-199 supplemented with 1%, 2.5% or 5% FBS in either 5% or 20% oxygen from day 8 to 11 post-IVF. Concentration of bioactive IFN τ in conditioned medium at day 11 post-IVF was normalized by cell number. Two independent replicate studies were completed with a total of 6 to 12 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

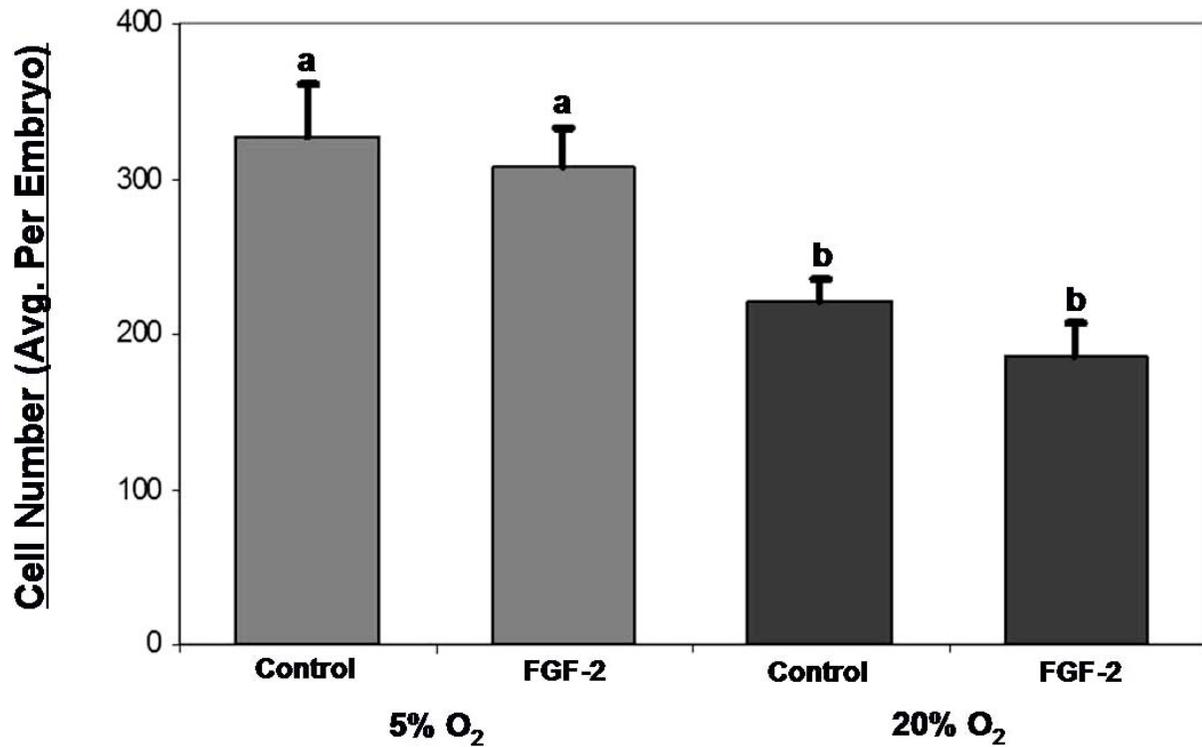


Figure 4-13. Effect of FGF-2 supplementation on cell numbers in embryos on day 11 post-IVF. Blastocysts were cultured individually from day 8 to 11 post-IVF in M-199 supplemented with 0 or 100 ng/mL boFGF-2 under 5% or 20% atmospheric oxygen conditions. Cell number was determined at day 11 post-IVF by staining nuclear DNA (Hoescht 33342) and counting nuclei using epifluorescent microscopy. Three independent replicate studies were completed with a total of 25-30 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

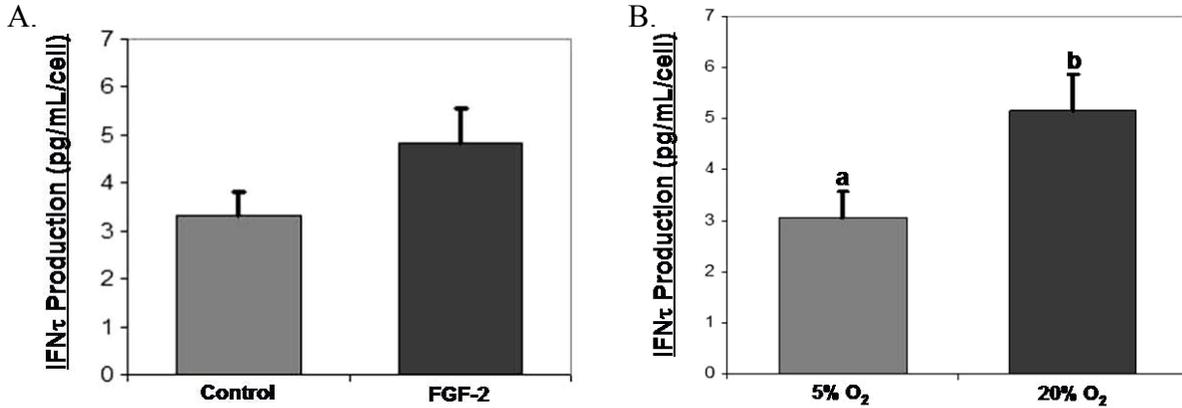


Figure 4-14. Main effects of FGF-2 supplementation and oxygen concentrations on IFN τ secretion from bovine blastocysts. Blastocysts were cultured individually from day 8 to 11 post-IVF in M-199 supplemented with 0 or 100 ng/mL boFGF-2 under 5% or 20% atmospheric oxygen conditions. The quantity of bioactive IFN τ in conditioned medium on day 11 post-IVF was determined and normalized for embryo cell number. Data represent the main effect of FGF-2 supplementation (A.) and oxygen condition (B.) on IFN τ concentrations in conditioned medium. Three independent replicate studies were completed with a total of 25-30 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

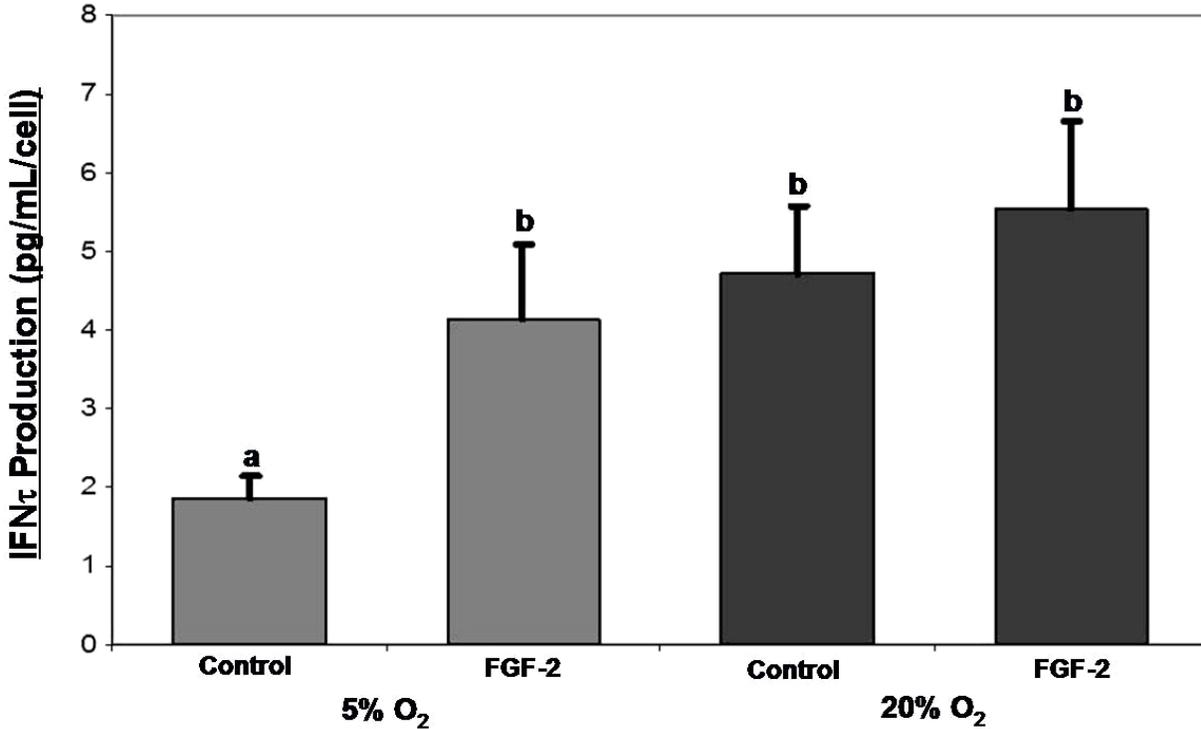


Figure 4-15. Treatment with FGF-2 and 20% oxygen increases IFN τ production from bovine blastocysts. Embryos were cultured individually from day 8 to 11 post-IVF in M-199 supplemented with 0 or 100 ng/mL boFGF-2 under 5% or 20% atmospheric oxygen conditions. The quantity of bioactive IFN τ in conditioned medium on day 11 post-IVF was determined and normalized for embryo cell number. Three independent replicate studies were completed with a total of 25-30 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.06$). Error bars indicate mean standard error.

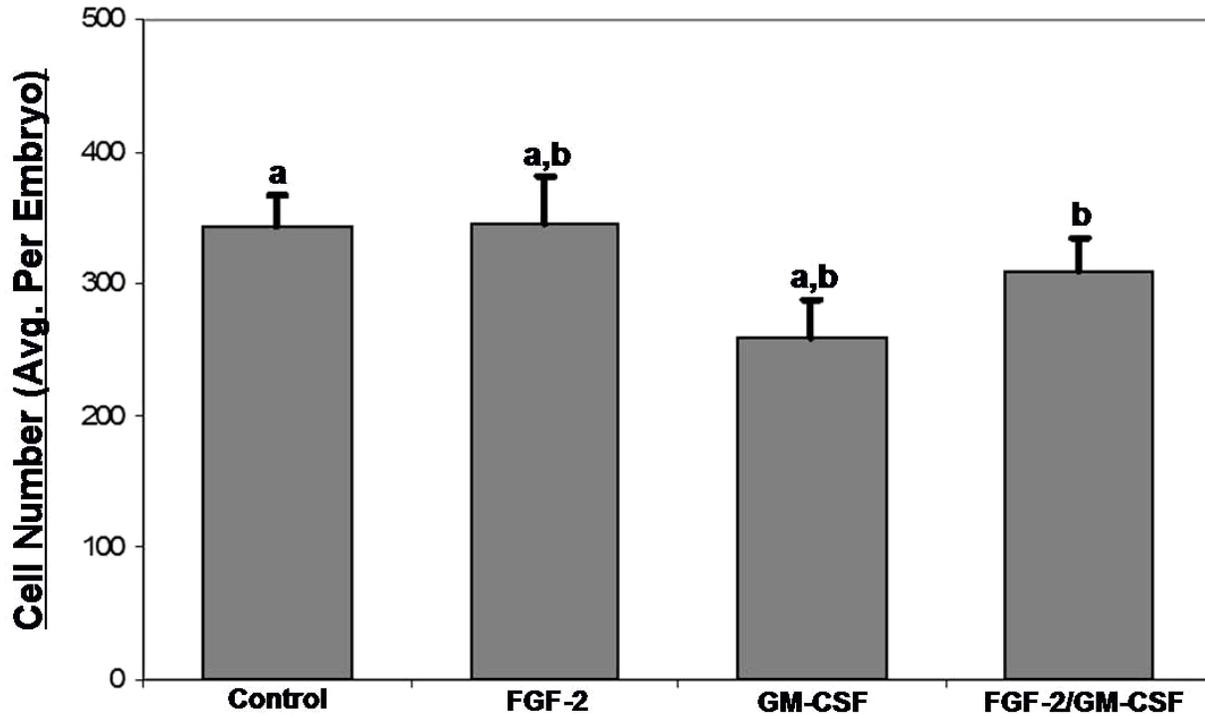


Figure 4-16. Effect of FGF-2 and GM-CSF supplementation on embryo cell number. Blastocysts were cultured individually in drops of M-199 containing 2.5% FBS and 100 ng/ml boFGF-2, 100 ng/ml poGM-CSF, both FGF-2 and GM-CSF (100 ng/ml of each), or no growth factor treatment (control) from day 8 to 11 post-IVF. Cell number was determined at day 11 post-IVF by staining nuclear DNA (Hoescht 33342) and counting nuclei using epifluorescent microscopy. Three independent replicate studies were completed with a total of 24-28 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

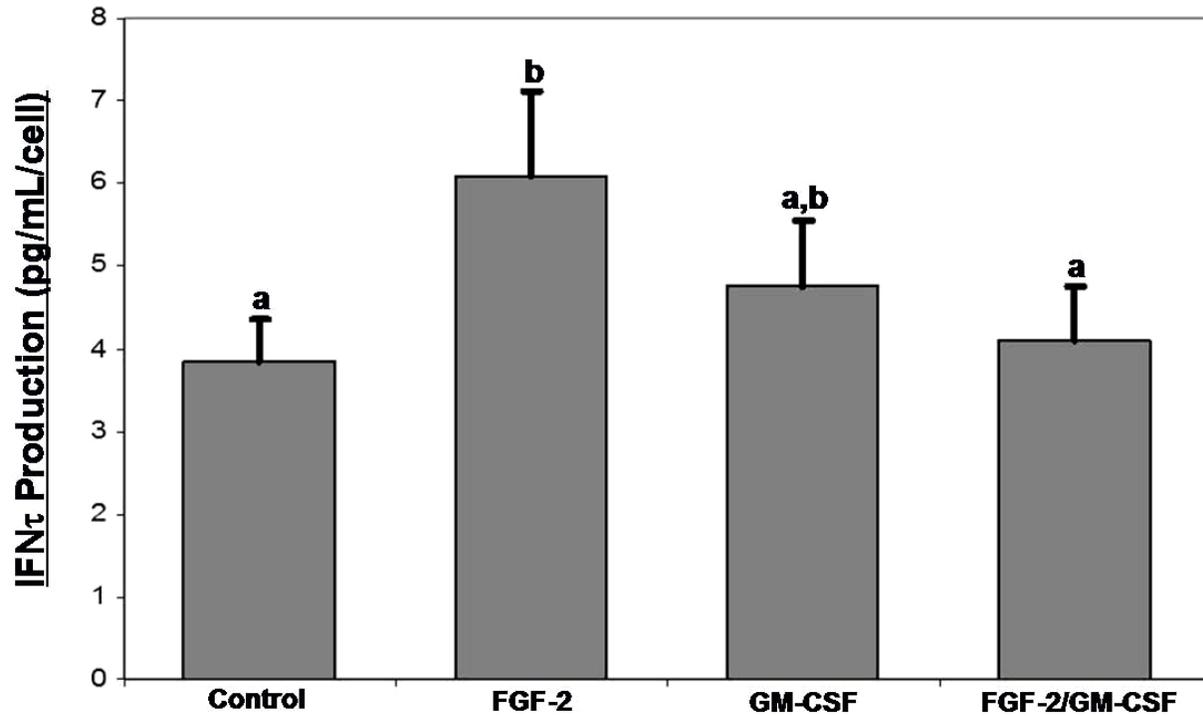


Figure 4-17. Supplementation with FGF-2 but not GM-CSF increases IFN τ production from bovine embryos. Blastocysts were cultured individually in drops of M-199 containing 2.5% FBS and 100 ng/ml boFGF-2, 100 ng/ml poGM-CSF, both FGF-2 and GM-CSF (100 ng/ml of each), or no growth factor treatment (control) from day 8 to 11 post-IVF. Quantity of bioactive IFN τ in conditioned medium at day 11 post-IVF was determined and normalized for embryo cell number. Three independent replicate studies were completed with a total of 24-28 blastocysts/treatment. Different superscripts above bars represent differences ($P < 0.05$). Error bars indicate mean standard error.

CHAPTER 5 DISCUSSION

Reproductive failure is a considerable issue in the beef and dairy industries, with early embryonic mortality being responsible for a substantial proportion of reproductive insufficiencies. A more articulate understanding of the series of events of embryonic development may lead to possible resolutions to these issues. The most valuable method of studying embryonic development is through *in vitro* production and culture of embryos. Conventional bovine embryo culture systems are not designed to adequately support blastocyst development. This is because the full extent of components provided by the natural uterine environment has yet to be defined, which proves to be problematic in developing embryo culture systems, particularly during post-hatching development. The work presented here establishes that blastocysts can be sustained in culture until at least day 11 post-IVF with the proper medium formulation, by supplementing medium with serum, and by exposing embryos to a low oxygen environment.

The medium formulation used in these experiments had a major impact on blastocyst viability and development between days 8 and 11 post-IVF. A majority of the components in the modified KSOM (with non-essential amino acids) and M-199 were fairly well balanced except for their glucose levels (5.5 mM for M-199; 0.2 mM for KSOM). Glucose can be toxic to bovine embryos, and especially female embryos, during initial cleavage stages [336-338]. As embryos become more advanced, rate of glycolysis and glucose uptake increases [336, 337], and medium containing glucose is required for propagating bovine embryos during and beyond the blastocyst stage [6, 7, 10, 12, 13]. However, since glucose withdrawal and/or replacement studies were not completed during the course of this work, it remains possible that minor differences between media formulations also contributed to the superiority of M-199 as a blastocyst culture medium.

Serum supplementation, previously shown to be a required supplement in culture medium [210, 211], limited the degeneration of bovine blastocysts between days 8 and 11 post-IVF in these experiments. Attempts to supply M-199 with defined agents known to substitute for serum in several somatic cell lines (*i.e.* insulin, transferrin, selenium, BSA) failed to provide adequate conditions for blastocyst development and survival. Others have noted the same requirement for serum in extended bovine embryo cultures [9, 12, 13]. In one study [9], FBS was superior to mature cow serum as a medium supplement and both serum types were better than media formulations lacking serum at sustaining bovine embryonic outgrowths formed on collagen-coated plates. In the present work, 2.5% FBS limited degeneration and sustained a modest amount of embryonic growth during culture. An individual blastocyst culture system was used throughout our studies to remove the confounding effects of group embryo culture on outcomes and to permit using blastocyst cell numbers for normalizing IFN τ production data. The minimum serum requirement for blastocysts cultured in groups remains unknown, but serum supplementation is routine during group culture [6-8]. Taken together, serum, and preferably FBS supplementation is required during bovine blastocyst culture when using M-199 or a related medium formulation.

Traditional embryo culture systems incubated embryos at an atmospheric oxygen level (approximately 20%) is greater than that found in the uterus and oviducts (approximately 2-9%) [227]. Recent work from several groups investigating bovine embryo development indicate that reducing the oxygen levels during *in vitro* culture to between 5 and 7% improves proportion of embryos developing to the blastocyst stage and in several species [215, 228-231, 339-342]. In this work, a 5% oxygen atmosphere prevented degeneration and sustained cell proliferation between days 8 and 11 post-IVF. It is generally accepted that reducing the oxygen tension during

embryo culture limits the production of reactive oxygen species that cause oxidative damage in embryos [229, 230, 343]. Level of oxidative stress was not measured in these studies, but day 8 to 11 bovine blastocysts were sensitive to *tert*-butylhydroperoxide (tBH), an agent that oxidizes various intracellular molecules, including glutathione, in mammalian embryos [344, 345]. This oxidant produced a dose responsive increase in the proportion of degenerated blastocysts and decrease in cell numbers but did not affect IFN τ levels in conditioned medium [Cooke et al., Unpublished observations]. In related work, addition of hydrogen peroxide to culture medium negatively affected developmental competence of bovine embryos but did not affect IFN τ production [346]. The agent tBH mimicked some but not all of the effects of 20% oxygen on blastocysts, thereby suggesting that oxidative stress probably is at least partial responsible for the inferior development of blastocysts cultured in 20% oxygen.

It was surprising that incubation in 20% oxygen increased IFN τ concentrations in conditioned medium. This oxygen tension compromised the overall fitness of blastocysts, as evidenced by reduced cell numbers and increased the incidence of degeneration, but increased the amount of IFN τ produced. Work by others did not detect differences in IFN τ mRNA or protein content in bovine blastocysts cultured in 5% versus 20% oxygen [215, 346]. In previous studies, exposure to different oxygen environments began on the day following fertilization (1-cell stage) [215] or on day 3 post-IVF (8-cell stage) [346] and IFN τ levels were evaluated either on day 7 post-IVF by measuring IFN τ mRNA abundance [215] or after 24 or 48 h culture of individual day 8 blastocysts by measuring the quantity of bioactive IFN τ in conditioned medium [346]. Early exposure to high oxygen also compromised the developmental potential of bovine embryos in these studies. One explanation for why outcomes differ between this and previous studies is that the early exposure to a high oxygen environment may provide embryos with an

opportunity to adapt to high oxygen conditions prior to the blastocyst stage, and embryos that reached the blastocyst stage represented the subset of embryos that overcame the limitations of this environment. In the present study, embryos were not given an opportunity to adapt to high oxygen before the blastocyst stage. Alternatively, various genetic and environmental factors, including gender, paternal genotype, medium composition, and time of blastocyst formation, influence IFN τ production in blastocysts [6, 7, 338, 347], and any one of these factors or variables that remain undefined could have contributed to this novel response to the high oxygen environment.

The 20% oxygen environment also precluded blastocysts from responding to FGF-2 supplementation. FGF-2 stimulates IFN τ mRNA and protein production in bovine trophoctoderm and IVF-derived blastocysts, and its presence in the uterine lumen throughout the estrous cycle and early pregnancy implicate it as an uterine-derived mediator of IFN τ production during early pregnancy in cows and ewes [8, 312]. Detecting IFN τ responses to FGF-2 treatment in blastocysts cultured individually in 5% oxygen supports previous findings, where groups of embryos (n=8-11 blastocysts/50 μ l drop of medium) produced more IFN τ in response to FGF-2 supplementation than non-supplemented controls [8]. FGF-2 did not contain detectable mitogenic activity in bovine blastocysts in a previous study [8] and likewise this activity could not be detected in present work when embryos were cultured individually. The biochemical processes that induce basal IFN τ levels but prevent FGF-2-induced IFN τ production in blastocysts exposed to 20% oxygen remain unclear, but the induction of oxidative stress is not directly involved with this event.

The failure of GM-CSF to increase IFN τ production and cell numbers from *in vitro*-derived bovine embryos is in direct disagreement with previous reports indicating that GM-CSF

supplementation increases IFN τ mRNA and/or protein production in bovine and ovine trophectoderm [314, 326-328] and stimulates embryo development [329]. Reasons for these unexpected outcomes remain uncertain. The activity of the poGM-CSF may have been compromised within the particular lot used in the experiment. Additionally, perhaps the dose of GM-CSF tested was inappropriate. In an earlier study, 150-300 pM (3.3-6.6 ng/mL) stimulated IFN τ production whereas 600 pM did not [326]. GM-CSF was not tested at different doses in this experiment and the greater concentration utilized (100 ng/mL poGM-CSF) may have prevented a beneficial effect to be detected. Conversely, this dose was effective when used on a bovine trophectoderm cell line (CT-1 cells) [328]. It was interesting to observe that GM-CSF prevented FGF-2 from stimulating IFN τ production. This study is the first to combine FGF2 and GM-CSF treatments, and the presumptive block in FGF-2 effects by GM-CSF suggest that 1) the GM-CSF preparation contained biological activity and 2) perhaps these two uterine-derived factors do not cooperate with one another but rather conflict with each other in mediating conceptus development and IFN τ production. Further study is needed to resolve these issues before definitive roles of GM-CSF on blastocyst development and IFN τ production can be described.

It remains unclear if the effect of high oxygen on IFN τ production has any physiological relevance or if it merely is an artifact of the culture system. Even so, this aberrant production of IFN τ may reflect the inferiority of these embryos for producing viable pregnancies. Bovine blastocysts producing lower amounts of IFN τ prior to transfer generate more pregnancies than blastocysts producing greater amounts of IFN τ before transfer [348]. Also, bovine blastocysts forming early in culture, which are superior at producing pregnancies after transfer than embryos lagging in development [197], produce lower amounts of IFN τ than blastocysts forming two to

three days later [6]. However, although early forming blastocysts produce less IFN τ initially, they produce much more IFN τ after three to four days than blastocysts forming later in culture [6]. Taken together, several genetic and environmental factors influence IFN τ production in bovine embryos, and these variables complicate the potential use of IFN τ as a predictor of pregnancy success in cattle. But, the contention that producing more IFN τ could predict embryos will be less able to produce pregnancies is a provocative concept that may explain why blastocysts cultured in 20% oxygen produce more IFN τ but have a greater incidence of degeneration and fewer cell numbers by day 11 post-IVF.

In summary, studies presented here provide evidence that M-199 containing $\geq 2.5\%$ FBS and a 5% oxygen environment is able to sustain bovine blastocyst viability until at least day 11 post-IVF. This system also permits blastocysts to respond to putative embryotrophic factors, such as FGF-2. It is anticipated that this system will be used by others to resolve some of the key issues relating to conceptus wastage in cattle.

Implications: The extension of this research must continue and further expand upon recent discoveries. This group continues to investigate this research area and limits of the culture system developed. Utilizing slight modifications to this culture system resulted in successfully lengthening its boundaries and obtaining healthy bovine blastocysts until day 14 post-IVF (Cooke et al., Unpublished observations). Extension beyond this point met with little success. Further expansion of this system will include utilizing these methods in conjunction with treatment of other currently known uterine-derived factors and those that have yet to be discovered, as well as developing an agarose tunnel mold, which will induce embryonic elongation in culture, under treatment with FGF-2 and GM-CSF to evaluate for further progress upon previous work.

Future work examining embryotrophic factors FGF-2 and GM-CSF involve the transfer of embryos treated with these growth factors into recipients to evaluate filamentous embryonic development, placentation and pregnancy rates compared to *in vivo* embryos. The analysis of growth factor treatment effects on *in vivo* derived embryos flushed from donors on day 8 post-insemination and comparisons between their growth factor treated *in vitro* counterparts for development and IFN τ secretion will provide further insight on the research presented here. Further interests include the effects of these growth factors at various stages of embryonic development between *in vitro* and *in vivo* derived embryos beginning at the time of *in vitro* fertilization to day 8 post-IVF, day 8-11 post-IVF and then day 11-14 post-IVF. Finally, evaluating the effects of intrauterine infusion/injection of these growth factors post-insemination will bring this research full circle, completely evaluating FGF-2 and GM-CSF effects from a strictly *in vitro* environment to an entirely *in vivo* one.

A more complete understanding of the events associated with embryonic development is a vital component in the complex series of events surrounding the fetus and mother, which results in either the gain or loss of a pregnancy. The work presented in this thesis is but one piece to the puzzle and must continue so that methods for the ultimate resolution of embryonic loss and reproductive failure may be devised.

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BIOGRAPHICAL SKETCH

Teresa Marie Rodina was born and raised in Ridge, New York on Long Island where she grew up with her twin sister Kristin, older brother James and her parents, Barbara and Robert. At an early age she developed a passionate interest in animals and sought to one day find a career in the field. She spent her undergraduate career in Kingston, Rhode Island at the quaint campus of the University of Rhode Island as an animal science and technology major. After graduating Cum Laude in 2005, she accepted a position with Dr. Alan Ealy as a graduate research assistant at the University of Florida. For the next two years she worked under Dr. Ealy pursuing her Master of Science in Animal Sciences. She hopes to one day attend the College of Veterinary Medicine at the University of Florida and conquer the final challenge in her ultimate goal of developing a career as a veterinarian.