Abstract

Gast, Lauren Renae. Reversal of the Effects of TNF-α on *In Vitro* Derived Bovine Embryos by Addition of a Prostaglandin Synthesis Inhibitor, Indomethacin. (Dr. Scott Whisnant)

Tumor Necrosis Factor alpha (TNF- α) is a cytokine released by macrophage cells in response to local infection or disease. Mastitis, a common infectious disease in cattle, has been shown to increase the serum concentration of TNF- α. This immune response pathway leads to an increase in other cytokines and prostaglandins which can compromise oocyte maturation and embryo development. The objective of this study was to investigate the possibility that the addition of TNF-α after fertilization of bovine oocytes *in vitro* will decrease embryo development to the blastocyst stage and that this effect can be reversed by the addition of a prostaglandin synthesis inhibitor, indomethacin. Ovaries were obtained from a local abattoir and immature cumulus oocyte complexes (COC) were aspirated from 2-10mm diameter follicles. For maturation, COCs were placed in M199 including fetal bovine serum (FBS), LH, FSH, estradiol, pyruvate, and gentamycin and incubated at 39^0 C, 100% humidity, and 5% CO_2 for 18-20 hours. After maturation and fertilization, groups of 20-25 presumptive zygotes were randomly placed in either control development medium (M199 plus FBS), development medium containing 25 ng/ml of TNF- α or development medium containing 25 ng/ml of TNF- α plus 1 μg/ml indomethacin. All embryos were cultured at 39^0 C in an atmosphere of 5% CO_2 in air and 100% humidity. Developmental data was collected by observation of blastocyst stages (early blastocyst, mid-blastocyst, late blastocyst, or hatching blastocyst) at day seven of culture (168 hours post fertilization [hpi]) and day nine

of culture (216 hpi). At 168 hpi, bovine embryos exposed to 25 ng/ml TNF-α after fertilization showed a decrease in the percent of embryos that developed to the blastocyst stage (17.15 ± 2.36 % $p<0.005$) compared to the control (30.45 ± 2.36% $p<0.005$). The results collected on day seven of development (168 hpi) were significant ($p<0.005$). At day nine of development (216 hpi) the proportion of embryos developing to the blastocyst stage was not affected by treatment and was not significant, however a definite trend was detected ($p< 0.08$). There was no significant difference between the TNF-α treatment plus indomethacin group (25.75 ± 2.89% $p<0.005$) and the controls (30.45 ± 2.36% $p<0.005$), however these two groups were significantly different from the TNF-α treatment group alone (17.15 ± 2.36% $p<0.005$) on day seven of development. These results indicate that the inhibitory effect of 25 ng/ml TNF-α on the development of bovine embryos to the blastocyst stage operates through the increase of $PGF_{2\alpha}$ and this effect can be reversed by the addition of a prostaglandin synthesis inhibitor, indomethacin which blocks the biosynthetic pathway of $PGF_{2\alpha}$ from arachidonic acid.

Reversal of the Effects of TNF-α on *In Vitro* Derived Bovine Embryos by Addition of a Prostaglandin Synthesis Inhibitor, Indomethacin

by

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Dedication

Graduate school has been a great personal journey for me and has opened up new doors and presented many opportunities. I could not have come so far without love and personal cheerleading from my mom. I would also like to thank Ashley Jackson for his continuous love, support, and optimistic attitude, even when helping me study for exams!

Biography

Before entering North Carolina State University as a graduate student I completed my undergraduate degree of Bachelor of Science in biology at East Carolina University in 2000. There I worked part time at a veterinary clinic while attending classes. Other than the four years spent in Greenville, North Carolina, I have lived in Raleigh, NC since 1989. I attended Millbrook High School from 1992-1996 and was active in sports medicine during those four years. Previous to moving to North Carolina, I followed my dad's relocation plan. I was born in Thomasville, Georgia and moved to Coral Springs, Florida by the time I was two years old. After Florida, my family set up residence in the Midwest. We lived in a small suburb of Chicago called Algonquin. In the middle of my fourth grade year, my family moved again to Cumberland, Rhode Island. I attended yet one more elementary school for a year and a half before settling down in Raleigh.

After graduating college, I worked at NC State University College of Veterinary Medicine as a research technician in the Center for Cutaneous Toxicology Research and Pharmacokinetics. Three years later, I realized I did not want my lifelong career to be in dermatology research and I applied to graduate school with hopes to continue my education in science and explore the field of reproductive physiology.

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List of Symbols and Abbreviations

BSA	Bovine Serum Albumin
CL	Corpus Luteum
COC	Cumulus Oocyte Complex
E_2	Estradiol
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
hpi	Hours Post Fertilization
IL	Interleukin
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
$PGF_{2\alpha}$	Prostaglandin-F_2 alpha
TNF-α	Tumor Necrosis Factor alpha

Review of Literature

Infectious Disease and Reproduction:

Immune and inflammatory responses linked to infectious disease can cause embryonic mortality, fertilization failure, and anovulation (Hansen, et. al. 2004). Maternal infections have also been associated with preterm labor and an increased risk of fetal neurological injury (Gayle, et. al. 2004). Mastitis, an infection of the mammary gland caused by bacteria, occurs often in cattle. Mastitis reduces milk production, causes an increase of leukocytes in the milk, and alters milk composition and appearance. It also reduces reproductive performance as well as increases veterinary costs and damage to the mammary tissue itself (Hansen, et. al. 2004). It has been recorded that development of clinical mastitis in cows between first service and the establishment of pregnancy increased the number of days open (Hockett, et. al. 2000).

Mammary glands exhibit innate immunity, or a non-specific response, to defend the body against early stages of infection. This immunity is mediated by the physical barrier of the teat, macrophages, natural killer cells (NK cells), and neutrophils. The cell defenses involved are predominately macrophage activity. Macrophages are the main cell type found in the milk and healthy tissues of involuted and lactating glands. Phagocytic cells are also present and work to ingest bacteria and cellular debris. Natural killer cells involved in immune response cause destruction of the target cells and secrete toxic substances such as Tumor Necrosis Factor- alpha (TNF-α), a cytokine and inflammatory mediator, which can initiate apoptosis in the disease altered cells (Sordillo, et. al. 1997). Other inflammatory mediators, or cytokines, involved in infectious diseases are Interleukin-1 (IL-1) and IL-6.

These two cytokines plus TNF-α help to initiate the recruitment of leukocytes to the site or sites of inflammation. They begin by activating adhesion molecules on the leukocytes in circulation and endothelial cells of the vasculature next to the inflamed site. Cytokines can also increase the bacterial engulfing activity of phagocytes (Shuster, et. al. 1993).

An experiment completed in 2004 showed that cows with clinical mastitis had reproductive complications as well as an increase in the concentrations of IL-1, IL-6, and TNF-α in blood samples. The mRNA concentrations of IL-1α, IL-1β, and TNF-α in the milk-derived cells from infected mammary glands were also increased. Mammary infusion of LPS from *Escherichia coli* elevated concentrations of IL-1β, IL-8, and TNF-α in milk samples. The infusion of LPS caused a subsequent increase in circulating serum concentrations of TNF-α and concentrations of Prostaglandin-F_{2}- alpha ($PGF_2\alpha$) in milk. The increase in the number of services required to achieve pregnancy in cows with mastitis may be due to the fact that these cows are predisposed to reproductive problems caused by stress or are more physiologically debilitated. This impairment of reproductive processes could occur via the hypothalamic-pituitary axis or through ovary, oocyte, and embryo pathways. The release of cytokines during infection can affect endometrial or oviductal tissue causing a decrease in the development of an embryo. Cytokines also cause a release of PGF_2-α in the endometrium, which can cause premature luteolysis (Okuda, et. al. 2002). Without a corpus luteum or high progesterone concentrations, establishment or maintenance of pregnancy will not occur. Administration of PGF_2-α to cows receiving progesterone supplements also had a negative effect on pregnancy rate and embryo development (Hockett, et. al. 2004).

Ovarian abnormalities also occur in cattle diagnosed with clinical mastitis. Huszenicza, et. al. in 2005 evaluated several cases of mastitis to determine when damage to

the ovary occurred and what type of abnormality was present. Aseptic milk samples were taken from infected mammary quarters and a definite pathogen was identified. The cows were then placed into two groups: Gram positive (GP) and Gram negative (GN) plus no detected pathogen (NDP). The percentage of cows that ovulated within twenty-eight days after calving was lower when mastitis occurred between fifteen to twenty-eight days post-partum (pp) compared to healthy controls and those with mastitis cases occurring between one to fourteen days pp. This delay of ovulation caused by mastitis is induced by suppression of the existing dominant follicle followed by atresia. The next dominant follicle selected from the new follicular wave is ovulated. Most of these data were recorded in the GN and NDP group though the numbers were not significantly different from the GP group. The incidence of ovarian cysts did not increase with the development of mastitis; however, cows with ovarian alterations did show a more severe case of mastitis. In GN mastitis cases, the endotoxin does not move from the mammary gland into the blood, but the immune response within the gland can cause a release of cytokines, such as TNF-α and IL-1 β, from macrophages and monocytes (Huszenicza, et. al. 2005). It is the local production of these cytokines and the kinetics involved that may cause some variation in clinical signs of mastitis. Intrauterine injection of endotoxin causes the release of eicosanoids such as PGF_2-α, which can induce premature luteolysis. TNF-α released by mastitis can also stimulate the release of PGF_2-α from bovine endometrial cells. Just before luteolysis occurs, an increase of TNF-α gene expression occurs. The CL itself also shows an increase in the release of TNF-α at this time leading to the hypothesis that TNF-α may cause an increase in PGF_2-α secretion from the uterus to initiate luteolysis (Okuda, et. al. 2002). In this study both GP and GN plus NDP mastitis induced luteolysis, but the occurrence was significantly higher in the GN plus

NDP infections. This may be due to the higher quantity of cytokines released during severe cases of mastitis which would trigger the release of PGF_2-α. Cows involved in this experiment showed no influence of mastitis on pregnancy rate or calving-to-conception interval. This may be because the severity of GN and NDP mastitis decreases with time (Huszenicza, et. al. 2005).

Attempts to decrease cytokine concentrations during an infection or disease may save the pregnancy or life of the cow. On the other hand, some recombinant cytokines are being used to treat mastitis. These engineered cytokines recruit effector cells to the mammary gland to increase the number of phagocytic cell populations to regulate acute inflammation reactions (Sordillo, et. al. 1997).

In humans, infection or disease can have repercussions on pregnancy as well. In these cases, disease usually causes pre-term labor or neurological damage to the growing fetus (Gayle, et. al. 2004). Cytokines are potentially transferred from mother to fetus through amniotic fluid, therefore, directly causing damage to the fetus. To prevent reproductive failure, cytokine concentrations, specifically TNF-α, can be measured to detect the early stages of infection or disease (Gayle, et. al. 2004). In contrast, previous studies revealed nanogram levels of TNF-α to be non-toxic to rat granulosa cells. TNF-α release is also stimulated by IVF induced ovulation and therefore may be non-toxic to embryos. This information is based on the fact that during infection, TNF-α concentrations range from 0.1 to 10 ng/ml in women. Also, mice injected with TNF-α during pregnancy did not abort the fetus unless they were previously exposed to an infection and had existing increased macrophage activity. TNF-α may aid in the development of the embryo as well its survival by inhibiting NK cells' cytotoxic response to fetal antigens. Overall, the beneficial or

damaging affects of TNF-α on reproduction depend on the presence or absence of other immune mediators (Witkin, et. al. 1991).

Infertility and sterility occurs in humans and some cases can be attributed to antiovarian antibodies (AOA) as well as cytokines. For women undergoing in vitro fertilization (IVF) and embryo transfer (ET), success depends on age, ovulation status, oocyte quality, and endometrial receptivity. Failure of pregnancy mainly results from immunologic disturbances at all oocyte and embryonic developmental stages. An experiment concerning AOA was conducted with five groups of women. Anti-ooplasm (OO-Ab), anti-zona pellucida (ZP-Ab), anti-granulosa cell (MG-Ab), anti-theca cell (TI-Ab), and anti-corpus luteum (LC-Ab) antibodies were measured in the follicular fluid of retrieved oocytes. Results speculated that an increase in OO-Ab lead to failure in the first step of IVF, oocyte recovery. Elevated concentrations of ZP-Ab affected fertility rates of harvested oocytes. High concentrations of TI-Ab and LC-Ab were indicative of a defective luteal phase which is important for implantation. High concentrations of IL-1β were also detected in the follicular fluid of women with no viable oocytes. It was concluded that this cytokine has an effect on the differentiation of oocytes during IVF hormone treatments since cytokines are under pituitary hormone and gonadal steroid regulation (Horejsi, et. al. 2000).

Cytokines:

At present date, approximately thirty cytokines have been identified, purified, and characterized by their regulatory activities. They have been referred to as hormones because they are produced locally and can emit potent biological activity at low doses (Sordillo, et. al. 1997). Cytokines are difficult to define because of their wide range of synthesis and

activities. One author uses the following definition: “Cytokines are a class of inducible, water soluble, heterogeneous protinaceous mediators of animal origin that exercise specific, receptor mediated effects in target cells and/or in the mediator producing cells themselves” (Meager 1991). Cytokines exhibit some general characteristics. First, they are redundant, meaning several cytokines have the same function such as TNF-α and IL-1. Second, they are pleiotropic or can encompass multiple activities for one cytokine. This may lead to some uncertainty for a specific cytokine’s role which can ultimately depend on the condition(s) surrounding the cytokine. Finally, cytokines produce different effects at different concentrations. For example, TNF-α during early pregnancy at physiological concentrations will promote embryonic development yet later in pregnancy, if the concentration is high, it can be lethal to the fetus (Hill 2000).

Cytokines are mainly involved in innate and adaptive immune responses. Various cytokines help protect a developing fetus from rejection by the mother whose own body will recognize the fetus as a foreign object. Others control the growth of microorganisms on newborn humans’ skin and in the gut. However, when disease or infection does occur, cytokine production is increased and they can act as protective agents that amplify the immune system to prevent the spread of the disease within the host body. The concentration of cytokines released during this immune response can determine if these molecules will help treat the disease or produce toxic effects on organs and tissues (Meager 1991).

Innate inflammatory response involves four activities: initiation, immune effector cell attraction, immunodilation, and finally, inhibition or resolution (Hill 2000). After a stimulus such as bacterial infection or disease occurs within the body, the disease-damaged cells produce IL-1 or TNF-α. These cytokines can act as endogenous pyrogens, or molecules that

raise the body temperature. In addition, cytokines can mediate shock responses, trigger acute phase response, stimulate fibroblast proliferation, or promote the gene for prostaglandin H synthase (COX-2) which increases the production of prostaglandins. Chemokines, or small cytokines, are activated after the release of cytokines. They have influence on mast cells, T-cells, and neutrophils, and can activate macrophages and monocytes. The influence of chemokines is based on the establishment of a concentration gradient between the site of infection and normal tissue. Effector cells will then migrate with the gradient and become activated against the imposing antigen in specific or nonspecific ways depending on the cell type of the damaged tissue. This collective work of both cytokines and chemokines help define the normal innate inflammatory pathway that is an organism's first stage of defense. If these responses are disturbed in any way, pathological injury to the host can occur (Hill 2000).

TNF-α has been investigated because of its role as an initiator of the immune response pathway and as a causative agent for damage to a host body. TNF-α was discovered in 1975 by Caswell and colleagues in the sera of mice that were treated with an endotoxin. It was named tumor necrosis factor because of its ability to induce selective tumor death. This action of TNF-α was first observed in the 1880s when patients recovering from local bacterial infections or those injected with bacterial endotoxins also showed a regression in tumor cells. TNF-α was found to be produced by mononuclear phagocytes such as monocytes and macrophages when an organism was exposed to bacterial endotoxin (LPS). TNF-α can also be produced in response to other substances such as cell wall components of bacteria, viruses, and other cytokines or chemokines. Other cell types such NK cells, T-

lymphocytes, mast cells, and fibroblasts can also help induce production of TNF-α (Meager 1991).

The primary function of TNF-α is to initiate a cascade of cytokines associated with the inflammatory response. It is produced by macrophages which also secrete TNF-β, IL-1, IL-6, and IL-8 (Janeway, et. al. 1999). These cytokines are involved in the second type of immune response: acute phase response or host defense. Bacteria first bind to the macrophage inducing release of cytokines. Subsequently the macrophage will engulf the pathogen. The cytokines, now involved directly with the tissue, will cause inflammation symptomatically characterized as pain and heat. They will also cause vasodilation and edema portrayed by the body as redness and swelling. During this phase, an increase in body temperature results in fever. Fever helps adaptive immune responses work better and can slow the growth of most pathogens which usually reproduce at lower temperatures (Janeway, et. al. 1999). The cells of the host body are protected from this increase in temperature and cytokine actions. Initiation of acute phase response begins when proteins such as C-reactive protein and Mannan-binding lectin (MBL) are secreted from the liver into the blood stream. This results from direct contact of cytokines on hepatocytes. These acute phase proteins mimic the actions of antibodies yet have less specificity to individual pathogens. C-reactive protein and MBL are made only in response to a stimulus that triggers the release of TNF-α and IL-1 (Janeway, et. al. 1999). The next step in acute phase response is leukocytosis which is an increase in circulating neutrophils that occurs by cytokine activation of phagocytes. Leukocytes are generated by bone marrow and gather in sites in blood vessels where they are attached to endothelial cells. TNF-α stimulates the migration of neutrophils and leukocytes to the lymph node for maturation into co-stimulatory antigen-presenting cells that play a major

role in adaptive immunity. The sequential transfer of leukocytes to the site of infection is often the cause of pain during infection or disease. This type of immune response does not occur until four to seven days after a pathogen or endotoxin has entered a host body, proving the importance of the cytokines initial involvement in innate immune responses (Janeway, et. al. 1999). After acute phase response has been induced, if the infection is local, TNF-α will activate the expression of molecules within endothelial cells to trigger blood clotting to prevent the flow of the pathogen from entering the rest of the body via the blood stream. Fluid from the infected tissue then carries the bacteria in phagocytic cells (macrophages and neutrophils) to the lymph node where the pathogen is degraded. If the infection becomes systemic sepsis occurs. Sepsis allows the release of TNF-α from the liver, spleen, and other sites causing an increase in the concentration of the cytokine throughout the body. Systemic TNF-α release causes vasodilation which can lead to loss of plasma volume and eventually shock. TNF-α also consumes clotting proteins within the blood and the ability of the host body to clot becomes lost. This will lead to failure of vital organs such as the heart, lungs, liver, and kidneys because of abnormal blood perfusion. Septic shock is rare and the benefits of TNF-α on local infection far outweigh the damaging effects of high concentrations of the cytokine released during systemic infection (Janeway, et. al. 1999). TNF-α inhibitors can help prevent systemic effects of the cytokine. These inhibitors are generally binding proteins such as TNF-R55-BP and TNF-R75-BP that occupy the receptor to prevent TNF-α from binding and causing damaging effects (Baxter, et. al. 1991).

The activities of cytokines depend on receptor type. Generally cytokine receptors are dimers or trimers with membrane bound subunits that will change in conformation during ligand binding. The receptor will trigger a signal by an intracellular component of itself. The

intracellular signal will then couple with G-proteins and cellular activity is altered. The signal is then transmitted to the nucleus by binding of proteins to a specific gene promoter. TNF-α have two types of receptor: Type I or B which is 55kDa and Type II or A which is 75kDa. They mediate intracellular responses via proteins called TNF Receptor Associated Factors (TRAFs). These proteins help transmit signals to the nucleus, some of which are apoptotic signals, causing cell death (Hill 2000). TNF-α receptors can also stimulate production of arachidonic acid and prostaglandins by increasing the permeability and fluidity of the plasma membrane allowing an influx of calcium which activates Phospholipase A_2; all of which occurs directly after ligand binding. Overall, the effect of TNF-α binding to its receptor is typical of growth promoting cytokines (Meager 1991).

Even with all the information described above, cytokine definition and activity is still not fully understood. However, there is one fact about all cytokines that is still reliable. They are indeed pleiotropic molecules whose beneficial and damaging effects depend on the quantity produced, the localization of production and secretion, and the activity of their receptors and binding proteins (Hill 2000).

Cytokines and Reproduction:

Cytokines have been found in reproductive tissues and fluids leading to the suggestion that they have a direct role in pregnancy, parturition, oocyte development, and oocyte maturation. In humans, placental and extra-placental membranes as well as amniotic fluid are sources of cytokines (Keelan, et. al. 2003). During labor an upregulation of inflammatory responses such as an increase in IL-1α, IL-1β, and TNF-α mRNA in the

placenta and amnion has been observed. Cervico-vaginal fluids also showed an increase in IL-1β and TNF-α during labor and pre-term labor associated with infection. This rise in concentration seems to hit a maximum level when the cervix is completely dilated. IL-6 is also present with these two cytokines in the amniotic fluid during an intrauterine infection. Pre-term delivery is often associated with an infection in the mother and her host response to the pathogen(s). Tissue samples taken during pre-term labor expressed high concentrations of cytokines that correlated with the level of leukocyte infiltration into the tissues (Keelan, et. al. 2003). Leukocytes typically migrate into the gestational membranes to fight infection and release cytokines.

Anti-inflammatory cytokines, such as IL-10, are also present in the choriodecidua and amniotic fluid. IL-10 is normally expressed during late gestation, but its production can be activated in response to TNF-α release in association with LPS infection. It has been recorded in rats that treatment with IL-10 prevents pre-term labor normally induced by LPS. The anti-inflammatory cytokines withdraw before parturition to allow inflammation which tends to aid in labor. In the normal labor process, matrix metalloproteinases (MMP) digest the extracellular matrix of fetal membranes. MMP increases with production of cytokines. Cytokines such as IL-8 increase with the stretching of membranes and are activated themselves by IL-1β and TNF-α. An increase in TNF-α also initiates apoptosis which leads to the thinning and rupture of membranes. These results speculate an indirect mechanism of cytokines involved with extracellular matrix remodeling. Pre-term labor associated with infection occurs by an increase in TNF-α, which will trigger activation of MMP to rupture fetal membranes (Keelan, et. al. 2003).

The effect of cytokines on IVF and embryo development has been researched. IL-1β was added to the culture media for bovine embryos after fertilization at various concentrations to observe its effect on embryo development (Paula-Lopes, et. al. 1998). In the first trial, concentrations of 0 or10 ng/ml of the cytokine were added to development medium 8-10 hours after fertilization and embryos were cultured until day nine. IL-1β had no effect on cleavage rate, but it did increase the proportion of embryos that developed to the blastocyst stage. IL-1β was then added to the development medium five days after fertilization. IL-1β at 1 ng/ml concentration showed no effect, but the 10 ng/ml concentration inhibited blastocyst development at day nine. In the third trial, IL-1β was added eight to ten hours after fertilization at low doses between 0 and 10 ng/ml. The cytokine stimulated embryo development in a dose dependent manner. The greatest effect was seen at days seven and nine with 0.1 ng/ml of IL-1β. This however depended on embryo density within the 50 µl drops of culture medium. For optimum growth stimulation, 25-30 embryos were cultured in 50 µl drops with 0.1 ng/ml of IL-1β. This cytokine may be more effective at low doses due to receptor down-regulation via activation of receptor inhibitor. Or IL-1β at high concentrations may inhibit a metabolic pathway or gene expression which is beneficial for embryo development. After recording these results, the authors suggest that the actions of this particular cytokine at low doses on the promotion of embryo development *in vitro* are different from those damaging effects involved with higher concentrations that occur during an immune response (Paula-Lopes, et. al. 1998).

TNF-α has many roles in reproduction. As stated before, the main producer of this cytokine is the macrophage cell. Macrophages are found in follicles and the corpus luteum (CL). The concentration of this cell type depends on the stage of development of each type of

structure. Macrophages are increased in number during ovulation suggesting that TNF-α may play a role in the apoptosis of follicles. Macrophages are also more prominent during CL regression and all throughout the luteal phase of the reproductive cycle (Hill 2000). This information proposes another role of TNF-α in the reproductive cycle and its relative pathways involving other hormones such as progesterone which is produced by the CL and PGF_2-α which causes lysis of the CL. It has been recorded in experiments that TNF-α is present in the ovaries of sheep, cows, rabbits, and humans (Hill 2000). In humans this cytokine is present in granulosa cells and during culture of such cells, TNF-α inhibits progesterone and estradiol biosynthesis. The effects of TNF-α may depend on the differentiation stage of the cells or the species type (Hill 2000). The importance of monocytes and inflammatory response during the follicular phase of the reproductive cycle was examined by Bouman, et. al.(2004). The authors hypothesized that monocytes in the blood would decrease in number during the follicular phase to allow the maternal immune system to prepare for implantation of blastocysts after ovulation and fertilization regardless of infection with LPS. Results indicated that even though LPS activated TNF-α secretion, the concentration of the cytokine decreased in whole blood samples incubated with estradiol at physiologic concentrations *in vitro*. This leads to the conclusion that during the follicular phase and ovulation, which can cause an inflammatory response itself, suppression of monocyte function, or suppression of immune response occurs (Bouman, et. al. 2004).

Based on the fact that immune responses can cause infertility or reduce embryo survival, the involvement of cytokines, specifically TNF-α, was suspected. In women, the value of TNF-α in maternal serum may be used as a predictive agent in determining the outcome of pregnancy using IVF since this cytokine is undetectable in the serum of healthy

women (Witkin, et. al. 1991). Witkin, et. al. found that TNF-α was present in the serum of women involved in hormone therapy as a part of the IVF procedure. However, their pregnancy rates were unaffected by this elevated concentration. They also found an increase in the serum concentration of TNF-α eight days after ET that resulted in both successful and unsuccessful pregnancies. The presence of TNF-α at this stage of IVF suggests it is being secreted from the CL or the granulosa cells. TNF-α was measured in embryonic culture medium with and without the presence of maternal serum and was detected in 24.5% of serum cultures even though the success rate of pregnancy was unaffected again (Witkin, et. al. 1991). Increased TNF-α concentration observed in the embryonic medium before ET supplied evidence that the embryo itself may also secrete the cytokine. They concluded that the hormones injected into the IVF patients to optimize fertility may stimulate cytokine production from ovaries as well as macrophages, therefore disproving the hypothesis that maternal serum concentrations of TNF-α can be used as a predicative measure for the success of IVF procedures (Witkin, et. al. 1991). In later experiments, TNF-α was added to maturation medium in IVF and was shown to reduce the potential of the treated cumulus oocyte complexes (COCs) of cattle to develop to the blastocyst stage after fertilization (Soto, et. al. 2003). This could result from TNF-α binding to its receptors in the granulosa cells causing apoptosis in a dose-dependent manner. Alternatively, the decrease in development may result from an increase of PGF_2-α caused by TNF-α secretion from the cumulus cells of the COCs ultimately leading to the disruption of maturation followed by inhibited blastocyst development of embryos exposed to PGF_2-α (Soto, et. al. 2003).

Another study to concentrate on the effects of cytokines and oocyte development was completed in 1999 by Mendoza, et. al. Human oocytes were obtained transvaginally and kept

in their follicular fluid. The hypothesis was that oocyte quality was directly related to concentrations of steroids, cytokines, and hormones present in the individual follicular fluid. It is believed that TNF-α is regulated by IL-1 and both enhance vascular permeability and are involved with angiogenesis. Working together they act to allow gonadotropins to enter the follicles. Results obtained support this hypothesis as an increase in TNF-α in follicular fluid coincided with an increase in luteinizing hormone (LH), growth hormone (GH) and prolactin. Oocytes from follicles containing high concentrations of TNF-α and IL-1 in the follicular fluid were associated with successful fertilization and post-fertilization development. Unsuccessful IVF patients may contain low concentrations of these cytokines in their follicles which would cause lack of direct stimulation of the oocyte by intrafollicular TNF-α, resulting in poor oocyte quality. A proposed method for future successful fertilization may be the use of recombinant TNF-α during the oocyte incubation and maturation periods (Mendoza, et. al. 1999).

The majority of these studies examining the effect of TNF-α during IVF have concluded that cytokines can directly affect the maturation of COCs and the development of the embryos. An indirect mechanism has also been proposed in which cytokines can affect the endometrial and oviductal tissue which can lead to a decrease in embryo development. Direct or indirect, cytokines' damaging effects on embryos may work through an increase in PGF_2-α. This increase in PGF_2-α is initiated by an increase in TNF-α (Skarzynski, et. al. 2000) and IL-1β (Davidson, et. al. 1995). An increase in the concentration of PGF_2-α promotes luteolysis, causes disruption of oocyte maturation (Soto, et. al. 2003) and inhibits embryo development (Scenna, et. al. 2004).

Cytokines and PGF_2-α:

Prostaglandins, especially PGF_2-α, are a major factor in regulating reproduction and pregnancy. PGF_2-α production can cause regression of the CL during normal reproductive cycles to allow new ovulatory follicles to mature and regulate cyclicity and fertility. PGF_2-α is secreted from the inter-caruncular regions of the epithelium in the uterus (Okuda, et. al. 2002). Prostaglandins are arachidonic acid metabolites that are released in a pulsatile manner to achieve maximum lysis of the CL compared to sustained absolute levels of secretion. When a bovine conceptus is present in the uterus, release of progesterone from the intact CL is maintained to establish pregnancy. This usually occurs between days fourteen and seventeen of pregnancy (Okuda, et. al. 2002). The puslatile release of prostaglandins within the uterus causes release of an embryonic signal, usually interferon-tau, which prevents luteolysis by down-regulating the oxytocin receptor blocking oxytocin-stimulated production of PGF_2-α (Okuda, et. al. 2002). In general, PGF_2-α is regarded as a luteolytic agent, but its biosynthetic pathway becomes uncoupled from the endometrium of the uterus causing its release during pregnancy to decrease, encouraging maternal recognition of pregnancy (MRP) and implantation of the embryo (Okuda, et. al. 2002, Hwang, et. al. 1988, Thatcher, et. al. 2001).

PGF_2-α is controlled by cyclooxygenase (COX), the first catalyzed step in its synthesis from arachidonic acid. COX-1 is present in most prostaglandins synthesized by tissues and is involved in cell function regulation. COX-2 is an inducible enzyme that remains undetectable in tissues but will increase in expression in response to stimulation by cytokines. Both COX-1 and COX-2 use different pools of arachidonate which are regulated by different cellular stimulus to produce prostaglandins (Markenson 1999). The COX gene

found in the bovine endometrial cells is directly under the influence of ovarian steroids (Okuda, et. al. 2002). COX-2 expression is induced in granulosa cells by increased concentrations of gonadotropins and is associated with the presence of PGF_2-α in the follicular fluid. It has been recorded that female COX-2 null mice exhibit a defective ovulatory process as well as inhibited oocyte maturation (Nuttinck, et. al. 2002). Based on this information, the COX gene is involved in many reproductive processes. Its regulation of the production of prostaglandins can either benefit or harm these processes depending on the time and place in the reproductive system in which the prostaglandins appear.

Prostaglandins can also be regulated by cytokines. TNF-α receptors have been located in bovine endometrial cells where prostaglandins are synthesized, showing possible action of cytokines on the biosynthetic pathways of prostaglandins (Okuda, et. al. 2002). It has been recorded that prostaglandin production by tissues such as chorion, amnion, decidua, and myometrium can be enhanced by IL-1β and TNF-α (Keelan, et. al. 2003). The enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH) catalyzes the breakdown of prostaglandins to inactive metabolites and is found in the chorion and placental trophoblasts. PGDH is inhibited by these two cytokines as well, supporting the theory that prostaglandin concentrations are increased by cytokines (Keelan, et. al. 2003).

TNF-α also affects PGF_2-α synthesis during both the follicular phase and mid to late luteal phases of the reproductive cycle. TNF-α gene expression is increased and it is released from the CL just prior to luteolysis. This release from the CL initiates PGF_2-α release from the uterus to aid in the regression of the CL. It has also been recorded that TNF-α dramatically increases during the second stage of luteolysis due to the increase of macrophage cells during this phase (Okuda, et. al. 2002).

A recent study observed the effects of perfusion with TNF-α on sheep CL function *in vivo*. Results indicated that between twenty-four and forty-eight hours after perfusion of the cytokine, progesterone release was decreased. TNF-α decreased progesterone concentrations even more when the CL was pretreated with PGF_2-α and endothelin-1(ET-1). During luteolysis, TNF-α acts as a type of progesterone suppressor when the luteal cells are stimulated by an increase in PGF_2-α and ET-1. In cattle, this cytokine is only secreted locally after completion of the lysis of the CL. A positive feedback loop exists between ET-1 and TNF-α in the luteolytic pathway. TNF-α stimulates ET-1 and since endothelial cells are targets of cytokines, ET-1 can stimulate TNF-α. This feedback loop causes an acceleration of the luteolytic cascade (Ohtani, et. al. 2004).

Prostaglandin and Cytokine Blockers:

As stated before, TNF-α's actions can be either beneficial or detrimental depending on the quantity produced, the activity of TNF-α binding proteins, and the localization of the affected tissue. Infliximab is a TNF-α blocker and was recently studied in women with endometriosis (Noriega, et. al. 2004). Women with this disease have an increased concentration of TNF-α in their peritoneal fluid which correlates to the stages of the disease. Endometriosis is associated with immune or inflammatory disorders that generally affect reproduction and fertility, so blocking TNF-α may be a form of treatment. Infliximab is form of a monoclonal antibody that binds to membrane and soluble forms of TNF-α to reduce its biological effects. It is known to be non-toxic to cleaving embryos, but its consequences on early embryonic development are unknown and therefore the objective of this study. Mouse

embryos exposed to 400 ng/ml of Infliximab in development medium during *in vitro* culture exhibited arrested growth and fragmentation. In conclusion, a negative effect of Infliximab is biodilution or bioconcentration, leaving toxic concentrations in the reproductive tract. However, the anti- cytokine properties of this antibody did slow TNF-α induced proliferation of endometrial stroma and inhibited the development of endometrial lesions and adhesions (Noriega, et .al. 2004).

Prostaglandin release and inhibition of gonadal steroidogenesis can be stimulated by cytokines. Release of cytokines such as TNF-α and IL-1β during and inflammatory response or infection may lead to anovulation, poor ooctye quality, and sometimes resorbtion of the embryo (Hansen, et. al. 2004). A proposed pathway for this action is the stimulation of PGF-$_{2\alpha}$ production by TNF-α which leads to the luteolytic cascade thereby reducing the amount of progesterone needed to maintain pregnancy or initiate a new reproductive cycle. PGF-$_{2\alpha}$ produces a direct negative effect on embryo development. Prostaglandin administered in vivo along with a progestogen feed additive in cattle undergoing artificial insemination showed a decrease in embryo quality in the treatment groups. These embryos from the treatment cows were obtained by flushing of the uterine horn eight days after insemination. A decrease in the rate of development of the embryos past the morula stage was also noticed (Hockett, et. al. 2004). An *in vitro* experiment with bovine embryos gave similar results. Treatment of embryos produced *in vitro*, or pre-compacted embryos, with PGF-$_{2\alpha}$ twenty-four hours after placement in development medium produced a decrease in the percent of embryos developing to the blastocyst stage. Compacted embryos, or those derived from *in vivo* fertilization, were also treated with PGF-$_{2\alpha}$ twenty-four hours after placement in development medium. These treated embryos did develop to the blastocyst stage but showed

a decrease in the proportion of embryos developing to the expanded or hatched blastocyst stages. A possible mechanism for these deprived embryos treated with prostaglandin may occur through apoptosis or alterations in embryonic gene transcription (Scenna, et. al. 2004).

Indomethacin stops PGF-$_{2\alpha}$ synthesis by blocking the COX pathway of arachidonic acid metabolism to prostaglandins (Fairchild, et. al. 1991). This chemical has been examined in luteal cell culture studies to measure the production of progesterone. In one experiment, luteal cells were exposed to progesterone, indomethacin, or interferon- γ (IFN-γ). IFN-γ is a cytokine that generally enhances immune responses but can also increase arachidonic acid metabolism and prostaglandin production in macrophages and fibroblasts. Luteal cells exposed to IFN-γ for more than twenty-four hours exhibited a reduction in PGF-$_{2\alpha}$ production. Cells treated with progesterone blocked the effect on PGF-$_{2\alpha}$ secretion on day seven of culture. Indomethacin also decreased PGF-$_{2\alpha}$ synthesis in luteal cell culture both alone and with IFN-γ stimulation allowing progesterone secretion to increase (Fairchild, et. al. 1991).

Luteal cell cultures have also been treated with IL-1β, another stimulator of PGF-$_{2\alpha}$ synthesis, as well as indomethacin. PGF-$_{2\alpha}$ production occurred in luteal cells and accessory cells and was increased by addition of IL-1β to the culture medium. When indomethacin was added to the cultures containing the IL-1β treatment or to control cultures, the prostaglandin concentrations in both groups decreased. In conclusion, indomethacin deters IL-1β from activating the COX pathway of PGF-$_{2\alpha}$ synthesis (Del Vecchio, et. al. 1997).

Because of the data presented here, it seemed appropriate to investigate whether or not indomethacin could block the effects of increased prostaglandin production caused by cytokines on the inhibition of bovine embryo development.

Introduction

Mastitis and other inflammatory diseases occur often in cattle and can lead to a decrease in milk production, altered milk appearance, damage to the mammary tissue, increased veterinary costs, and a reduction in reproductive performance (Hansen, et. al. 2004). Cytokines, or inflammatory mediators, are secreted at the site of infection by macrophage cells. Specifically, tumor necrosis factor alpha (TNF-α) helps to initiate the recruitment of leukocytes to the site of inflammation (Shuster, et. al. 1993). Hansen, et. al. (2004) reviewed an increase in the concentration of TNF-α as well as interleukin 1 (IL-1) and IL-6 in the blood of cows diagnosed with clinical mastitis. This release of cytokines can also have detrimental effects on endometrial and oviductal tissue leading to a decrease in embryo development (Hansen, et. al. 2004). It has been demonstrated that disease damaged cells produce IL-1β and TNF-α and these two cytokines promote the expression of the gene for cyclooxygenase-2 (COX-2), which is an inducible enzyme that is upregulated by inflammatory mediators such as cytokines (Markenson 1999) and is responsible for the production of prostaglandins from arachidonic acid (Hill 2000). Therefore, TNF-α can also cause a release of prostaglandin-F_2-alpha (PGF-$_2$α), which can lead to early luteolysis (Hansen, et. al. 2004).

In previous studies, TNF-α has been shown to be present in the ovaries of sheep, cows, rabbits, and humans (Hill 2000). Using this information, Mendoza, et. al. (1999) hypothesized that oocyte quality was directly related to concentrations of steroids, cytokines, and hormones present in the individual follicular fluid. It is believed that TNF-α is regulated by IL-1β and both are involved with angiogenesis and vascular permeability. Working together they act to allow gonadotropins to enter the follicles. Results obtained support this

hypothesis as an increase in TNF-α in follicular fluid coincided with an increase in luteinizing hormone (LH), growth hormone (GH) and prolactin within the follicle. Oocytes from follicles containing high concentrations of TNF-α and IL-1β were associated with successful fertilization and post-fertilization development in humans (Mendoza 1999).

In later experiments, TNF-α was added to maturation medium in IVF and was shown to reduce the potential of the treated cumulus oocyte complexes (COCs) from cattle to develop to the blastocyst stage after fertilization (Soto, et. al. 2003). Soto, et. al.(2003) also added TNF-α to development medium after fertilization of bovine embryos and found that extremely high or low concentrations of the cytokine, 100 ng/ml or 0.1 ng/ml, respectively, inhibited embryo development to the blastocyst stage. Moderate concentrations such as 1 ng/ml and 10 ng/ml actually increased embryo development. Directly or indirectly, cytokines' damaging effects on embryos may work through an increase in PGF_2-α. TNF-α binds to receptors in the endometrium and increases PGF_2-α production causing premature luteolysis which leads to a decrease in the concentration of progesterone which is required to maintain pregnancy (Hansen, et. al. 2004). The increase in the concentration of PGF_2-α and TNF-α can also lead to the disruption of oocyte maturation by two possible mechanisms. First, TNF-α can bind to its receptor in the granulosa cells causing apoptosis of the oocyte. Or, as Soto, et. al. hypothesized (2003), an increase in TNF-α may cause production of PGF_2-α by the cumulus cells, leading to disruption of maturation followed by reduced development of these embryos exposed to PGF_2-α (Soto, et. al. 2003). PGF_2-α also affects embryo development by reducing the quality of the embryos recorded in *in vivo* experiments (Hockett, et. al. 2004) as well as inhibiting the development of embryos to reach the blastocyst stage in *in vitro* experiments (Scenna, et. al. 2004). Based on these previous

experiments, the objective of this study was to investigate the possibility that the addition of TNF-α after fertilization of bovine oocytes *in vitro* will decrease embryo development to the blastocyst stage and that this effect can be reversed by the addition of a prostaglandin synthesis inhibitor, indomethacin. Our hypothesis is that TNF-α inhibits embryonic development through prostaglandin mediated mechanisms, therefore, indomethacin should block the inhibition of development of bovine embryos caused by TNF-α.

Materials and Methods:

Reagents, Cytokines, and Hormones

Tissue culture media (M199) was purchased from Gibco BRL (Grand Island, NY). β-estradiol, gentamicin, porcine pituitary FSH (50mg/vial Standard), equine pituitary LH (11.5 NIH LH-S1 units/mg), and fetal bovine serum (FBS) (heat inactivated), BSA Fraction V, heparin sodium salt, pyruvic acid sodium salt, and indomethacin were all obtained from Sigma Chemical Company (St. Louis, MO). Indomethacin was reconstituted to a 1000x stock solution of 1 μg/μl in sterile absolute ethanol according to directions. Recombinant Bovine TNF-α was purchased from Pierce Endogen (Rockford, IL) and reconstituted in sterile saline to a final concentration of 4 ng/ml. All other reagents used were of tissue culture grade and were obtained from Sigma Chemical Company.

In Vitro Embryo Production

Ovaries from cows were obtained from a local abattoir and held in saline and 0.75 μg/ml penicillin for up to seven hours. The ovaries were washed in sterile saline upon arrival to the laboratory. Follicular fluid was aspirated from follicles 2-10 mm in size with an 18 gauge needle and syringe and then placed into a 50 ml centrifuge tube held in a water bath at a temperature of 39°C. The debris was allowed to settle to the bottom of the tube for at least fifteen minutes after aspiration. The sediment was then transferred to a search plate (Fisher Scientific Pittsburg, PA). Immature COCs were selected and moved to a divided petri dish (Fisher Scientific Pittsburg, PA) containing Tyrode Medium (TL-Hepes, Crosier, et. al. 2001) and washed four times. The COCs in groups of 50 were then randomly placed into individual wells of a 4-well plate (Nalge Nunc Rosklide, Denmark) containing maturation medium

which consisted of M199, 1 mg/ml E_2 , 50 μg/ml gentamicin, 200 μM pyruvate, 5 μg/ml FSH, 10 μg/ml LH, and 10% FBS (Farin & Farin 1995). Cultures were maintained in an atmosphere of 5% CO_2 in air, 100% humidity, and at a temperature of 39°C for 18-20 hours. After the maturation period, COCs were washed and held in fertilization medium containing heparin-supplemented Tyrode Albumin Lactate Pyruvate medium with 6 mg/ml BSA (Crosier, et. al. 2001). Frozen spermatozoa were obtained from American Breeders Service (DeForest, WI). Swim-up procedure was used for selection as it enhances the percentage of motile sperm (Parrish, et. al. 1986). Briefly, straws (0.5 ml, 50 x 10^6 sperm) were thawed in a 39°C water bath for 45 seconds, dried, and contents placed in empty 12 X 75 mm plastic tube (Fisher Scientific, Pittsburgh, PA) resting in a water bath of the same temperature. Thawed sperm (0.25 ml per tube) was layered under 0.5 ml pre-warmed Sperm-TL medium (Parrish, et. al. 1986) kept in the same water bath and allowed to swim up for at least one hour at 39°C. The top 0.5 ml of medium was drawn off, placed into pre-warmed centrifuge tubes containing Sperm-TL medium, and centrifuged at 2500 rpm and 39°C for 8 minutes. The supernatant was removed and the sperm pellet resuspended in 1ml of Sperm-TL. Sperm were then counted using a Brightline Hemacytometer and used to inseminate the matured COCs at a final concentration of 1 x 10^6 spermatozoa/ml. Gametes were co-incubated for 18-20 hours in fertilization medium at 39°C in an atmosphere of 5% CO_2 in air. After fertilization, presumptive zygotes were washed 6 times in TL-Hepes using a 24-well culture plate (Becton Dickinson, Franklin Lakes, NJ) and approximately 25-30 embryos were randomly placed into one well of a 4-well tissue culture plate containing one of five treatments. All treatments were contained within developmental medium containing M199, 10% FBS, and 50 μg/ml gentamicin. All treatment media were sterile filtered after the addition of treatment using a

0.2 μm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI). Treatments were as follows:

1) Control 2) 25 ng/ml TNF-α 3) 1 μg/ml Indomethacin reconstituted with absolute ethanol 4) 25 ng/ml TNF-α and 1μg/ml Indomethacin 5) Absolute ethanol for vehicle control. All embryos were cultured in wells containing 1ml medium at 5% CO2, 100% humidity, and at 39°C for up to 216 hpi. Treatments 1, 2, and 4 were replicated four times. Culture medium was changed at 48 hour intervals and the monocell layer was detached from the bottom of culture plate to allow proper development. Embryo development was recorded on days seven (168 hpi) and nine (216 hpi) and evaluated for quality and stage of development. The number of embryos in each stage of development was recorded and compared to the total number of embryos in each well to calculate the percentage of total blastocysts. Developmental stages were classified as compact morula, early blastocyst, mid-blastocyst, expanded blastocyst, and hatched blastocyst (Tables 1& 2, Figures 3 & 4). The total numbers of oocytes used in the experiment were control 225, TNF-α 232, and TNF-α plus Indomethacin 225.

Statistical analysis:

Data for the percentage of embryos developing to the blastocyst stage with treatment were arcsin transformed and analyzed by one way ANOVA using PROC GLM. A Duncan's Multiple Range Test was used for mean separation. Data are presented as LSM ± SEM.

Results

Preliminary Studies

50 ng/ml as well as 100 ng/ml of TNF-α were added to the development media after fertilization as described above and 25-30 embryos were randomly placed in each treatment well. All embryos were cultured as described in the previous section. Embryo development was recorded on days 7 (168 hpi) and 9 (216 hpi) and evaluated for quality and stage of development. Embryonic development stages were counted and blastocyst percentage calculated as described above. On day 9 of culture with the addition of 50 ng/ml and 100 ng/ml of TNF-α, the percentage of embryos developing to the blastocyst stage in the control group was 29%, treatment of embryos with 50 ng/ml of TNF-α was 30%, and treatment of 100 ng/ml of TNF-α was 14%. In two other trials, 10 ng/ml and 25 ng/ml of TNF-α were used. At day 9 of culture, the percentage of control embryos developing to the blastocyst stage in the first experiment was 40%. Treatment of embryos with 10 ng/ml elicited a 26% blastocyst development and treatment with 25 ng/ml resulted in 33% blastocyst development. The percentages of blastocysts in the second experiment were as follows: Control 25%, 10 ng/ml TNF-α 29%, and 25 ng/ml TNF-α 17%.

The 1 μg /ml dose of indomethacin was chosen as a non-toxic active dosage based on previous literature in which it was used with bovine embryos and luteal cells *in vitro* along with the addition of blood platelets to the cultures (Battista, et. al. 1989, Thibodeaux, et. al. 1993). This dosage of indomethacin was tested in two replicates in our IVF system and had no effect by itself on embryo development with a mean of 25.56 % blastocysts on day seven of culture and a mean of 36.11% blastocysts on day nine of culture when compared to the control embryos with a mean of 39.10% blastocysts on day nine.

A vehicle control treatment group was also tested within our IVF system using 1μg/ml absolute ethanol in two replicates. This dosage of ethanol had no effect on the development of bovine embryos to the blastocyst stage with a mean of 29.71% blastocysts on day seven of culture and a mean of 35.87% blastocysts on day nine of culture compared to the controls with means of 30.4% and 24.0% blastocysts on days seven and nine respectively.

Present Study

In contrast to previous reports (Soto, et. al. 2003), bovine embryos exposed to TNF-α after fertilization showed a decrease in the percent of embryos that developed to the blastocyst stage. Treatment with 25 ng/ml TNF-α decreased the percentage of embryos to developing to the blastocyst stage (17.15 ± 2.36 $p<0.005$) compared to the controls (30.45 ± 2.36 $p<0.005$) on day seven of culture (168hpi) (Figure 1). There was no significant difference between the TNF-α plus indomethacin treatment group (25.75 ± 2.89 $p<0.005$) and the controls (30.45 ± 2.36 $p<0.005$), however these two groups were significantly different from the TNF-α treatment group alone (17.15 ± 2.36 $p<0.005$, Duncan Multiple Range Test grouping, $p=0.05$) on day seven of development (Figure 1).

Data from day nine of development (216 hpi) was not significant (Figure 2), but a definite trend was detected ($p< 0.08$). Fewer embryos tended to develop to the blastocyst stage when cultured with 25 ng/ml TNF-α (21.46 ± 2.72 $p<0.08$) compared to the controls (30.21 ± 2.72 $p<0.08$). The mean percentage of embryos developing to the blastocyst stage when treated with 25 ng/ml plus 1 μg/ml indomethacin (29.45 ± 3.33) was closer to the control group mean (30.21 ± 2.72) but was not significantly different from the mean of the

TNF-α treatment group alone (21.46 ± 2.72 , Duncan Multiple Range Test grouping, p=0.05) on day nine of development.

Data on embryo classification was also recorded and analyzed. There was no significant difference in the percentage of embryos within a particular stage of blastocyst development compared to the treatments (Figures 3 & 4).

Discussion

Preliminary Studies

Preliminary studies were completed to determine the appropriate concentration of TNF-α to be used in the main experiment. Based on the literature, concentrations of TNF-α from 0.1 ng/ml to 100 ng/ml was used on bovine oocytes during maturation and embryos during and after fertilization (Soto, et. al. 2003 and Hansen, et. al. 2004). When added after fertilization, 10 ng/ml of TNF-α enhanced the percentage of embryos developing to the blastocyst stage whereas, 100 ng/ml inhibited this development (Soto, et. al. 2003). Using this information, several concentrations of TNF-α were tested under our in vitro embryo production conditions.

Based on the preliminary results, the concentration of 25 ng/ml of TNF-α was chosen as the treatment dosage for the experiment involving indomethacin. TNF-α at 25 ng/ml decreased the ability of the embryos to develop to the blastocyst stage versus the control embryos. Using indomethacin as a prostaglandin synthesis inhibitor, the concentration of TNF-α that produced the most consistent ability to slow embryo development was needed to verify the results after the addition of indomethacin to the embryo cultures.

Present Study

Results of this study support the hypothesis that 25 ng/ml TNF-α added to culture medium after fertilization inhibits bovine embryo development to the blastocyst stage. This decrease in development was significant at day seven (168 hpi), but only tended to be significant at day nine of development (216 hpi). Our results contrasts findings that 0-100

ng/ml of TNF-α added after fertilization to bovine embryos did not reduce the percentage of embryos developing to the blastocyst stage when data was collected on day eight of culture (Soto, et. al. 2003). At 216 hpi, the embryos treated with 25 ng/ml TNF-α recovered to the range of development seen in the control embryos. However, at $p<0.08$, there was a tendency for these treated embryos to show a decrease in blastocyst development. This may be due to the fact that TNF-α is dependent on other present immunological mediators and 25 ng/ml of TNF-α is slightly higher than physiological concentrations observed in women during infection (0-10 ng/ml) (Witkin, et. al. 1991). The concentration of plasma TNF-α in cattle during an infection such as mastitis ranges from 0-8 ng/ml and has been observed to peak at seven to ten hours after LPS administration. Milk concentrations of TNF-α are higher during LPS induced mastitis and range from 0-250 ng/ml with a peak around six to ten hours after initiation of infection (Hoeben, et. al. 2000). It should also be taken into consideration that the concentrations observed in milk and plasma may not reflect the actual concentration of TNF-α within the reproductive tract of the animal.

Cytokines, such as TNF-α, as well as macrophages have been shown to increase during luteolysis (Fairchild, et. al. 1991). This increase in cytokines is also present in circulating serum concentrations during infections such as mastitis which leads to an increase of $PGF\text{-}_{2\alpha}$ in milk samples (Hansen, et. al. 2004). This increase in $PGF\text{-}_{2\alpha}$ will then cause luteolysis, disruption of oocyte maturation (Soto, et. al. 2003), and inhibition of embryo development (Scenna, et. al. 2004) and can directly reduce embryo quality and survival (Hockett, et. al. 2004). Based on these observations, TNF-α can work within a positive feedback loop of $PGF\text{-}_{2\alpha}$ and luteolysis.

PGF-$_{2\alpha}$ can be detrimental to IVF embryo development to the blastocyst stage when added after fertilization with the most effective dose being 1 ng/ml. This effect was reversed when recipient cows received an injection of Flunixin Meglumine, a COX-1 and COX-2 inhibitor, at the time of embryo transfer (ET). The COX inhibitor blocks the production of prostaglandins from arachidonic acid. It was recorded that in the flunixin treated animals, the rate of successful pregnancy increased after ET due to the decrease in production of PGF-$_{2\alpha}$ (Scenna, et. al. 2004). The COX gene is found in bovine endometrial cells (Okuda, et. al. 2002) and is activated to produce prostaglandins by an increase in both IL-1β and TNF-α which are produced by macrophage cells and production was increased by luteolysis (Hill 2000).

Authors discussed previously suggest that an increase in IL-1β influences the increase of TNF-α, especially in follicular fluid (Mendoza, et. al. 1999). When added to CL accessory cell culture, IL-1β increases the production of PGF-$_{2\alpha}$ and this effect is decreased with the addition of indomethacin, a prostaglandin synthesis inhibitor (Del Vecchio, et. al. 1997). Therefore, TNF-α induced production of PGF-$_{2\alpha}$ may also be regulated by indomethacin.

In the present study, exposure of embryos to 25 ng/ml TNF-α plus 1 μg/ml indomethacin after fertilization resulted in an increase in the proportion of embryos developing to the blastocyst stage, when compared to the TNF-α alone treatment group. The addition of indomethacin to the embryos exposed to TNF-α provided reversal of the inhibited development and was not significantly different from the control groups on day seven of culture (Duncan Multiple Range Test grouping, $p=0.05$). These results speculate that the inhibitory effect of 25 ng/ml TNF-α on the development of bovine embryos *in vitro*

to the blastocyst stage operates through the increase of PGF-$_{2\alpha}$ and this effect can be reversed by the addition of a prostaglandin synthesis inhibitor, indomethacin.

Conclusion

In summary, IVF produced bovine embryos treated with 25 ng/ml TNF-α had a significantly decreased percentage of embryos developing to the blastocyst stage on day seven (168hpi) of culture ($p<0.005$) and there was a trend for inhibited development to the blastocyst stage at day nine (216hpi) of development ($p<0.08$). The percent of embryos developing to the blastocyst stage within the treatment group of 25 ng/ml TNF-α plus 1 μg/ml indomethacin was increased compared to the 25 ng/ml TNF-α alone treatment group and was not significantly different from the percent of blastocysts in the control embryos at day seven of culture. Indomethacin, a prostaglandin synthesis inhibitor, seems to decrease the inhibition of bovine embryo development caused by 25 ng/ml TNF-α by blocking synthesis of $PGF_{2\alpha}$ from arachidonic acid. Based on this information, a general model can be deduced: mastitis or infection can lead to an increase in TNF-α in circulation, which can bind to its receptors within the endometrial tissue causing an increase in $PGF_{2\alpha}$ (Figure 5) (Okuda, et. al 2002). The increase in $PGF_{2\alpha}$ can cause direct effects on oocyte maturation (Soto, et. al. 2003) or premature luteolysis resulting in decreased concentrations of progesterone and embryo resorbtion.

Research in this area is still needed to determine if different concentrations of TNF-α would produce the same developmental effect on IVF produced bovine embryos. A larger number of embryos per treatment group as well as pairing each well of treated embryos with one well of control embryos should be used. Indomethacin also needs to be investigated further. The addition of indomethacin alone during oocyte maturation and after fertilization as well as the addition of various concentrations of TNF-α to the maturation and developmental IVF culture mediums within this design should be pursued. Another

prostaglandin synthesis inhibitor, specifically a phospholipase-A_2 inhibitor, should also be investigated to determine if the production of $PGF_{2\alpha}$ from the embryo is regulated by earlier steps in its biosynthetic pathway.

Possible *in vivo* studies could examine the effects of prostaglandin synthesis inhibitors on embryo survival and premature luteolysis in cows with naturally occurring clinical mastitis or LPS induced mastitis if the blood and milk sample from these animals display an increase in the concentrations of TNF-α or other inflammatory cytokines.

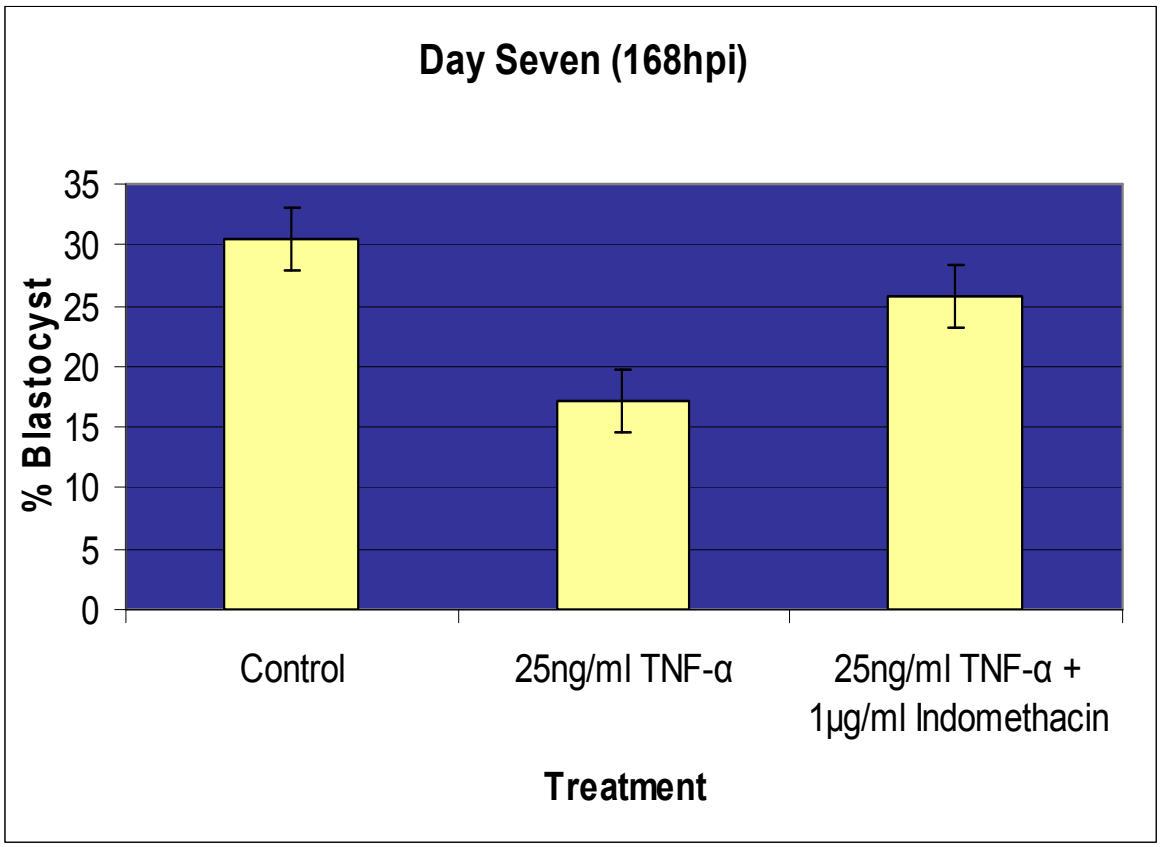

Figure 1: Effects of 25ng/ml TNF-α and 25ng/ml TNF-α plus 1µg/ml indomethacin added after fertilization on bovine embryo development to the blastocyst stage on day seven of culture. Results are least squares means ± SEM. (Control= 30.45 ± 2.36, TNF-α= 17.15 ± 2.36, TNF-α + 1µg/ml indomethacin=25.75 ± 2.89 $p<0.005$).

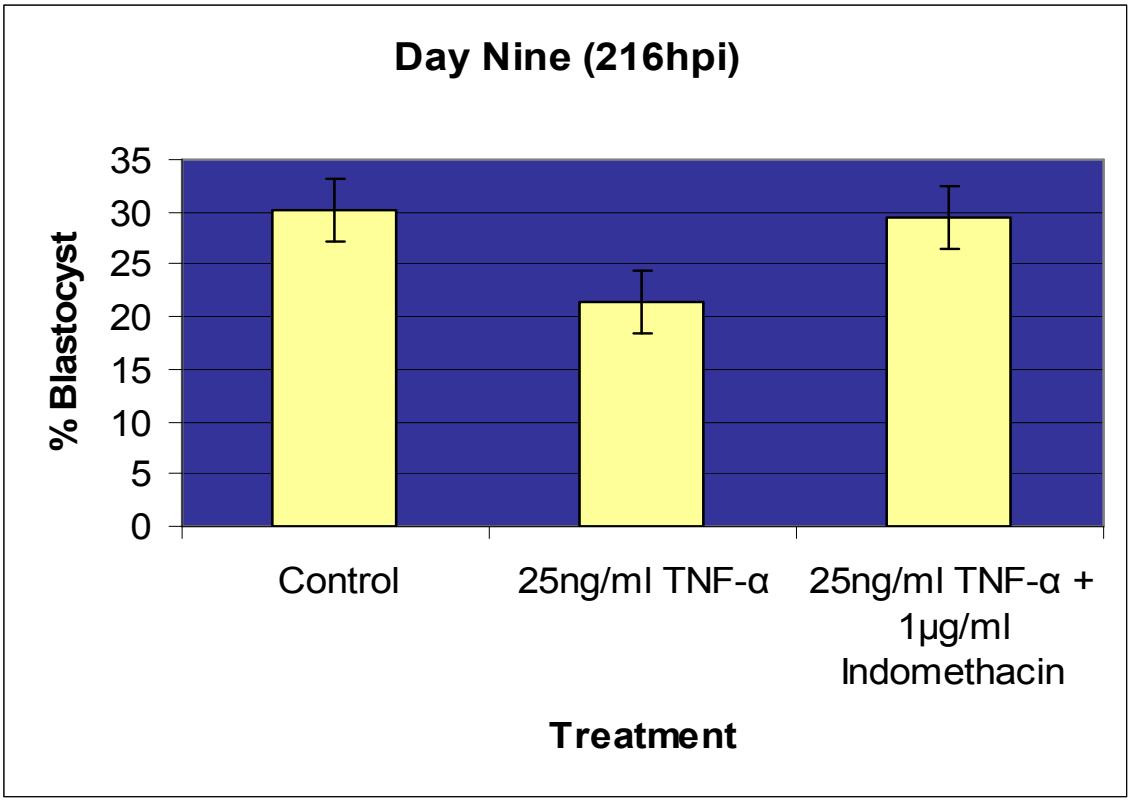

Figure 2: Effects of 25ng/ml TNF-α and 25ng/ml TNF-α plus 1µg/ml indomethacin added after fertilization on bovine embryo development to the blastocyst stage on day nine of culture. Results are least squares means ± SEM. (Control= 30.21 ± 2.72, TNF-α= 21.46 ± 2.72, TNF-α + 1µg/ml indomethacin=29.45 ± 3.33 $p<0.08$).

Table 1: Embryo classification between treatment groups on day seven of culture. Embryo numbers are percentages of embryos within each classification group from the last four trials. EB=early blastocyst, MB=medium blastocyst, ExB=expanded blastocyst, HB=hatched blastocyst.

Day 7	EB	MB	ExB	HB
Control	64.29	26.19	9.52	0.00
25ng/ml TNF-α	68.75	31.25	0.00	0.00
25ng/ml TNF-α + 1μg/ml indomethacin	40.32	43.55	16.13	0.00

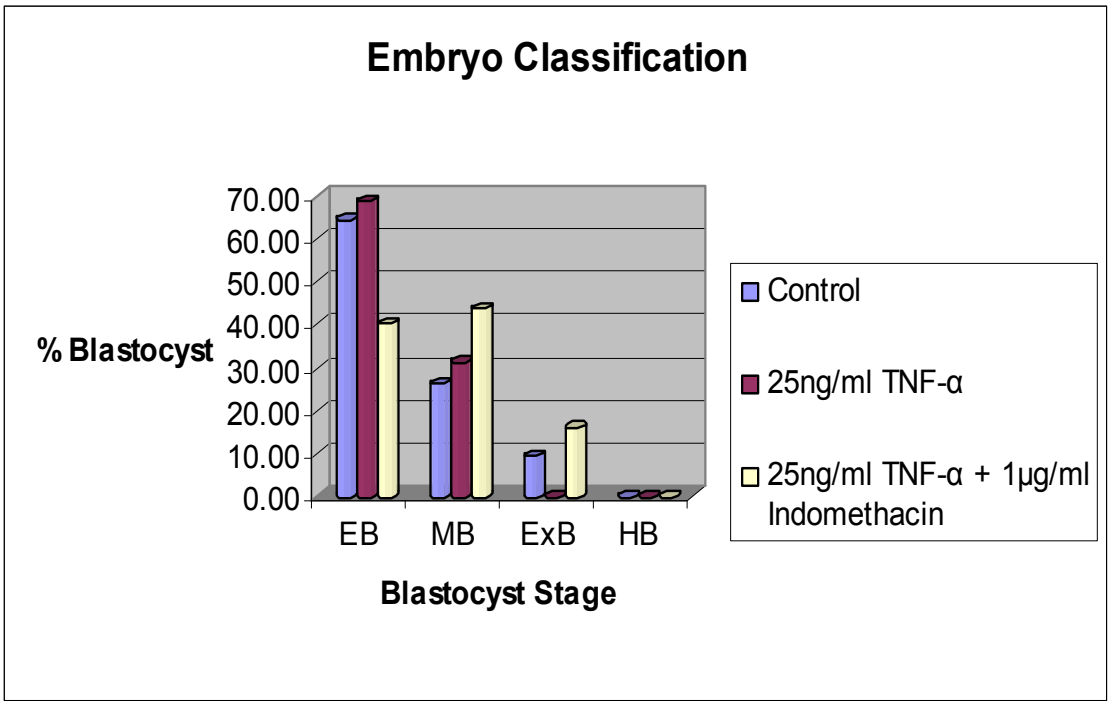

Figure 3: Embryo classification between treatment groups on day seven of culture. Embryo numbers are percentages of embryos within each classification group from the last four trials. EB=early blastocyst, MB=medium blastocyst, ExB=expanded blastocyst, HB=hatched blastocyst. (Graphical depiction of Table 1).

Table 2: Embryo classification between treatment groups on day nine of culture. Embryo numbers are percentages of embryos within each classification group from the last four trials. EB=early blastocyst, MB=medium blastocyst, ExB=expanded blastocyst, HB=hatched blastocyst.

Day 9	EB	MB	ExB	HB
Control	56.76	16.22	27.03	0.00
25ng/ml TNF-α	51.28	30.77	17.95	0.00
25ng/ml TNF-α + 1μg/ml indomethacin	20.29	23.19	56.52	0.00

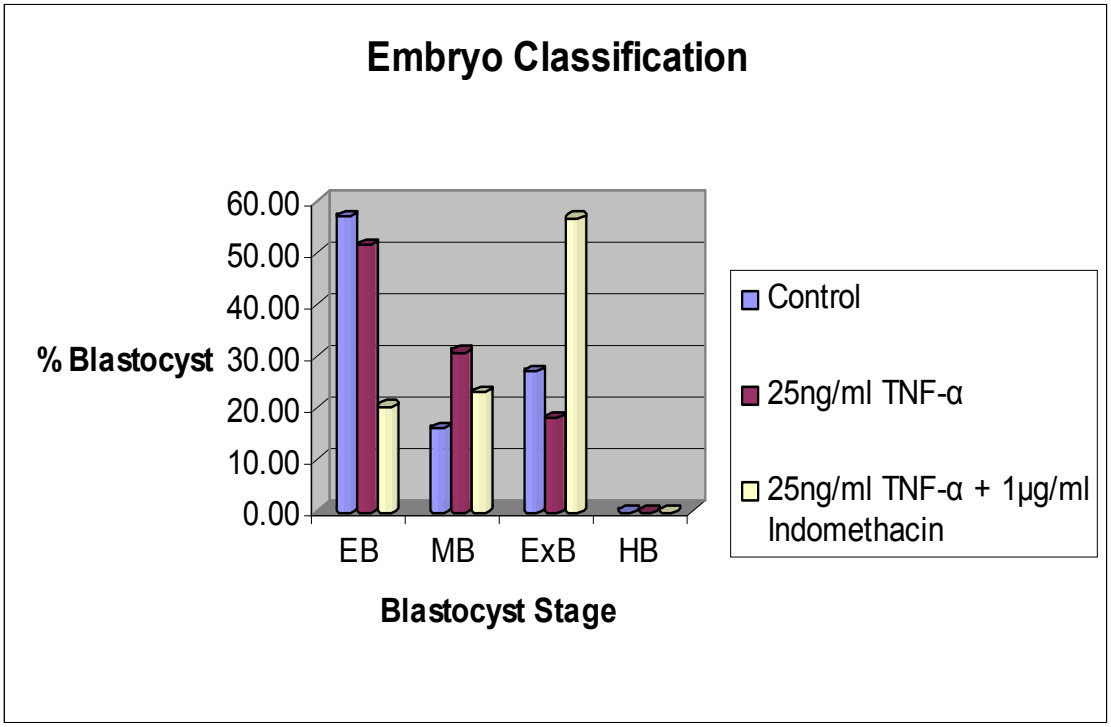

Figure 4: Embryo classification between treatment groups on day nine of culture. Embryo numbers are percentages of embryos within each classification group from the last four trials. EB=early blastocyst, MB=medium blastocyst, ExB=expanded blastocyst, HB=hatched blastocyst. (Graphical depiction of Table 2).

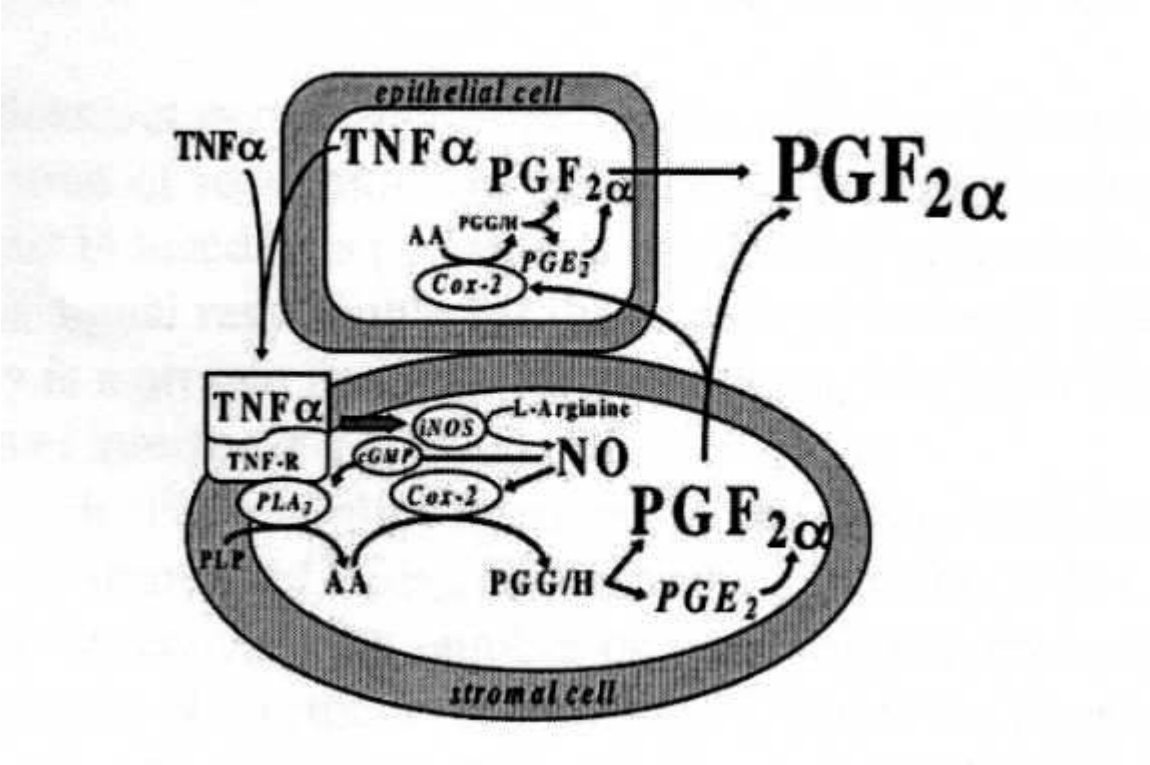

Figure 5: Hypothetical model of TNF-α induced production of PGF-$_{2\alpha}$ from bovine endometrial cells. TNF-α produced by endometrial cells or by uterine macrophages causes prostaglandin production from arachidonic acid within the stromal cells by activating phospholipase-A_2 and COX-2 (Okuda, et. al. 2002).

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Appendices

A. Media Base Stocks

I. TL-Hepes Base	For 500ml
NaCl	3.3313g
KCl	0.1193g
$NaHCO_3$	1.0500g
NaH_2PO_4	0.0204g
Na Lactate	0.4310ml
$CaCl_2$	0.1110g
$MgCl_2$ $6H_2O$	0.0509g
Hepes	1.1916g

Use ¾ volume of distilled dionized (d/d) water

Mix $CaCl_2$ in 10ml d/d water separately

Mix $MgCl_2$ $6H_2O$ in 10ml d/d water separately

Bring to volume and filter

Store at 4°C, good for two years

II. M-199	For 1 Liter
M-199	1 packet (Gibco-BRL, Invitrogen)
L-glutamine	100mg
$NaHCO_3$	29.3ml

Bring M-199 to 800ml, then add other reagents

Bring to 1 Liter

pH to 7.3, then filter

Store at 4°C, good for two weeks.

III. Sperm-TL Base	For 250ml
NaCl	1.4611g
KCl	0.0578g
$NaHCO_3$	0.5250g
NaH_2PO_4	0.0087g
Na Lactate	0.2155ml
$CaCl_2$	0.0555g
$MgCl_2$ $6H_2O$	0.0254g
Hepes	0.5958g

Use ¾ volume of d/d water

Mix $CaCl_2$ in 10ml d/d water separately

Mix $MgCl_2$ $6H_2O$ in 10ml d/d water separately

Bring to volume and filter

Store at 4°C, good for two years

IV. Fertilization Media Base	For 250ml
NaCl	1.6656g
KCl	0.0597g
$NaHCO_3$	0.4499g
NaH_2PO_4	0.0102g
Na Lactate	0.2155ml
$CaCl_2$	0.0556g
$MgCl_2$ $6H_2O$	0.0210g

Use ¾ volume of d/d water

Mix $CaCl_2$ in 10ml d/d water separately

Mix $MgCl_2$ $6H_2O$ in 10ml d/d water separately

Bring to volume and filter

Store at 4°C, good for two years

B. IVF Media

I. TL-Hepes Wash	For 125ml
TL-Hepes Base Stock	125ml
BSA Fraction V	0.375g
Pyruvate	0.00275g
Gentamicin	125µl

Mix and adjust pH to 7.2

Filter and store at 4°C

II. Maturation Media	For 10ml
M-199	9ml
FBS (heat inactivated)	1ml
LH stock	100µl
FSH stock	50µl
Gentamicin	10µl
Estradiol stock	10µl
Pyruvate	100µl

Mix under laminar flow hood and filter with 0.22µm Acrodisk®.

III. Fertilization Media	For 10ml
Fertilization Base	10ml
BSA Fraction V	0.06g
Pyruvate	100µl
Heparin	20µl

Mix under laminar flow hood and filter with 0.22µm Acrodisk®.

IV. Sperm-TL	For 30ml
Sperm-TL Base	30ml
BSA Fraction V	0.18g
Gentamicin	30µl
Pyruvate	0.003g

Adjust pH to 7.1, and then filter with 0.22µm Acrodisk®.

V. Development Media	For 10ml
M-199	9ml
FBS (heat inactivated)	1ml
Gentamicin	10µl

Add treatments, and then filter with 0.22µm Acrodisk®.

C. SAS Programs

I. Raw data

```
data TNFD7indobs;
input date obs TRT D7Blpcnt D9Blpcnt  ;
cards;

add data

;

proc print;
proc glm;
class trt;
model D7Blpcnt d9Blpcnt= trt;
lsmeans trt /stderr;
means trt /duncan ;

run;

quit;
```

II. ArcSin data

```
data TNFD7 D9obs;
input date TRT D7Blpcnt D9Blpcnt  ;
D7Blpcnttran=arsin(sqrt(D7Blpcnt/100));
D9Blpcnttran=arsin(sqrt(D9Blpcnt/100));
cards;
add data;

proc print;
proc glm;
class trt;
model D7Blpcnt D7Blpcnttran D9Blpcnt D9Blpcnttran= trt;
lsmeans trt /stderr;
means trt /duncan ;

run;

quit;
```