Investigating the Supplementation of IL-6, IL-11, & LIF During *In Vitro* Maturation to Improve Oocyte Competency

Endya McKinley

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Alan D. Ealy
Michelle L. Rhoads
Fernando H. Biase
J. Lannett Edwards

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Scientific Abstract:

Bovine embryos produced *in vitro* consistently display decreased quality in terms of their potential to reach the blastocyst stage as well as post-transfer survival. Media formulations, oocyte quality, and inferior expression of needed transcripts are all causes of this reduced developmental potential commonly present in *in vitro*-produced (IVP) bovine embryos. Recently our lab has confirmed interleukin-6 as an embryokine whose capabilities include increasing inner cell mass (ICM) numbers and promoting bovine blastocyst development. LIF is another family member of the IL6 cytokine family and has been shown to produce several positive effects when supplemented during oocyte *in vitro* maturation. IL6 has predominantly been studied as being supplemented post-fertilization. However, published transcriptomic work described receptors for IL6, IL11, and LIF as present in cumulus cells at the time COCs are removed from their follicles. Consequently, we wanted to investigate if supplementing 25 ng/ml of IL6, IL11, or LIF would improve IVM bovine oocyte competency. Several experiments were completed (≥4replicates/experiment; 30-60 cumulus-oocyte complexes (COCs)/replicate). In Experiment 1, COCs were *in vitro* matured for 16 or 22 hours, then meiotic stage was assessed after denuding, fixation, and DNA staining. No cytokine treatment influenced the percentage of oocytes that achieved metaphase II at either time point. In Experiment 2, COCs were *in vitro* matured for 4 hours before snap freezing. and processing to examine changes in five cumulus-expressing transcripts associated with oocyte competency (*CX43, CX37, AREG, TNFAIP6, HAS2)*. Our chosen housekeeping gene, *HPRT1*, served as the internal control. An increased abundance of *AREG* occurred following exposure to LIF but not with the other treatments. Supplementation with IL6 and IL11 but not LIF tended to increase *TNFAIP6* abundance ($P<0.10$). No other transcript differences were detected. In Experiment 3, we examined whether supplementing these cytokines during IVM affects subsequent fertilization and blastocyst rates. No effects were detected on cleavage rates but at day 8 increases in blastocyst yield were detected for LIF and IL11, but not IL6. LIF showed a tendency to increase hatching rates. In Experiment 4, we aimed to assess how the cytokine treatments affected cryosurvivability. Blastocysts (5-10/replicate, 9 replicate studies) were frozen at a rate of -0.6°C/min until reaching -32°C, then were stored in liquid nitrogen for 4-8 weeks before being thawed and incubated in conventional embryo culture medium (SOF-BE1) for 3 days. No treatment effects were noted for re-expansion, hatching, and overall survivability. In summary, these results implicate IL11 and LIF as potential mediators of oocyte competency. However, the evidence presented here suggest that IL6 and IL11 may function differently than LIF when provided during COC maturation.

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General Audience Abstract

The numerous similarities in the regulation of early embryonic development between human and cow has made bovine embryos an excellent model for exploring how to optimize assisted reproductive techniques (ARTs) and other methods for improving and preserving fertility in humans. Pregnancy loss is also very similar in both cattle and humans. In beef cattle, more than 50 percent of reproductive failures occur before day 16 of gestation. In women, approximately 15 percent of all clinically recognized pregnancies result in spontaneous loss, however, several more pregnancies fail prior to ever being clinically recognized. Various ARTs are used to treat sub-fertile conditions in cattle, and these technologies are generally deemed as a viable way to improve fertility. However, IVP embryos are inferior in their ability to properly fertilize and develop to the blastocyst stage, the stage when embryos are normally transferred. Furthermore, IVP-generated embryos are inferior at maintaining pregnancies. There are two primary restraints to the IVP process: a low percentage of oocytes that become fertilized and produce transferable embryos and transferred IVP embryos have decreased chances of maintaining a viable embryo than embryos produced *in vivo.*

Very little is known about the various hormone and molecular factors that promote oocyte and embryo development. Therefore, a primary objective for bovine oocyte and embryo research is to classify these factors and implement them into their maturation and culture media to improve overall IVP efficiency. My lab studies members of the IL6 cytokine family as potential factors that might play a role in the development of oocytes and embryos. The aim of this work is to assess the capacity of three molecules within this family, IL6, IL11, and Leukemia inhibitory factor (LIF) to improve oocyte development, fertilization rate, and blastocyst yield when supplemented during *in vitro* maturation (IVM).

This work revealed that both IL11 and LIF improved IVP bovine blastocyst development at day 8. Unfortunately, none of the treatments had any effect on fertilization rates. LIF increased the expression of a cumulus-specific transcript known to aid in cumulus expansion. Cumulus cells are the somatic cells immediately surrounding the oocyte. Cumulus expansion is a key indicator of proper oocyte maturation. We did not observe any treatment effect on post-thaw survival of cryopreserved bovine embryos. This indicates that our treatments did not help the embryos maintain viability after undergoing a slow-freeze cryopreservation protocol followed by thawing and culture.

In summary, this work showed that IL11 and LIF have potential benefits to the *in vitro* production of bovine embryos when supplemented at IVM. However, future work is needed to assess how these molecules are causing these improvements. Our results indicate that IL11 and LIF may function differently from IL6 when supplemented during IVM.

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ART Assisted Reproduction Technologies
AREG Amphiregulin
CAMKII Calmodulin-dependent protein kinase II
cAMP Cyclic adenosine monophosphate
Cdx2 Caudal-related homeobox 2
CL Corpus luteum
COC Cumulus-oocyte complex
DAG Diacylglycerol
DNA Deoxyribonucleic acid
EGA Embryonic genome activation
ERK Extracellular receptor kinase
FGF Fibroblast growth factors
FGFR Fibroblast growth factor receptor
EPL Early pregnancy loss
GnRH Gonadotropin-releasing hormone
Grb2 Growth factor receptor-bound protein 2
GV Germinal vesicle
ICM Inner cell mass
IL6 Interleukin-6
IL11 Interleukin-11
IP3 Inositol triphosphate
IVP In vitro embryo production
JAK Janus kinase
LH Luteinizing hormone
LIF Leukemia inhibitory factor
MAPK Mitogen-activated protein kinase
MPF M-phase promoting factor
mRNA Messenger ribonucleic acid
MII Metaphase II
OCT Ornithine carbamoyltransferase
Oct4 Octamer-4
PAG Pregnancy-associated glycoproteins
PrE Primitive endoderm

PI3K Phosphatidylinositol 3-kinase
PIP2 Phosphatidylinositol 4,5-bisphosphate PKC
PLCζ Phospholipase C zeta
STAT Signal transducer and activator of transcription
TE Trophectoderm
Tead4 TEA domain transcription factor 4
TET Ten-eleven translocation methylcytosine dioxygenases
TGF-β Transforming growth factor-beta
Yap1 Yes associated protein

Chapter 1–Literature Review

Introduction

Assisted reproductive technologies (ART) comprise all fertility treatments involving oocytes, sperm, or embryos. In cattle, artificial insemination (AI), embryo transfer (ET), and *in vitro* production (IVP) are the primary methods used to increase genetic gain of economically important phenotypes (Boerjan et al., 2000). In the human, *in vitro* fertilization (IVF), Gamete Intrafallopian Tube Transfer (GIFT), and Intra Cytoplasmic Sperm Injection (ICSI) are utilized to assist sub-fertile couples with having children (Boerjan et al., 2000). My review will focus primarily on cattle, but I also will mention various facets of ART in humans because of my personal interest with ART in humans and because women and cows have various common reproductive characteristics. Both species are mono-ovulatory, allowing for the selection of one dominant follicle that will ultimately ovulate (Sirard, 2017). Additionally, the estrous cycle of the cow persists for approximately 21 days, while the human menstrual cycle is moderately longer averaging around 28 days (Sirard, 2017). Also, cow and humans share very similar gestation lengths. Cows gestate around 283 days while a female human's gestation period lasts approximately 280 days. Pregnancy loss in both cattle and human is very prominent within the first 30 days of gestation. In beef cattle, more than 50 percent of reproductive failures occur before day 16 of gestation (Reese et al., 2020). Early pregnancy loss (EPL) in human females is defined as a spontaneous pregnancy loss occurring prior to 20 to 24 weeks of gestation (Ford and Schust, 2009). Around 15 percent of all clinically recognized pregnancies result in spontaneous loss, however, several more pregnancies fail prior to ever being clinically recognized (Ford and Schust, 2009). In women, a mere 30% of all natural conceptions will lead to live birth (Ford and Schust, 2009).

Various ART procedures are similar between humans and cattle. These similarities make the cow embryo an excellent model for ART in the human. IVF procedures in both human and cow are nearly identical. Additionally, both cow and human share similar optimal media compositions for embryo culture (Duranthon and Chavatte-Palmer, 2018). Utilizing bovine embryos as a model to assess the cause of reduced developmental potential of human embryos resulting from in vitro production (IVP) and unassisted means is needed, given the increasing legal pressures scientists face using human embryos for research.

In humans, there are various benefits in utilizing ART. For example, ICSI allows for all the natural obstructions of fertilization to be bypassed. This form of ART is typically used when there is a sperm-related infertility issue. This typically means the male has a semen ejaculate sample with insufficient concentration of sperm. The primary benefit of ICSI is its enhanced fertilization rate allowing for increased numbers of fertilized eggs to freeze or transfer. However, several disadvantages of ART exist in humans. Perhaps most notably pertains to IVF in females and their potential for multiple pregnancies. This is becoming less of an issue now that the industry has mastered embryo cryopreservation techniques so that multiple rounds of single embryo transfer can occur between superstimulation and IVF cycles. Another limitation pertains to low egg retrieval rates in some women. To rectify this issue, some physicians might recommend advancing hormonal stimulation to increase the potential number of eggs retrieved.

In cattle, AI was the first ART used. While this method is still heavily used, the power of genetic selection lies mainly on males when utilizing this method. Oocyte and embryo-based technologies allow us a genetic selection from both cow and bull. Superovulation is also a common ART used in cattle. The objective of superovulation in embryo transfer programs is to obtain maximum numbers of transferable embryos with an increased probability of pregnancy. However, this technique is being surpassed and replaced by ovum pick-up (OPU) and IVF. This is because 5-10 times more transferable embryos can be generated using OPU-IVP instead of by superovulating cattle. Indeed, IVF has become a common ART in cattle. IVF allows producers to efficiently maximize their herd's genetics. There is a shorter interval between procedures, producing more calves in a shorter period. IVF can be utilized to produce offspring from genetically superior animals that are non-productive due to physiological problems. These technologies improve fertility in the cow, however, the best environment for developing bovine embryos is within the uterus of the cow. IVP bovine embryos are not as healthy as ones produced *in vivo*, resulting in decreased pregnancy rates and compromised freezability.

Various ARTs are used to treat sub-fertile conditions in cattle, and these technologies are generally deemed as a viable way to improve fertility. For example, during warmer months, heat-stressed cows can benefit from ET if they were struggling to naturally conceive. Embryos used for ET are better able to withstand the stresses associated with elevated ambient temperature than younger embryos. Unfortunately, IVP embryos are inferior in their ability to properly fertilize and develop to the blastocyst stage, the stage when embryos are normally transferred. Additionally,

IVP-generated embryos are inferior at maintaining pregnancies. There appear to be several reasons for this problem, and there certainly is a critical need to improve the understanding of developmental processes happening in the embryo during early gestation so that adjustments can be made to improve the efficiency of IVP. We know there are two main limitations to the IVP process. There is a low percentage of oocytes that become fertilized and produce transferable embryos (Wooldridge et al., 2022). Additionally, transferred IVP embryos have decreased chances of maintaining a viable embryo than embryos produced *in vivo.* Over one million IVP bovine embryos were produced across the globe in 2019 (Viana, 2018). However, only 20% to 40% of oocytes will yield transferable embryos (Ealy et al., 2019; Hansen, 2020). Due to the increasing utilization of ART, there is grave need to improve the efficiency of IVP.

To sum things up thus far, there is a dire need to better understand developmental processes and the biology of the oocyte, sperm, and embryo so that intervening steps can be made to promote *in vitro* embryo production efficiency and encourage post-ET pregnancy retention and neonatal health. This literature review will delve into this discussion by beginning with a discussion of key events in oocyte maturation, fertilization, and preimplantation embryo development. I then will describe key bioactive factors normally found in the follicle that assist with oocyte maturation. These factors are termed "ookines" herein. Cytokines serving as ookines will be the primary focus of this discussion point as these ookines are the primary focus of my MS research efforts.

Key Events in Preimplantation Development:

Oocyte Maturation

Oocyte maturation entails cytoplasmic and nuclear changes that prepare the oocyte for fertilization and the early stages of embryonic development. Nuclear maturation involves preparing female chromatin to couple with the male chromatin at the time of fertilization. The first key event in this process is progression to metaphase II. Bovine oocytes are arrested in prophase I of meiosis in both preantral and large antral follicles from birth, although each follicle type has its own mechanism by which they maintain this arrest (Eppig, 1996). Oocytes within antral follicles are competent to spontaneously resume meiosis after being excised from their follicles, however, preantral follicles do not share this competency (Edwards, 1965). Oocytes from the antral follicles maintain meiotic arrest via factors from surrounding follicular somatic cells, while oocytes from

preantral follicles arrest at prophase by independent regulatory mechanisms (Eppig, 1996). This causes oocytes of preantral follicles to be incompetent to resume meiosis. Prophase I is also known as the germinal vesicle stage, and the breakdown of the germinal vesicle is required for completing nuclear maturation. The first step in achieving competence to complete nuclear maturation is oocytes progressing from the meiotic arrest at the dictyate stage of prophase I (Eppig, 1996). Mammalian oocytes reach this stage before or at birth and remain arrested until germinal vesicle breakdown (GVBD). The second step includes oocytes achieving competence to advance past metaphase I, entering anaphase and later metaphase II (Eppig, 1996).

Signaling factors from surrounding somatic cells will regulate the attainment of meiotic resumption. Cyclic AMP (cAMP) is a signaling factor found in follicular cells and oocytes (Guixue et al., 2001). High levels of cAMP in the ooplasm have downstream consequences that maintain a meiotic block (Larose et al., 2019). This is achieved by cAMP acting through signaling molecules to maintain elevated levels of Cyclin B, a key element of a two-part protein complex that is commonly referred to as maturation-promoting factor (MPF) (Norbury and Nurse, 1992). Specifically, elevated cytoplasmic levels of cAMP impede MPF activation through protein kinase A and the phosphorylation of CDK1, making it inactive (Larose et al., 2019). CDKs require the binding of a cyclin subunit and limited levels of CDK1 and/or the cyclin will prevent the kinase activity (Holt et al., 2013). Cyclin B is the primary binding unit of CDK1 during meiosis, both proteins develop a greater capacity to associate with each other as the oocyte matures (Holt et al., 2013). Limited CDK1 and Cyclin B play an essential role in maintaining the germinal vesicle (GV) arrest of larger mammalian species (Holt et al., 2013). The prophase arrest is also maintained via cyclic guanosine monophosphate (cGMP) produced from granulosa cells that immediately surround the oocyte (Mehlmann, 2005; Jaffe and Egbert, 2017). These cells are referred to as cumulus cells. cGMP diffuses into the oocytes where they block phosphodiesterase 3 (PDE3), the enzyme that breaks down cAMP (Sun et al., 2009).

Nuclear maturation is initiated with the luteinizing hormone (LH) surge. LH is produced by gonadotropin cells in the anterior pituitary gland. LH production is regulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. In females, a drastic rise in LH, or the LH surge, stimulates ovulation. One of the primary actions of LH is to interrupt cumulus cell and oocyte communication so that meiosis can resume. This cell-to-cell communication is mediated by Connexin 43 (Cx43) channels that create gap junctions between granulosa and cumulus cells

as well as between cumulus cells and the oocyte (Granot and Dekel, 2002). Increased LH concentrations have an inhibitory effect on Cx43 synthesis in large preovulatory follicles (Granot and Dekel, 2002). LH signals via the cAMP-dependent PKA pathway. Upon binding to its receptor, LH interrupts cell-to-cell communication via phosphorylation and conformational altering of the Cx43 protein—leading to closure of the channels (Granot and Dekel, 2002). cGMP levels in the somatic cell compartment of the follicle decreases in response to (LH). This action prevents cGMP transfer from the cumulus cell to the oocyte, and this decrease in oocyte cGMP permits PDE3 to be active and degrade cAMP, thereby reducing the cAMP blockage of MPF (Larose et al., 2019). Interestingly, the LH receptor is a G-protein-coupled receptor that activates adenylyl cyclase, leading to increases in intracellular cAMP concentrations within granulosa cells (Segaloff and Ascoli, 1993; Davis, 1994). This is intriguing because an increase in cAMP within granulosa and cumulus cells is needed to drive down cAMP in the oocyte. Epidermal growth factor receptor (EGFR) activation is another crucial component in triggering LH-induced meiotic resumption (Norris et al., 2010). The rapid release of EGFR ligands epiregulin and amphiregulin from granulosa and cumulus cells is an important catalyst of LH receptor activation (Norris et al., 2010). The EGFR kinase-dependent components rapidly drive down cGMP concentrations in these cells (Norris et al., 2010). Additionally, there is data showing the LH receptor is coupled to phospholipase C-β and this factor could signal resumption of meiosis by causing an increase in intracellular Ca^{2+} (Davis, 1994). Increased intracellular calcium catalyzes the release of LH and FSH—leading to meiotic resumption.

While nuclear maturation primarily involves chromosomal segregation, cytoplasmic maturation is a second important facet of the oocyte maturation process. Cytoplasmic maturation involves organelle reorganization within the oocyte along with the storage of mRNAs, proteins, and transcription factors that contribute to maturation, embryogenesis, and fertilization (Ferreira et al., 2009). The proteins and transcripts stored in the cytoplasm of the oocyte are essential for maturation and for the progression of the early embryo to the eight-cell stage in cattle—when the embryonic genome is activated (Ferreira et al., 2009). The reorganization of mitochondria to areas of high energy consumption is essential for the oocytes and the embryo blastomere during critical points in the cell cycle (Ferreira et al., 2009). The oocyte mitochondria move from a peripheral position to a more dispersed distribution throughout the cytoplasm around 12-18 hours into maturation (Hyttel et al., 1986). After reaching metaphase II (MII), the mitochondria along with

lipid droplets maintain a central position in the cell—primed to metabolize stored triglycerides providing the zygote with energy until EGA occurs (Hyttel et al., 1986). Another organelle that is reorganized are the cortical granules. Cortical granules are derived from the Golgi complex. Oocytes in the GV stage have distributed clusters of cortical granules throughout the cytoplasm (Wang et al., 1997). Once oocytes reach the MII stage, the granules are distributed close to the plasma membrane—waiting to be released after fertilization and oocyte activation to prevent polyspermy (Conner et al., 1997).

One last important concept we need to discuss before proceeding is to mention that oocytes undergo a second meiotic arrest. This arrest occurs at metaphase II, and this second meiotic arrest is maintained by elevated levels of cyclin-dependent protein kinase 1 (CDK1) activity in the oocyte (Larose et al., 2019). Fertilization is the signal that re-starts the meiotic process. More detail about this process is provided next.

Fertilization:

Fertilization is the process by which eggs and spermatozoa fuse so that their haploid chromosomes may combine and generate a new diploid organism. Ovulation occurs 24-30 hours after the LH surge in cattle, and the infundibulum of the oviduct will capture the ovulated cumulus-oocyte complex (COC) and direct it into the oviduct. Fertilization occurs soon thereafter at the ampullary-isthmic junction of the oviduct. The mammalian oviduct allows for an environment suitable for storage and capacitation, sperm transport, oocyte pickup, fertilization, and eventually early embryo cleavage (Rodríguez-Martínez, 2003). Before fertilization can occur, spermatozoa need to obtain competency for successful fertilization. Sperm capacitation is defined as the events leading up to the mammalian spermatozoa gaining the developmental capacity to penetrate the oocyte (Chang, 1984). It's a multiplex physiological process involving biophysical, biochemical, and metabolic modifications of sperm (Harrison, 1996). Capacitation leads to altered plasma membrane architecture and permeability, regulation of flagella activity, the removal of the glycoprotein coat, and shedding of certain surface proteins. This allows for recognition and binding to the zona pellucida of the oocyte (Rodriguez-Martinez, 2007).

The first step in fertilization requires that a capacitated spermatozoa traverse through cumulus cells and contact the zona pellucida. The binding of the spermatozoon to the oocyte's zona pellucida happens via distinct receptors concentrated over the head region of the

spermatozoon. These receptors are a zona pellucida glycoprotein, termed zona protein 3 (ZP3). Spermatozoon binding to the zona pellucida initiates the acrosomal reaction, where the release of various enzymes from the sperm head degrades the region of the zona pellucida in close contact with the sperm head (Bedford, 2011). Sperm-egg fusion is defined as a cell-to-cell membrane fusion event between sperm and oocyte that is critical for the advancement of sexually reproducing beings and results in a single diploid cell or zygote (Stein et al., 2004). This process involves fusion between the outer acrosome and overlying cell membrane (Bedford, 2011). The reaction leads to the acrosome releasing soluble lysins which in turn promotes the modeling of a path in the zona pellucida. Additionally, the acrosome reaction allows exposure of the inner acrosomal membrane to the outside by breaking the organelle (Kaji and Kudo, 2004). IZUMO1 is the singular sperm protein required for sperm-egg fusion (Satouh and Ikawa, 2018). The oocyte receptor for IZUMO1 is termed JUNO (Satouh and Ikawa, 2018). There is a rapid loss of JUNO on the oocyte membrane right after fusion. This gave rise to the notion that JUNO is critical for the polyspermy block in mammals. Both IZUMO1 and JUNO are conserved in eutherian mammals. Additionally, Fertilin β (ADAM2) participates in sperm-egg membrane binding, and integrin ligands and cellular disintegrins will interact with one another on the oocyte and sperm (Evans, 2001). Once the coupling of IZUMO1 and JUNO is complete, stimulation of fused sperm prompts the egg to sustain changes in egg cytosolic Ca^{2+} concentrations (Satouh and Ikawa, 2018). Also, changes in global degradation of maternal mRNAs and proteins, changes in protein phosphorylation, and new protein synthesis begin (Satouh and Ikawa, 2018). These processes are broadly termed oocyte activation.

After fusion, an exocytotic event occurs in the oocyte, where contents of the cortical granules are released into the extracellular environment (Stein et al., 2004). Within these granules are enzymes that modify ZP3 proteins in ways that prevent further sperm binding and penetration (Stein et al., 2004). This exocytosis event is triggered by the oscillating pulsation of cytosolic Ca^{2+} (Stricker, 1999). This begins with the sperm for most species, where the sperm releases phospholipase C zeta (PLCζ) into the cytoplasm, and this causes PIP_2 to cleave into DAG and IP_3- the latter of which triggers intracellular Ca release from the endoplasmic reticulum (Saunders et al., 2002). DAG activates protein kinase C (PKC) leading to exocytosis of the cortical granules into the perivitelline space (Sun, 2003).

A second critical action of sperm-egg fusion and the resulting calcium oscillations is to reactivate the meiotic cycle and so that meiosis can be completed (Sanders and Swann, 2016). Prior to the Ca^{2+} pulsing, this second meiotic arrest is maintained by high levels of MPF (Sanders and Swann, 2016). The Ca^{2+} surges activate calmodulin-dependent protein kinase II (CAMKII), and this leads to the loss in MPF activity so that meiosis can be completed (Markoulaki et al., 2004). The completion of meiosis and entry into interphase of the first mitotic division also relies on the decline in cytoplasmic MAPK activity, and this event is triggered by the decline in MPF (Moos et al., 1996).

Sperm nuclear decondensing activity is regulated via glutathione (Perreault et al., 1988). Glutathione reduces the disulfide bonds of protamines and replaces them with histones (Perreault et al., 1988). Fertilization results in the combining of two specific chromatin sets. Sperm chromatin is highly compacted with protamines instead of histones, while oocyte chromatin is loosely packaged into nucleosomal arrays and arrested in metaphase II of meiosis (Mann and Bartolomei, 2002). The remodeling of the two sets of parental chromatins is primarily coordinated by the levels in oocytes arrested at metaphase II of meiosis (Mann and Bartolomei, 2002).

Female and male pronuclei are formed soon after sperm-egg fusion and completion of meiosis. Pronuclei are the nucleus of sperm or egg cells up until the time of fusion with another gamete in the process of fertilization. Upon entering the ooplasm, the sperm nucleus begins to decondense. Following pronuclei formation, the pronuclei greatly increase in size and move together to come into close contact (Austin and Bishop, 1957). The pronuclei lose their nuclear envelope, and the two haploid genomes become joined in the center of the zygote. The sperm nucleus will undergo nuclear envelope breakdown, the substitution of protamines with histones, and chromatin decondensation and pronuclear formation (Wright, 1999). The oocyte chromatin continues through the second meiotic division and a maternal pronuclei is formed.

At the completion of meiosis in the oocyte and the presence of a male pronucleus that rapidly develops from the engulfed male DNA, an event termed syngamy occurs, where the union of two gametes will form a new haploid organism referred to as a zygote. This organism has a unique DNA portfolio that closely resembles its parental DNA but is distinct thanks to DNA recombination and chromosomal crossing-over events that occur during prophase of meiosis I in both the male and female and again as syngamy occurs.

Early Cleavage Events:

Cleavage is the process that is unique to early-stage embryos because it involves cellular division without growth of the organism. Rather, the cytoplasm is divided equally between the sister cells, so each is approximately half the size of the parent cell. Blastomeres are cells produced by cleavage divisions of the early embryo. The early development of bovine embryos is driven by proteins stored in the oocyte and protein synthesized from stored transcripts (Barnes and Eyestone, 1990). In humans, the timing of first zygotic cleavages varies between 22- and 30 hours post insemination (Lechniak et al., 2008). In bovine, the timing of the first zygotic cleavage varies between 24- and 48-hours post-insemination (Lechniak et al., 2008). Bovine blastomeres will divide approximately once every 24 hours for the next several days, forming embryo stages characterized by blastomere numbers (e.g., 2-cell, 4-cell, 8-cell, 16-cell stages). The upcoming sections will discuss a few key events that occur in these early-stage embryos and in embryos that have progressed to later stages of development.

Epigenetic Changes: preimplantation development

Epigenetics is defined as heritable changes in gene expression that are not dictated by the primary DNA sequence (Shi and Wu, 2009). Epigenetics plays an essential role in the control of gene expression and the maintenance of cell identity (Xu et al., 2021). Precise epigenetic reprogramming is imperative for avoiding developmental deformities during the development of the pre-implantation embryo. These epigenetic modifications include histone modifications, DNA methylation, modifications affecting chromatin accessibility, and 3D chromatin organization (Xu et al., 2021).

In early development, male and female genomes undergo two waves of epigenetic reprogramming. The first wave is soon after fertilization and involves near-complete demethylation of both paternal and maternal genomes—leaving imprinted genes untouched (Larose et al., 2019). The second wave happens later once the embryonic germline is being established (Larose et al., 2019). I will focus solely on the first wave of reprogramming given its relevance to my focus on early embryogenesis. The maternal and paternal genomes are differentially demethylated at this time. The paternal genome experiences rapid and active demethylation while it is still housed in the male pronucleus by the dioxygenase enzyme, TET3 (Oswald et al., 2000). This rapid demethylation appears to occur because most of the paternal genome does not contain the DNA-Binding protein, STELLA, which exists on the maternal genome and the paternally imprinted loci and functions to protect DNA from TET3 demethylation

(Nakamura et al., 2007). STELLA binds demethylated histone H3 at lysine 9, a mark enriched on the maternal genome and at genomic imprinting regions (Nakamura et al., 2012). The maternal genome will undergo a slower demethylation process where TET3 remains inactive in the maternal pronucleus and demethylation does not begin until the pronuclei have joined together, where TET3 is activated and both paternal and maternal epigenomes undergo passive demethylation (Smith et al., 2012). Active demethylation is driven via TET3-mediated 5-methylcytosine (5mC) oxidation (Smith et al., 2012). Passive demethylation is a process where the maintenance methylation is inhibited and the genomes undergo gradual dilution over subsequent cleavage divisions in response to DNA replication (Smith et al., 2012). Passive demethylation is primarily mediated via replication, however, TET3 is also needed (Guo et al., 2014). Active demethylation can occur only in the absence of STELLA, and passive demethylation can occur when STELLA is present (Smith et al., 2012).

Asymmetric histone modifications are observed in the epigenome during early embryonic development. Another facet of the rapid changes made to the paternal genome involve a DNA decondensation event, where protamines are exchanged for maternal histones (Zhou and Dean, 2015). Histones will undergo various modifications, including acetylation, phosphorylation, ubiquitination, and ribosylation (Vastenhouw and Schier, 2012). These modifications adjust protein-DNA and protein-protein interactions and manage the interaction of transcriptional regulators with chromatin (Vastenhouw and Schier, 2012). After protamine removal, the paternal genome is replaced by newly synthesized and acetylated histones (Zhou and Dean, 2015). However, maternal chromatin will maintain its histone methylation pattern throughout early cleavage stages. This promotes epigenetic asymmetry between the paternal and maternal genomes (Zhou and Dean, 2015). I will discuss the process of DNA remethylation later in this review.

Embryonic Genome Activation

Maternal-to-embryonic transition (MET), maternal-to-zygotic transition (MZT), and embryonic genomic activation (EGA) are terms commonly used to describe the stage in embryogenesis when maternal RNAs and proteins stored in the oocytes are gradually degraded and transcription of the embryonic genome begins (Graf et al., 2014). This transition varies in its timing between species (Watson and Barcroft, 2001). MZT occurs at the end of the one-cell stage until the two-cell stage in mice (Vigneault et al., 2004). Bovine MZT occurs at the 8- to 16-cell

stage (Vigneault et al., 2004). In macaques and humans, MZT occurs between the 4- to 8-cell stages (Tesarik, 2022).

EGA starts gradually in all mammals. An initial, minor embryonic transcription event usually precedes the major EGA events (Graf et al., 2014). In bovine, this minor activation occurs at the 4-cell stage (Graf et al., 2014). Activated genes related to regulation of transcription, RNA processing, protein biosynthesis, protein degradation, and protein modification or transport are first detected during minor activation. This prepares the embryo for ensuing major EGA where genes from a broader spectrum of functions are activated (Graf et al., 2014). In bovine, major EGA occurs at the 8- to-16-cell stage (Graf et al., 2014). While certain processes have already been initiated at the four- and eight-cell stages, the functions of the genes activated at the 16-cell stage become diversified and targeted (Graf et al., 2014). These genes can be classified into categories relating to RNA splicing, regulation of glycolysis, ATP biosynthesis process, negative regulation of transcription, protein synthesis, and cell fate decisions (Graf et al., 2014). Each of these have a role in the maintenance of transcription and translation as well as the beginning of various metabolic processes occurring at EGA (Graf et al., 2014).

Alteration in chromatin structure is a primary characteristic of EGA. Chromatin alterations modulate the activity of transcription factors by allowing or restricting their access to regulatory elements of the genome, however, they are not singularly sufficient to activate transcription (Graf et al., 2014). A primary factor governing what genes are available for transcription and when those genes become available is the temporal control of chromatin structure (Latham and Schultz, 2001). A key determining factor of chromatin structure is the type of histones associated with the DNA (Latham and Schultz, 2001). Core histones package the DNA into nucleosomes, these include histones 2A, 2B, 3, and 4 (Latham and Schultz, 2001). Linker histones such as B4 and HMG1 associate with the DNA in between nucleosomes and are responsible for condensing chromatin (Latham and Schultz, 2001). The close connection of DNA with core histones and the linker histones can leave the DNA effectively unusable to transcription factors (Latham and Schultz, 2001). Nucleosome placement along the DNA strand can be altered by other DNA binding proteins, and this placement can consequently affect the ability of other proteins to bind (Latham and Schultz, 2001). Various linker histones intermingle with the chromatin of oocytes, early zygotes, and later somatic cells (Latham and Schultz, 2001). Alterations in linker histones affect chromatin structure because the different linker histones vary in their overall basicity and tightness

of association with the DNA (Latham and Schultz, 2001). Binding of core histones to DNA can be controlled via acetylation and phosphorylation (Latham and Schultz, 2001). However, acetylation of the lysine residues in the histone tails reduces the contact of the DNA with the core histones and allows access to the DNA by other DNA-binding proteins (Latham and Schultz, 2001). During MET, nuclear reprogramming is required to activate the transcriptionally inactive embryonic genome (Graf et al., 2014). Consequently, selection of the initial genes transcribed and the timing of EGA is due to restrictive mechanisms of chromatin and altering chromatin structure to a more open state allowing transcription to occur (Graf et al., 2014).

Formation of the Blastocyst

It becomes difficult to distinguish individual blastomeres from one another at and after the 16-cell stage. The embryo is called a morula when this occurs, and the packing of blastomeres in close proximity to one another is termed blastomere compaction. The timing of this event varies between species. Morula compaction begins approximately 5 days post-ovulation at the 32-cell stage in the cow (Soom et al., 1997). As compaction occurs, two distinct cell populations begin to emerge. These populations are initially termed outer and inner cells, and they eventually will form into an outer layer of trophectoderm (TE) and an inner clump of cells referred to as the inner cell mass (ICM). These are the first cell lineages to differentiate in mammalian embryos.

The embryonic stage that follows the morula stage is termed the blastocyst stage. This stage contains a clear distinction between TE and ICM cells and a prominent fluid-filled cavity, called the blastocoel cavity. This cavity emerges from an action of the TE cells (Frankenberg et al., 2016). This process begins during compaction, where compacting embryos experience an increase in interblastomeric contact that conceals the distinct individual cell boundaries (Watson and Barcroft, 2001). These cells develop Ca2+ dependent adhesion, establish of gap junction mediated interblastomeric cell communication, and begin to form focal tight junctions (Watson and Barcroft, 2001).

The TE adopts standard epithelium morphology to surround a fluid-filled cavity. The trophectoderm gains the capacity to initiate and regulate the events of cavitation by expressing gene products that promote the transport and retention of the blastocoelic fluid (Watson and Barcroft, 2001). The tight junction (or zonal occludens) includes several complex proteins including ZO-1, ZO-2, 7H6, cingulin, occludin, and claudins (Watson and Barcroft, 2001). TE-

specific tight junctions serve two primary functions: they regulate paracellular transport, and they maintain epithelial cell polarity (Watson and Barcroft, 2001). For cavitation to take place, the embryo depends on the development of a polarized epithelium (Watson and Barcroft, 2001). Additionally, cavitation requires the tight junction gene family, the Na/K-ATPase gene family, and the aquaporin gene family (Watson and Barcroft, 2001). The blastocoelic fluid is mostly water, consequently, the means of its production relies on the ion transport properties of the trophectoderm (Biggers et al., 1988).

It has been hypothesized that the trans-trophectoderm Na^+ gradient is completed by the active transport of Na^+ out of the cell into the blastocoelic cavity via a basolaterally localized Na/K-ATPase (Watson and Barcroft, 2001). Immense evidence collected supports this hypothesis due to its confirmation that the NA/K-ATPase is present in the basolateral plasma membrane domain of the trophectoderm for mouse, rabbit, pig, and cow (Watson and Barcroft, 2001). Cavitation is controlled by an ion gradient created by Na/K-ATPase across the trophectoderm, furthering the movement of water through aquaporins across the epithelium to construct the blastocoel in the extracellular space of the blastocyst (Watson and Barcroft, 2001). Aquaporins are a family of small hydrophobic membrane channel proteins that aid in the accelerated passive movement of water (Huang et al., 2006). Aquaporins are present in the trophectoderm membrane and are functional in the early embryo (Barcroft et al., 2003).

Transcriptional Control of ICM and TE Formation

As mentioned previously, the initial cell fate decision during mammalian development is the differentiation of the TE from the ICM. The TE is the first epithelium to arise during mammalian development and will contribute to the fetal portion of the placenta. The TE differentiation process was initially described in the mouse. Hippo signaling plays an essential role in TE and ICM lineage segregation. The foundational components of this signaling pathway include the protein kinases mammalian sterile 20-like 1 and 2(MST1/2), large tumor suppressor kinase 1 and 2 (Lats1/2), their transcriptional coactivators Yes-associated protein 1 (Yap1), transcriptional coactivator with PDZ-binding motif (Taz), and the TEA domain transcription factors TEAD 1-4 (Sasaki, 2015). Additionally, Angiomotin (Amot) and neurofibromin type 2 (*Nf2*) are apical polarity proteins known to be significant regulators of Hippo signaling (Sasaki, 2015).

The Hippo signaling pathway is heavily regulated via cell-cell adhesion and cell polarization (Sasaki, 2015). In apolar cells (aka inner cells), *Amot* is phosphorylated at adherens junctions at the plasma membrane. This allows for MST1/2 to act on Lats1/2 which then phosphorylates Yap and Taz. This cytoplasmic phosphorylation event inhibits Yap/Taz nuclear translocation and encourages their breakdown (Sasaki, 2015). The lack of Yap/Taz within the nucleus suppresses TEAD4 activity, and this limits the expression of genes needed to induce TE differentiation (Sasaki, 2015). Things are different in the outer, polarized cells. This cell polarization will cause *Amot* to be sequestered from basolateral adherens junctions, and this action will prevent MST1/2 and Lats1/2 activation (Sasaki, 2015). Yap/Taz phosphorylation, thus allowing them to translocate into the nucleus, interact with TEAD4, and stimulate TE-specifying gene expression (Sasaki, 2015).

Various transcriptional events occur once Yap and Taz enter the nucleus. Tead4 is a primary target of their actions, and Tead4 is the upstream transcriptional regulator of caudal type homeobox 2 (*Cdx2*), octamer-binding transcription factor 4 (*Oct4*), SRY-related HMG box 2 (*Sox2*), and GATA binding protein 3 (*Gata3)*, each of which play vital roles in TE lineage segregation (Sharma and Madan, 2020). Gata3 and Cdx2 are TE-promoting factors, and therefore are upregulated by Tead4 whereas Sox2 and Oct4 are ICM-specifying factors, and either Tead4 or Cdx2 represses their expression as the TE lineage is being created (Nichols et al., 1998). (Wu et al., 2010).

The Hippo signaling system outlined for the mouse is generally correct for other mammals, but there are a few signaling differences between mice and bovine that should be explained. First, the explicit role of E-Cadherin-mediated cell-cell contact in initiating the Hippo signaling pathway has not been fully revealed in the cow embryo (Sharma et al., 2021). Second, in mice blastocyst formation, knockdown of the *Amot* protein inhibits Hippo signaling pathway in the apolar ICM blastomeres but remains active in the polar TE blastomeres (Sharma et al., 2021). Third, Oct4 expression persists in bovine TE after their formation. It is expressed by both ICM and TE until day 11 (Berg et al., 2011). Fourth, unlike the mouse where Cdx2 is indispensable for blastocyst formation, removal of *CDX2* in bovine embryos results in blastocyst formation although there are subsequent adverse effects noted after blastocyst formation, including the loss of epithelial integrity and failure to elongate (Berg et al., 2011; Goissis and Cibelli, 2014). Fifth, the involvement of Yap/Taz in mediating ICM vs TE specification appears to be more complicated in

bovine embryos because YAP/TAZ is not excluded from the nucleus with the ICM. Rather, it has been proposed that additional phospho-activation events mediate YAP/TAZ signaling within the nucleus of the ICM and TE (Sharma et al., 2021).

Epiblast and Hypoblast Differentiation

The next cell fate decision made after forming a blastocyst is the development of the primitive endoderm (PrE) and the epiblast (Epi) within the ICM. The PrE will contribute to the endoderm layers of the yolk sac, which is integrated into the fetal gut (Artus and Chazaud, 2014). The Epi is a single-cell-layered epithelium that gives rise to the embryo proper and three embryonic germ layers generated during gastrulation (Artus and Chazaud, 2014). It was originally believed that cells located at the surface of the ICM in contact with the blastocoel should be classified as the PrE. However, this now has been proven to be wrong. The PrE – EPI determination is not location-restricted, but rather use of markers specific for each cell type have found that these cells exhibit a "salt and pepper" organization within the ICM (Chazaud et al., 2006). Lineage markers are co-expressed in the majority of blastomeres from the eight-cell stage onward, but as the ICM forms, cells will begin to preferentially express markers of PrE (e.g., Gata6) or Epi (e.g., Nanog). Fibroblast growth factor (FGF) signaling is involved in determining these two lineages (Lanner and Rossant, 2010). FGFs are an expansive family of polypeptide growth factors found in various organisms. FGFs and their receptor tyrosine kinases (RTKs) regulate several developmental processes including differentiation, proliferation, and migration. FGF4 is central to this discussion about PrE and Epi determination. Generally speaking, FGF4 is expressed in Epi cells and its receptor, FGFr2 is expressed in PrE cells, so the presence and absence of the ligand and receptor facilitates Epi-PrE determination (Lanner and Rossant, 2010). However, it is more complex than this. FGF4 and FGFr2 may differ in abundance between cell lineages, but initially, it is possible to artificially shift cells away from one lineage and towards another lineage by controlling the amount of FGF4 (to make more Epi cells) or the activity of FGFr2 (to make more PrE cells). So, this mechanism probably is not the only system mediating Epi-PrE specification. That said, the signaling events of this system have been described for the mouse. FGF4 binds to FGFr2, and this activates *Grb2* (growth factor receptor-bound protein 2)—causing downregulation of *Nanog* and active *Gata6* expression in PrE precursor cells (Lanner and Rossant, 2010). Epiblast precursor cells maintain an absence of FGFr2, inactive MAPK pathway, and *Nanog* transcription allowing for pluripotency (Lanner and Rossant, 2010). Each of these molecules are essential for

pregnancy in the mouse. FGF4 Mice carrying mutations in elements of the FGF/ERK pathway, including FGFR2, FGF4, and *Grb2*, collectively have peri-implantation lethality and insufficient PrE formation (Lanner and Rossant, 2010).

Epiblast and primitive endoderm specification are slightly different in the bovine. *Nanog* and *Gata6* remain excellent genetic markers of the Epi and PE. However, the mode of how these markers are activated and expressed deviates from what occurs in the mouse. First, *NANOG* expression is not present in bovine morula stage but then rapidly occurs as the blastocyst forms (Lanner and Rossant, 2010). At the expanded blastocysts stage, there is strong *Gata6* expression restricted to cells of the ICM (Lanner and Rossant, 2010). In mice, both *Gata6* and *Nanog* are co-expressed in all ICM cells at the early blastocyst stage (Schrode et al., 2014). Utilizing a MEK inhibitor, it was determined that embryonic cells are diverse in their response to MEK inhibition—proving that in certain cells *Gata6* expression is not dependent on the activation of MEK (Lanner and Rossant, 2010). The same MEK inhibitor showed the vitality of MAPK activation for *Gata6* transcription in mouse embryos (Lanner and Rossant, 2010). MAPK pathway inhibition via the MEK inhibitor did not entirely prevent PrE differentiation in bovine, although it still resulted in more *NANOG*-positive cells than *GATA6*-positive cells (Brinkhof et al., 2015). These results showed that *Gata6* expression could be regulated by pathways other than FGF signaling (Lanner and Rossant, 2010).

Embryo Hatching

The mammalian embryo is surrounded in a glycoprotein coat known as the zona pellucida (ZP) during the initial stages of preimplantation development. It is imperative that the blastocyst hatch out of its ZP so it can attach to the uterine lining. The hatching process is initiated by the development of a break in the ZP, produced by a hydrostatic pressure applied by the progressively expanding blastocyst (Seshagiri et al., 2009). The timing of hatching is species-specific and occurs in nearly all mammals. Hatching occurs in bovine at day 8 to 10 (Lonergan, 1994) mouse at day 4 to 4.5 (Bergstrom, 1972), and human at day 5.5 to 6.5 (Lopata and Lachlan Hay, 1989). Blastocyst hatching is regulated by advanced cellular elements such as actin-based trophectodermal projections (TEPs) compounded with a myriad of autocrine and paracrine molecules (Seshagiri et al., 2009). Additionally, release of proteolytic enzymes weakens the zona pellucida allowing for escape (Seshagiri et al., 2009). The cellular phenomenon involving TEPs was first described in guinea pig blastocysts by Ferdinand Von Spee and later confirmed in various other species

(Gonzales et al., 1996). Initial findings demonstrated that TEPs puncture the ZP at the abembryonal pole and display undulating motions during ZP piercing (Seshagiri et al., 2009).TEPs were confirmed in bovine, equine, and human blastocysts (Gonzales et al., 1996). However, the functional importance of TEPs associated with peri-hatching embryo developmental stages is not well established (Seshagiri et al., 2009).

Hormonal Regulation of Oocyte and Embryo Development:

In vitro-produced bovine embryos have two major limitations: weekly variation in embryo production rates and low efficiency of embryo production (Ealy et al., 2021). These shortcomings are attributed to the failure of in vitro culture media to effectively mimic the maternal environments. To more closely model what occurs in vivo within the uterus and oviduct, certain maternally-secreted regulatory molecules can be supplemented to IVP media. The term "embryokine" was coined by Hansen and colleagues to describe regulatory molecules or cytokines produced by the female reproductive tract that regulate embryonic development (Hansen et al., 2014). The term "ookine" has not been officially coined. However, I will be using it herein to refer to regulatory molecules produced by the female reproductive tract, and more specifically the follicle, that regulate or improve oocyte competency and development. These molecules are important because they provide molecular insights into the natural environment of folliculogenesis and early embryogenesis. I have dedicated a short narrative to describing a few of the best-studied embryokines, and then I will switch the focus to describing ookines, and more specifically cytokines that serve as ookines.

Known Embryokines:

Various potential embryokines have been identified utilizing uterine (endometrial) and embryo biopsy collection compounded with RNA sequencing (Sjöblom et al., 2005; Jousan and Hansen, 2007; Neira et al., 2010; Wooldridge and Ealy, 2019; Seekford et al., 2021; Wooldridge and Ealy, 2021). I want to focus solely on cytokines that are being studied as embryokines because this information will provide nice background information for the upcoming ookine section discussion. There are two main classes of cytokines to discuss, the ᵬ-common cytokines and the IL6 family of cytokines.

The β-common cytokine family:

Colony-stimulating factor 2 (CSF2)

The β-common cytokine family has three members. This family is characterized by sharing a β subunit receptor along with a ligand-specific α-subunit receptor. β-common cytokine signaling is mediated primarily via Signal Transducer and Activator of Transcription 5 (STAT5) activation as well as other intracellular signaling molecules such as phosphoinositide 3-kinase (PI3K) and MAPK (Ealy et al., 2021).

Among the three members of this family, colony-stimulating factor 2 (CSF2) has been the most noteworthy molecule to study in regard to improving bovine embryo production. Early in pregnancy, CSF2 is produced by the oviduct and the uterus(Becher et al., 2016; Tríbulo et al., 2017). CSF2 is confirmed to impact bovine embryogenesis, however, CSF-specific alpha-subunit receptor is not present in pre-implantation bovine embryos (Jiang et al., 2014). Although its receptor is not present, CSF2 is still able to yield feedback from bovine embryos (Wooldridge et al., 2022). Early studies supplementing bovine recombinant CSF2 generated conflicting data. Some studies showed that CSF2 supplementation improved blastocyst development, but more recent studies saw no improvement (Loureiro et al., 2009; Tríbulo et al., 2017). Evidence suggests that CSF2 acts as a pro-survival factor of the ICM (Dobbs et al., 2013) and advances peri-implantation conceptus elongation (Loureiro et al., 2011). The most notable embryokine-like feature of CSF2 supplementation to IVP embryos is its improvement in post-transfer pregnancy rates(Loureiro et al., 2009). Studies have also observed improvements in pregnancy retention rates (Loureiro et al., 2009) and postnatal health of calves (Kannampuzha-Francis et al., 2015) when CSF2 was supplemented prior to embryo transfer.

IL6 Cytokine Family

The IL6 cytokine family are functionally and structurally distinguishable from the β-common family, although this family is similar in the regard that they share a common B-subunit receptor. This receptor is distinct from the B-common receptor. It is called glycoprotein 130 (gp130) or IL6 signal transducer (IL6ST). Members of the IL6 cytokine family utilize MAPK, PI3K, STAT3, and nuclear factor-kappa B (NFkB) signaling pathways (Rose-John, 2018). Of the seven members of this cytokine family, IL6, and leukemia inhibitory factor (LIF) have been definitively confirmed as embryokines.

IL6

IL6 and its ligand-specific receptor are the most abundant among its family found in bovine blastocyst (Wooldridge et al., 2019). IL6 supplementation improves bovine embryo development when cultured individually (Wooldridge and Ealy, 2019) and increases ICM cell numbers in group-cultured bovine embryos (Wooldridge et al., 2022). Our lab has also noted that supplementing 100 ng/ml of recombinant bovine IL6 increases PrE cell numbers present in bovine blastocysts at day 8 and 9 post-fertilization (Wooldridge and Ealy, 2021). However, IL6 supplementation revealed inconsistent effects on EPI cells (Wooldridge and Ealy, 2021). Our lab also confirmed that IL6 activates the JAK/STAT3 pathway in bovine embryos. Utilizing an inhibitor or JAK1/2, AZD1480, on day 5 post-fertilization ICM cell numbers were reduced regardless of IL6 supplementation (Wooldridge and Ealy, 2021). Additionally, total blastomere cell numbers were decreased when treated with the JAK1/2 inhibitor in both IL6-treated and non- treated embryos (Wooldridge and Ealy, 2021). It's known that STAT3 signaling is needed for IL6 activity, however, the exact mechanism of IL6 action on the ICM is being further investigated.

Our lab also did a study examining the effects IL6 has on the bovine embryo transcriptome utilizing RNA sequencing. Results revealed that treatment with IL6 increased the ICM: TE ratio, ICM, TE, and total cell numbers (Wooldridge and Ealy, 2021). 91 genes were affected by IL6 treatment, and this dataset was used to detail the abundance of IL6 family member ligands and receptors in bovine blastocyst. Within the IL6 family, *IL6R, IL6ST,* & *IL11RA* expression was present (Wooldridge and Ealy, 2021). Gene expression in ICM and TE lineage markers revealed no changes after IL6 treatment (Wooldridge and Ealy, 2021). However, there were two PE lineages markers with increased expression (Wooldridge and Ealy, 2021).

My lab previously completed an ET study where IL6 was supplemented during *in vitro* embryo development and the embryos were transferred into estrous-synched non-lactating commercial beef and dairy recipient cows on day 7.5 (Seekford et al., 2021). A subset of cows from the herd underwent timed artificial insemination (TAI). Our group found that pregnancy rates were similar in each treatment group (Seekford et al., 2021). However, reduction in crown-rump length (CRL) and placental fluid volume (PFV) on day 28, crown nose length (CNL) and abdominal diameter (AD) on day 56, combined with a tendency for a reduction in PFV on day 35 was detected when comparing ET-control group with the ET-IL6 group (Seekford et al., 2021). Additionally, circulating pregnancy-associated glycoprotein (PAG) concentrations were similar in all treated groups (Seekford et al., 2021). Our lab concluded that IVP embryos treated with

IL6 before ET produced pregnancies more closely resembling TAI-generated pregnancies rather than those developed using standard culture systems (Seekford et al., 2021).

Ookines:

Various growth factors, cytokines, and other proteins, lipids and other molecules have been explored for their potential roles during oocyte maturation and fertilization, so it can be a bit challenging to develop a concise definition for the term, ookine. However, I propose that one or both of the following definitions capture much of what is needed when discussing ookines. The first definition would represent what occurs *in vivo*, where an ookine represents a paracrine, endocrine, autocrine, or intracrine factor that benefits some feature of oogenesis, granulosa and/or theca cell activity, cytoplasmic and/or nuclear oocyte maturation, or fertilization. A second definition, one that perhaps is more applicable to my research efforts, relates to *in vitro* oocyte maturation and fertilization, where an ookine would represent a factor that will directly or indirectly influence cytoplasmic and/or nuclear oocyte maturation in ways that improve fertilization success, embryo development to the blastocyst, and/or post-transfer pregnancy maintenance. These ookines can be produced by the cumulus cells or oocyte, or they can represent a factor that the COC would normally see *in vivo* but that is absent during *in vitro* culture. For example, thecal factors that mediate cumulus cell activity are prime examples of ookines. This section will introduce you to several of the common ookines, then I will focus on describing a few cytokines that also function as ookines.

FSH is arguably the best studied ookine. The primary role of FSH *in vitro in* oocyte developmental competence is well studied. FSH is commonly used in IVM protocols because evidence shows it improves fertilization, early embryonic development, and cumulus expansion (Calder et al., 2003). The action of FSH on oocytes maturation is not well understood but it is thought to be mediated via cumulus cells (Sirard et al., 2007). Studies show that the FSH receptor mRNA being present in granulosa cells and cumulus cells but not present in oocytes isolated from bovine antral follicles (Sirard et al., 2007). Being that FSH activates cumulus expansion *in vitro* in a similar manner as the LH surge behaves *in vivo*, I would say it is a necessary addition to *in vitro* maturation media when culturing embryos.

Another very well studied ookine is epidermal growth factor (EGF). EGF is part of the group of ligands that interact with the EGF receptor (EGFR) (Dreux et al., 2006). There are

inconsistencies when it comes to the effects of EGF in IVP embryo culture. Among the researchers who have studied EGF's effects on blastocyst formation, roughly one-half have observed that EGF increases blastocyst formation while others found no effect after supplementing EGF (Wooldridge et al., 2022). Interestingly, it has been stated that EGF is required in the maturation medium to enhance the effectiveness of follicle stimulating hormone (FSH) to induce oocyte maturation (Farin et al., 2007). The EGF family comprises 11 proteins including EGF, transforming growth factor-α (TGFα), heparin-binding EGF, epigenetic (EPGN), the neuregulins 1-4 (NRG1-4), amphiregulin (AREG), epiregulin (EREG) and beta cellular (BTC)—each containing similar structural and functional properties (Schneider and Wolf, 2009). As discussed earlier, EGF-like growth factors are necessary for FSH-induced COC maturation (Zhang et al., 2009). EGF-like peptides bind to EGFR and activate its tyrosine kinase, resulting in the autophosphorylation of specific tyrosine residues and activation of various signaling processes within the cell (Zhang et al., 2009). This signaling includes phosphatidylinositol 3-kinase (PI3K0, PLCγ/PKC), and the MAPK3/1 pathway that is thought to be chiefly involved in the regulation of downstream signaling pathways that control the expansion of cumulus cells, resumption of meiosis, and ovulation (Zhang et al., 2009). Conclusively, vast evidence suggests that EGF-life peptides are the mediators of LH signaling in the preovulatory follicle.

For my study, I will be focusing on cytokines that may also serve as ookines. There are various cytokine families that have roles in embryogenesis including interferons (IFN), colony-stimulating factors (CSF), interleukins (IL), transforming growth factors (TGF), and tumor necrosis factors (TNF)(Wooldridge et al., 2022). My research emphasized the IL6 cytokine family, so I will focus on these molecules.

Cytokines that serve as Ookines

Although members of the IL6 cytokine family have been supplemented during IVP of bovine embryos, they have not always been supplemented at IVM. For example, IL6 has been studied extensively when supplemented after fertilization whereas LIF has been supplemented at both IVM and after fertilization. We will now discuss what has been reported for exploring LIF, IL6, and IL11 as ookines. A table is included at the end of this chapter to provide an overview of the information available for LIF, IL6, and IL11 as ookines.

Leukemia Inhibitory Factor (LIF)

LIF is a member of the IL6 cytokine family (Field et al., 2014). Studies often utilize human LIF in bovine supplementation studies due to it having a high sequence homology to bovine LIF and increased levels of purity. Several LIF supplementation studies occurring during IVM have been completed, but they have yielded opposing results on blastocyst developmental rates. Supplementing LIF during in vitro maturation (IVM) of bovine oocytes showed inconsistencies in its effects on cleavage, blastocyst yield, and hatching rates (Sirisathien et al., 2003; Mo et al., 2014; Vendrell-Flotats et al., 2020a). One study supplemented LIF to bovine COCs and observed enhanced nuclear maturation in LIF-treated groups (Mo et al., 2014). Another group showed that LIF supplementation to murine cultured ovarian tissue inhibited the growth of primary, secondary, and antral follicles (Komatsu et al., 2015). A popular supplementation study utilized a cocktail of LIF, insulin growth factor 1 (IGF1), and FGF2, which is being termed as FLI medium (Yuan et al., 2017). This group observed improved nuclear maturation of oocytes derived from immature pig ovaries (Yuan et al., 2017). Additionally, they note after 22 hours the COCs in FLI medium continued to expand and displayed an over five-fold increase in apparent size (Yuan et al., 2017). Vendrell-Flotats and colleagues included LIF in IVM medium before vitrification and found that bovine oocytes treated with LIF presented gene patterns more closely resembling those from non-vitrified oocytes (Vendrell-Flotats et al., 2020a). The discrepancy in LIF supplementation may be due to differences in media formulations, varied concatenations of LIF, and protocol inconsistencies (Wooldridge et al., 2022). These varying results may also be due to the LIF concentrations tested, and the purity and biological activity of the protein preparations.

IL6 and IL11 as potential ookines

There is only one study that studied both IL6 and IL11 as potential ookines. Vendrell-Flotats and colleagues completed a series of studies that compared IL6, IL11, LIF supplementation during bovine oocyte IVM (Vendrell-Flotats et al., 2020b). Their primary endpoint was characterizing the expression of various microRNAs (miRNAs) that normally change their expression patterns from the GV oocyte up to the 3-cell embryo stage. They found that miR-146a expression was increased in oocytes matured with IL6 and IL11 (Vendrell-Flotats et al., 2020b). However, they did not assess how IL6 and IL11 treatment affected embryo development. LIF-exposed COCs showed a higher expression of miR-21 and miR-155 (Vendrell-Flotats et al., 2020b). LIF supplementation did not affect cleavage or blastocyst yield (Vendrell-Flotats et al., 2020b). Another study matured mouse COCs in the presence of IL6 and/or its soluble receptor

aiming to determine how IL6 alone affected cumulus expansion (Liu et al., 2009). This group confirmed that COCs matured in the presence of IL6 had improved embryo transfer rates than controls without IL6 and were comparable to those matured *in vivo* (Liu et al., 2009). Additionally, they were able to confirm that IL6 alone can induce cumulus expansion (Liu et al., 2009). IL6 was supplemented to mouse COCs in a different study to analyze its effects on oocyte spindles (Banerjee et al., 2012). This study concluded that exposure to IL6 at IVM resulted in a dose-dependent weakening in microtubule and chromosomal alignment (Banerjee et al., 2012). Furthermore, elevation in IL6 expression has been shown to promote meiotic progression during oocyte maturation. (Rispoli et al., 2019; Rowinski et al., 2020).

I am intrigued with studying IL6, IL11, and LIF as ookines because recent COC expression profiling studies indicate that these ligands and/or their receptors are present in bovine COCs (Walker et al., 2022). IL6 is sourced from both thecal and granulosa cells (Field et al., 2014). While IL11 is produced from the preovulatory follicle via theca cells (Vendrell-Flotats et al., 2020b). A more thorough description of the localization of receptors for these cytokines is needed for us to better predict which IL6 family member to pursue as potential ookines.

I have already introduced you to interesting roles that IL6 and LIF play in reproduction, so I want to devote some time to introducing a few ways that IL11 influence reproduction. There is very little known about IL11's role in reproduction. IL11 was first reported as having cytoprotective effects (Du and Williams, 1994). IL11 is a multifunctional cytokine known to manage cell cycle, invasion, and migration in several cell types (Winship et al., 2016). Each of these known functions of IL11 are essential in placental development and carcinogenesis (Winship et al., 2016). Among the IL6 family members, IL11 and LIF have confirmed roles in the cycling endometrium and in the initiation of pregnancy during implantation and endometrial stromal cell decidualization (Winship et al., 2016). Some studies have suggested IL11 has a role in placentation, detailing its effect on human trophoblast (Paiva et al., 2007; Paiva et al., 2009). IL11 and IL11Rα are detected in normal human ovarian tissues, but little is known about IL11 regulation of ovarian function in women. IL11 is known to be produced by the human endometrium (Tabibzadeh et al., 1995; Laird et al., 1997; Cork, 2002).

Summary and Concluding Remarks:

In vitro produced embryos allow for increased utilization of breeding programs for dairy and beef cattle. However, a major disadvantage of IVP in cattle and other species is that embryo

developmental competence is inferior when compared to embryos produced *in vivo*. Also, the number of oocytes that become fertilized and produce viable embryos via IVF is inferior to that of oocytes produced *in vivo.* These shortcomings of IVP embryos clearly indicate the need to gain a broader understanding of the biological and developmental processes happening during early embryogenesis.

Being that embryos originate from oocytes; oocyte maturation is a vital factor in creating a viable and healthy embryo. Nuclear and cytoplasmic maturation of oocytes must occur for potential fertilization. Sperm-egg fusion entails various processes critical for proper fertilization, including capacitation, acrosomal reaction, cortical reaction, calcium oscillations, and egg activation. Epigenetic changes and the two waves of epigenetic reprogramming are also vital for preimplantation development and in avoiding developmental deformities during the development of the preimplantation embryo. Additionally, MZT is essential in replacing maternal transcripts of the oocyte with early embryo and zygotic transcripts, as well as initiating EGA. For proper blastocyst formation, compaction and cavitation must occur as well as the development of tight junctions and aquaporins. Hippo signaling will drive the initial cell fate and differentiation of the TE from the ICM. The second cell fate decision, development of the PrE and Epi, are driven by FGF signaling pathways and their RTKs. Embryo hatching requires a break in the ZP mediated by TEPs.

We are beginning to delineate how embryokines may influence these various developmental events, and a focus of our lab has been to explore how members of the IL6 cytokine family may regulate embryogenesis and potentially be used to improve post-transfer embryo competency. However, LIF has been the only member of the IL6 family that has been explored as an ookine. When I entered the laboratory, I was tasked with exploring additional IL6 family members for their potential involvement with oocyte maturation and subsequent in vitro embryo production. As I will discuss in the next chapter, initial findings led me to focus my work on describing actions for IL6, IL11, and LIF during IVM and exploring how this scheme influences several key indicators of oocyte competency to be fertilized and generate a transfer-quality blastocyst. ***I hypothesized that supplementing IL6, L11, and/or LIF during IVM will improve overall oocyte competency, and this will lead to improvements in maturation, cleavage, and blastocyst rates.*** The objective of my work was to determine if supplementing IL6, IL11, or LIF during IVM will improve nuclear maturation, cleavage, and blastocyst rates,

post-thaw viability of blastocysts, and alter gene expression of cumulus-expressing transcripts associated with oocyte competency.

Table 1. Overview of studies exploring how supplementation with the IL6 family of cytokines during in vitro oocyte maturation influences the oocyte, fertilization, and embryo development.

Cytokine	Treatment	Oocyte Competence	Cleavage	Blastocyst	Cryo-preservation	Species	Paper
LIF	50 ng/mL rhLIF 10 ng/mL EGF LIF + EGF	LIF improved maturation rate	LIF + EGF Improved	Increased in LIF + EGF group	N/A	Mouse	(Amiri et al., 2009)
	50 ng/mL rhLIF 100 ng/mL ovine FSH LIF+FSH	LIF and/or FSH increased GVBD	N/A	N/A	N/A	Sheep	(Cadoret et al., 2021)
	1000 ng/mL LIF FSH LIF+FSH	LIF increased cumulus expansion	LIF+FSH improved cleavage rate	LIF + rFSH increased the number of 2 cell embryos	N/A	Human & Mouse	(De Matos et al., 2008)
	25 ng/mL LIF	LIF improved nuclear maturation	LIF increased cleavage rate	LIF increased number of total cells	N/A	Cow	(Mo et al., 2014)
	1 mg/ml insulin + 50 ng/mL LIF 50 ng/mL LIF	LIF increased primordial to primary follicle transition.	N/A	N/A	N/A	Rat	(Nilsson et al., 2002)
	25 ng/ml rhLIF	LIF influenced oocytes gene patterns after oocyte vitrification	LIF reduced cleavage rates after vitrification	LIF in the IVM medium prior to vitrification improved blastocyst development	LIF reduced cleavage rates after vitrification	Cow	(Vendrell-Flotats et al., 2020a)
	100 μM Cys 100 IU/ml LIF 10 μM Y27632	Cys +LIF increased maturation rate	Combined supplementation of all three treatments resulted in most improved cleavage rates	Combined supplementation of all three treatments resulted in most increased blastocyst yield	N/A	Goat	(An et al., 2018)
	pLIF 1,000 U/ml	Increased Phosphorylated STAT3 (p-STAT3) in both cumulus cells and oocytes	Tendency for increased cleavage rates	tendency for increased blastocyst development	N/A	Pig	(Dang-Nguyen et al., 2014)
FLI	40 ng/mL FGF2 20 ng/mL LIF 20 ng/mL IGF1	N/A	N/A	FLI during IVM improved blastocyst development	N/A	Sheep	(Tian et al., 2021)

	Porcine Follicular fluid (pFF) + FLI medium 20 ng/mL rhLIF 20 ng/mL rhIGF1 40 ng/mL rhFGF2	N/A	FLI increased cleavage rates	pFF + FLI resulted in increased development rates	N/A	Pig	(Currin et al., 2022)
FLI	LIF (20 ng/mL) FGF2(40 ng/mL) IGF1 (20 ng/mL)	FLI increased dissociation of transzonal projections & improved oocyte maturation	No effect	FLI increased development to the blastocyst stage, cytoskeleton integrity	FLI increased cryosurvival	Cow	(Stoecklein et al., 2021)
	LIF (20 ng/mL) FGF2(40 ng/mL) IGF1 (20 ng/mL)	FLI improved oocyte meiotic maturation & cytoplasmic maturation	N/A	No effect in blastocyst rate or cell numbers	N/A	Pig	(Albal et al., 2022)
	FGFG2 (40-80 ng/mL) LIF (10-40 ng/mL) IGF1 (10-80 ng/mL)	FLI medium improved nuclear maturation	N/A	FLI-treated oocytes advanced to the blastocyst stage more efficiently than controls	N/A	Pig	(Yuan et al., 2017; Vendrell-Flotats et al., 2020b)
IL6, IL11, LIF	rhLIF (25 ng/mL) rhIL6 (10 ng/mL) rhIL11 (5ng/mL)	-LIF increased expression of miR-21 and miR-155 in oocytes, IL6, IL11: increased miR-146a expression -In cumulus cells, elevated miR-155 expression by all treatments, LIF only - increased miR-21 expression	No effect	LIF did not affect blastocyst yield but did increase hatching rate	N/A	Cow	(Vendrell-Flotats et al., 2020b)
IL6	2μg/ml IL6 IL6/IL6SR (250ng/ml, 100 ng/ml, 1μg/ml)	IL6/IL6SR could induce COC expansion and stimulated phosphorylation of key kinase cascades in cumulus cells	N/A	COCs matured in the presence of IL6 had increased embryo transfer rates than ones without IL6 and more comparable to those matured *in vivo*	N/A	Mouse	(Liu et al., 2009)

IL6	Recombinant mouse 50 ng/ml IL6 100 ng/ml IL6 200 ng/ml IL6	IL6 caused dose-dependent deterioration in chromosomal and microtubule alignment	N/A	N/A	N/A	mouse	(Banerjee et al., 2012)
IL6 & LIF	IL6 and LIF transcript abundance and correlated IL6 transcripts were evaluated throughout IVM at both an elevated temperature (41 °C) & thermoneutral temperature (38.5°C)	Heat-induced shifts in temporal production of IL6 and IL6ST likely correlate with a heat-induced hastening of meiotic progression. Transcripts for LIF varied over time but was not affected by 41℃ exposure	N/A	Blastocyst development was not affected by 41℃ exposure	N/A	Cow	(Rowinski et al., 2020)

Chapter 2: Investigating the Supplementation of IL-6, IL-11, & LIF at *In Vitro* Maturation to Improve Oocyte Competency.

Introduction

Assisted reproduction technologies (ART) encompass the various fertility-enhancing strategies and techniques involving oocytes, sperm, and embryos. In the human, *in vitro* fertilization (IVF), gamete intrafallopian tube transfer (GIFT), and intra cytoplasmic sperm injection (ICSI) are utilized to assist sub-fertile couples with having children. In cattle, artificial insemination (AI), embryo transfer (ET), and *in vitro* embryo production (IVP) are the primary ART methods used, and the goal of these technologies is to increase genetic gain of economically important phenotypes. Humans and cattle share various reproductive commonalities when discussing the oocyte, fertilization, and early embryonic development. Also, pregnancy loss in both cattle and human is very prominent within the first 30 days of gestation. In beef cattle, for example, more than 50 percent of reproductive failures occur before day 16 of gestation (Reese et al., 2020). Approximately 26% of women's pregnancies end in miscarriage or spontaneous abortion, meanwhile, up to 10% of clinically recognized pregnancies end in miscarriage (Wilcox et al., 1988; Zinaman et al., 1996).

Both human and cows are mono-ovulatory, have similar gestation periods, and comparable reproduction cycle lengths (Sirard, 2017). The preparation of spermatozoa for IVF in domestic cattle is nearly identical to methods used for the human (Ménézo and Hérubel, 2002). This makes the cow an ideal model to study human IVF and learn how optimizations could be made. This study will focus on in *vitro* production (IVP) of bovine embryos being that both cow and human have a similar incidence of early pregnancy loss. Over one million IVP bovine embryos were produced across the globe in 2019 (Viana, 2018). However, only 20% to 40% of oocytes will yield transferable embryos (Ealy et al., 2019; Hansen, 2020). Due to the increasing utilization of ART, there is grave need to improve the efficiency of IVP. Numerous strategies can be implemented to overcome the limitations of culturing pre-implantation embryos *in vitro*; however, these embryos are still inferior to their *in vivo* counterparts. IVP embryos are inferior in their ability to properly fertilize and develop to the blastocyst stage, the stage when embryos are normally transferred (Wooldridge et al., 2022). Also, IVP-generated embryos are inferior at retaining pregnancies (Wooldridge et al., 2022). There appear to be several reasons for this problem, and there is a crucial

need to improve the understanding of developmental processes occurring in the embryo during early gestation so that modifications can be made to improve the efficiency of IVP. We know there are two main limitations to the IVP process.

My thesis project focused on exploring whether improvements in IVP success could be made by exposing oocytes to factors that may improve their quality. I and others have proposed that poor oocyte quality is the primary factor contributing to the number of oocytes that develop to the blastocyst stage during in vitro culture (Rizos et al., 2002; Lonergan et al., 2003). Oocyte maturation is typically defined as events correlated with the initiation of germinal vesicle breakdown (GBVD) and subsequent nuclear maturation. This is event happens differently *in vivo* than it does *in vitro. In vivo,* maturation of the ovulatory follicle is catalyzed by the pre-ovulatory surge of luteinizing hormone (LH). One of the primary actions of the gonadotroph LH is to interrupt cumulus cell and oocyte communication so that meiosis can resume. *Follicle-stimulating* hormone (FSH) is another gonadotroph and is associated with stimulating antral follicle growth *in vivo* (Sirard et al., 2007). High estradiol concentrations are present in the preovulatory follicle before and a few hours after the LH peak (Beker et al., 2002). Decrease in follicular estradiol concentrations are associated with GBVD and it is believed to play a role in meiotic resumption (Beker et al., 2002). Epidermal growth factor receptor (EGFR) activation is another crucial component in triggering LH-induced meiotic resumption (Norris et al., 2010). The rapid release of EGFR ligands epiregulin (EREG) and amphiregulin (AREG) from granulosa and cumulus cells is an important catalyst of LH receptor activation (Norris et al., 2010). Additionally, EGFs ligands are known to stimulate cumulus expansion, and disturbing this EGF complex *in vivo* impairs ovulation (Hsieh et al., 2009). The next 24-hour period after LH-induced meiotic resumption involves the oocyte's nuclear progression from prophase I to metaphase II (MII). Moreover, alterations at the cytoplasmic level occur involving organelle reorganization, storage of mRNAs, proteins, and transcription factors within the oocyte (Ferreira et al., 2009). *In vitro,* antral follicles are competent to spontaneously resume meiosis after being excised from their follicles (Edwards, 1965). During IVM, theca cell and endocrine-derived factors are not present in the media. FSH and LH effects are typically mediated via the surrounding somatic cells of the oocyte, and it is recognized that both gonadotrophs are pertinent in achieving proper oocyte maturation *in vivo* and *in vitro* (Gilchrist et al., 2004). Estradiol and EGF are also typically supplemented into IVM media to more closely mimic the *in vivo* follicular environment. My study will focus on a set of cytokines

whose ligands or receptors are present during *in vivo* maturation. Paracrine dialogue between the oocyte and surrounding granulosa/thecal cells is known to be mediated via cytokines and hormones. Supplementing these cytokines to IVM media could potentially bring us closer to mimicking the *in vivo* environment these oocytes would typically mature in.

Interleukin 6 (IL6) cytokine family members characteristically maintain both pro- and anti-inflammatory properties and members of this family have been identified to regulate embryonic cell development and maintenance. Of the seven members of this cytokine family, IL6, and leukemia inhibitory factor (LIF) have been studied the most. They both are known as "embryokines" or molecules secreted from the maternal oviduct and endometrium that regulate embryonic development. Several LIF supplementation studies occurring at IVM have been completed, but they have yielded opposing results on blastocyst developmental rates. The inconsistency in LIF supplementation may be due to differences in media formulations, varied concentrations of LIF, and protocol discrepancies (Wooldridge et al., 2022). These fluctuating results may also be due to the LIF concentrations tested, and the purity and biological activity of the protein preparations.

IL6 supplementation improves bovine embryo development when cultured individually (Wooldridge and Ealy, 2019) and increases ICM cell numbers in group-cultured bovine embryos (Wooldridge et al., 2022). There is minimal work where IL6 is supplemented at *in vitro* maturation (IVM). We are proposing the term "ookine" for when these cytokines are supplemented at IVM and can regulate or improve oocyte and embryonic development. One study completed by Liu and colleagues where IL6/IL6SR was supplemented at IVM resulted in increased COC expansion progression (Liu et al., 2009). However, most IL6 supplementation studies are conducted post-fertilization. IL11 is less studied than IL6 and LIF, however, both IL11 and LIF have confirmed roles in the cycling endometrium and in the initiation of pregnancy during implantation and endometrial stromal cell decidualization (Winship et al., 2016). Some studies have suggested IL11 has a role in placentation, detailing its effect on human trophoblast (Paiva et al., 2007; Paiva et al., 2009). IL11 and IL11Rα are detected in normal human ovarian tissues, but little is known about IL11 regulation of ovarian function in women. IL11 is known to be produced by the human endometrium (Tabibzadeh et al., 1995; Laird et al., 1997; Cork, 2002). I am intrigued with studying IL6, IL11, and LIF as ookines because recent COC expression profiling studies indicate that these ligands and/or their receptors are present in bovine COCs.

Based on these considerations, I hypothesized that supplementing IL6, IL11, or LIF during IVM will improve overall oocyte competency, and this will improve the efficiency of IVP embryo production. The objectives of this study were to determine if supplementing IL6, IL11, or LIF during IVM will improve nuclear maturation, cleavage, and blastocyst rates, post-thaw viability of blastocysts, and alter the abundance of cumulus transcripts associated with oocyte competency.

Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials that followed humane slaughter practices according to USDA guidelines. Reagents were purchased from ThermoFisher Chemical Company (Waltham, MA USA), unless otherwise specified.

In vitro maturation

Maturation procedures were completed as previously described (Wooldridge et al., 2019; Speckhart et al., 2023). In brief, cumulus-oocyte-complexes (COCs) were harvested from ovaries purchased from Brown Packing Company (Gaffney, SC, USA). COCs were incubated overnight for 22-24 h at 38.5 ℃ in 5% [v/v] CO_2 in air in groups of 25-35 COCs per 500 µl TCM-199 containing Earle's salts supplemented with 10% [υ/v] fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia, USA), 200 µg/ml Gentamicin, 1 mM sodium pyruvate, 2mM L-alanyl-L-glutamine (Glutamax), 5 µg/ml Folltropin (AgTech), and 20 ng/ml estradiol (Sigma-Aldrich; St. Louis, MO). These COCs were collected at various time points for analysis or were processed for in vitro fertilization and embryo culture.

Supplementation of IL6, IL11, & LIF

Preparations of recombinant human IL6, IL11, and LIF (R&D Systems; Minneapolis, MN, USA) were prepared in SOF containing 1% [w/v] bovine serum albumin (BSA; Sigma-Aldrich) and stored in – 80 ℃ for no longer than 6 months. Control treatments consisted of carrier only (1% BSA). Stocks were thawed only once and were used immediately after thawing. Treatments were supplemented into 500 µl drops of oocyte maturation media (OMM) containing 25-35 COCs.

Oocyte Maturation Assessment

Procedures are based on previous work by others (Eichenlaub-Ritter et al., 2008; Campen et al., 2018). At 16 or 22 h after beginning IVM, oocytes were denuded by vortexing. The denuded oocytes were fixed in freshly prepared 4% [w/v] paraformaldehyde in Dulbecco's phosphate-

buffered saline (DPBS) for 15 min at room temperature. The oocytes were permeabilized with 1% [v/v] Triton X-100 in DPBS for 1 h at room temperature and blocked in 10% [v/v] donkey serum for 1 h at room temperature. Oocytes were incubated at room temperature for 1 h or overnight at 4 ℃ with 5 µg/ml mouse-α-tubulin antibody and 5 µg/ml mouse anti-β-tubulin antibody (Campen et al., 2018). Oocytes were washed and then incubated for 1h with Alexa Fluor 488 donkey anti-mouse at a final concentration of 3.3 µg/ml. The cells were washed and then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml) for 5 minutes. Following three washes, oocytes were placed into imaging droplets and visualized and scored using ECHO Revolve 4 fluorescent microscope and associated software.

Oocytes were scored as to have undergone meiosis II (MII) if they contained one polar body and contained cytoplasmic chromosomes that aligned on the equator of the cell and formation of the first polar body.

Real-Time Quantitative RT-PCR

Total RNA was isolated from groups of COCs (n=20) after 4 or 22 h of maturation using the PureLink™ RNA Mini Kit (Thermofisher, Waltham, MA). Total RNA concentration and quality were determined using the NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). Only samples containing an $A_{260/280}$ ratio >1.8 were used. RNA was treated with RNase-free DNase I at 37℃ for 30 minutes, then the enzyme was inactivated by exposure to 75 ℃ for 10 min. Reverse transcription was completed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) 25 ℃ for 10 minutes, then 37 ℃ for 2 hours, followed by an infinite hold at 4 ℃.

The Applied Biosystems 7500 Fast PCR System (Applied Biosystems) was used in triplicate 10 µL samples containing SYBR Green Master Mix (Applied Biosystems) and primer pairs (Table 1). Reactions were completed with an initial 95 ℃ for 20 s step followed by 40 cycles of 95 ℃ for 3 s and 60 ℃ for 30 seconds. The quantification of each transcript was calculated relative to the housekeeping transcript, *HPRT1*. Transcript abundance relative to *HPRT1* was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *HPRT1* was chosen based on its recognized performance as a stable control for bovine cumulus cells (Caetano et al., 2019). We initially tested beta-2-microglobulin (B2M) in addition to HPRT1, however, it was variant with our transcript *TNFAIP6.*

In Vitro Fertilization

After 22 h maturation, COCs were washed and placed in groups of 25-30 in 500 µL SOF-FERT covered by paraffin oil (Origio; Malov, Denmark). Frozen semen from four Holstein bulls (donation form Select Sires, Plain City, OH, USA) was thawed, and spermatozoa were isolated through a biphasic (40 and 80%, [v/v]) Bovipure ™ density gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA) before addition to the fertilization media at a concentration of 1 million sperm/ml fertilization media. Day 0 (D0) indicates the day of fertilization.

In vitro Embryo Culture

After incubation with sperm for 14-18 h at 38.5 ℃ in 5% CO_2 in humidified air, presumptive zygotes were denuded of cumulus cells, washed in HEPES-SOF, and placed in groups of 20-30 in droplets of 50 µl of SOF-BE1 covered by paraffin oil and incubated at 38.5℃ in 5% O_2, 5% CO_2, 90% N_2 in humidified air (Fields et al., 2011).

Assessing Cleavage and Blastocyst Development

A zygote was considered to have "cleaved" if visible cellular division had taken place. A zygote was considered a blastocyst if its blastocoel cavity took up at least fifty percent of the entire embryo (Fragouli et al., 2014). A blastocyst was considered early if the blastocoel cavity accounted for less than one-half the volume of the embryo (Fragouli et al., 2014). Expanded blastocysts have blastocoel cavity's larger than the early embryo accompanied by a thinning of the zona pellucida (Fragouli et al., 2014). Hatched blastocysts have completely been removed from the zona pellucida. Advanced blastocysts were calculated as hatched plus expanded blastocysts.

Embryo Cryopreservation and Thawing

At D8 post-fertilization, a subset of non-hatched blastocysts were cryopreserved after three washes in HEPES-SOF by placing them in 500 µL of ethylene glycol with sucrose (MWI, Animal Health, Boise, ID) for 5-15 minutes, or until equilibration is met by blastocyst falling to the bottom of the well. Blastocysts were then transferred into a new dish containing 500 µL of ethylene glycol, then loaded into ET straws as previously described (Leibo, 1986; Gómez et al., 2020). Each straw was sealed with a sealing plug. the Crysalys Cryocontroller PTC 9500 (Biogenics, Harriman, TN) was used for slow freezing. Straws were placed into the cryo-bath and underwent two initial minutes at -6℃, then they were seeded with liquid nitrogen on the upper area of the straw to induce ice crystal formation. Slow freezing conditions were as follows: -6℃ for 8 minutes, followed by a steady decline of 0.6 ℃/min until reaching -32 ℃, and held at -32 ℃ until the end of the cycle and removal. After completion of the program, straws were stored in liquid nitrogen.

Embryos were thawed using a Cito Thaw (CITO Products, Watertown, WI) at 35.0 ℃ for 30 s. Embryos were expelled into HEPES-SOF and passed through three HEPES-SOF washes followed by two washes of SOF-BE1. Blastocysts were plated into 500 µL of SOF-BE1 (5 blastocysts/drop) covered in mineral oil and incubated at 38.5℃ in 5% CO_2, 5% O_2, and 90% N_2 in humidified air. Blastocyst re-expansion, hatching, degeneration, and survival were recorded at 24, 48, and 72 h post-thawing (hpt). Re-expanded blastocysts were classified as those whose blastocoel cavity fully re-expanded. Hatching was classified as a blastocyst hatching or hatched from their zona pellucida after being thawed. Degenerate embryos were classified as those that had visible atresia and no signs of a blastocoel cavity, (see Fig. 1).

Transcriptome Abundances for IL6 Family Member Ligands and Receptors in the Bovine COC

Tables (s) 2 and 3 describe the transcriptome abundance of all IL6 family member ligands and receptors in cumulus or oocyte cells immediately following COC excision from its follicle. Data set was collected and published previously (Walker et al., 2022). No statistics were run by our lab, the standard deviation and mean were derived from the previously mentioned data (Walker et al., 2022). Table 2 shows that most IL6 cytokine family member ligands are absent in oocyte or cumulus cells. However, Table 3 displays most of the IL6 cytokine family member receptors being present in cumulus or oocyte cells.

Statistical Analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc.). When necessary, individual comparisons were partitioned further by using the probability of difference (PDIFF) test of SAS. Relative mRNA abundance data were log-transformed before analysis, but data are presented as fold change with standard error. Study replicate was used as the experimental unit in all analyses. This replicate refers to the mean developmental percentages observed on each *in vitro* embryo production occasion (i.e., each IVP run). Percentage data (e.g., blastocyst formation rates) were arcsine-transformed before an analysis but are presented as non-

transformed means and SEM. Statistical significance was determined at $P \leq 0.05$ and a tendency in statistical significance was defined at $P \leq 0.1$ and > 0.05.

Results

Experiment 1: Nuclear progression of oocytes

An analysis of the effect of IL6, IL11, and LIF supplementation during IVM of bovine oocytes on the progression of meiosis was completed based on previous findings proposing that IL6 and IL6ST aid in the heat-induced hastening of meiotic progression (Rowinski et al., 2020). Mature and immature nuclear stages are shown in Figures 2-5. The effect is expressed as the percentage of oocytes observed to be in metaphase II (MII) after 16h or 22h of maturation. Cytokine treatment had no improved effect on the percentage of oocytes that reached MII at either of these time points. However, IL6 tended to decrease the number of oocytes that reached MII at 22 hours as seen in Figure 6.

Experiment 2: Transcript Abundance Assessments

A set of cumulus competency markers were chosen based on their role in cumulus expansion and their capacity to stimulate meiotic resumption (Park et al., 2004; Downs, 2010; Conti et al., 2012). Cumulus expansion-related transcripts were upregulated upon supplementation of cytokines at IVM as seen in Figure 7. An increased abundance of *AREG* occurred following exposure to LIF for 4h but not with the other treatments. At 4 h IL11 and IL6 tended to increase *TNFAIP6* abundance ($P<0.10$), however, LIF did not. No other transcript differences were detected for 4h or at 22h (See Fig. 8).

Experiment 3: IVF and embryo development outcomes

An analysis of the effects of IL6, IL11, and LIF supplementation during IVM of bovine oocytes on embryo development was completed. LIF has been supplemented at IVM in various studies resulting in discrepant outcomes, whereas IL6 and IL11 effects at IVM have not been evaluated. No effects were detected on cleavage rates, (see Fig. 9). At day 7 IL6 tended to decrease day 7 expanded blastocysts ($P=0.06$), (see Fig. 10). IL11 decreased the number of advanced blastocysts on day 7 ($P=0.03$), whereas IL6 tended to decrease the amount of advanced blastocyst on day 7 ($P=0.07$), (see Fig. 10). IL11 tended to increase day 7 blastocysts out of zygotes ($P=0.07$), (see Fig. 11). IL11 increased the number of regular blastocysts over cleaved zygotes on day 7 ($P<0.006$) (see Fig. 12). IL6 tended to increase the number of regular blastocysts over cleaved

zygotes on day 7 (P=0.06) (see Fig. 12). IL11 (P=0.04) and LIF (P=0.046) increased day 8 blastocyst yield over total zygotes (see Fig. 11). Also, IL11 (P=0.08) and LIF (P=0.06) tended to increase day 8 blastocyst yield over cleaved zygotes (See Fig. 13). IL6 tended to increase the number of regular blastocysts over zygotes on day 8 (P=0.07) (See Fig. 14). Also, IL6 tended to increase the number of regular blastocysts over cleaved zygotes at day 8 (P=0.06) (See Fig. 15). LIF showed a tendency to increase day 8 hatching rates (P=0.11) (See Fig. 14). There were no significant differences in Day 8 morula development (See Fig 16). Day 7 and 8 total regular, expanded, and hatched yields are grouped in Figure 17. Day 8 total blastocyst development yield is displayed in Figure 18.

Experiment 4: Cryo-survivability

The effects of IL6, IL11, and LIF supplementation during IVM of bovine oocytes on the cryo-survivability of embryos was assessed. We observed no treatment effects for re-expansion, hatching, and overall survivability (see Fig. 19).

Discussion

The IL6 cytokine family includes a group of pleiotropic cytokines that act on various cells to aid in differentiation, proliferation, apoptosis, and pro or anti-inflammatory responses (Kang et al., 2020). A well-known member of this family, IL6, has been previously studied by our group. Our group noted IL6's role as an "embryokine" due to its ability to improve bovine embryo development, including improvements in ICM cell numbers via JAK/STAT3, total and primitive endoderm cell numbers, and post-transfer embryonic development (Wooldridge et al., 2019; Wooldridge and Ealy, 2019; Seekford et al., 2021; Wooldridge and Ealy, 2021). In each of these studies, IL6 was supplemented post-fertilization—leaving a gap in knowledge on how IL6 and its other family members may affect IVP embryo development when supplemented prior to fertilization.

I began my quest for exploring the role of IL6 family members in oocyte maturation by identifying the IL6 family member ligands and receptors that were being expressed in bovine COCs. The primary RNA-sequence profiling study I used is from (Walker et al., 2022). This data shows ligand and receptor abundance of IL6 cytokine family members in bovine cumulus and oocyte cells immediately following removal from their follicles (Walker et al., 2022). It is worth noting that other studies have documented *IL6* expression levels nearly absent immediately

following COC removal from its follicle but increases thereafter with peak expression being at 4 hours (Liu et al., 2009; Zhao et al., 2012). Additionally, LIF's relative expression in COCs immediately following COC removal is very low and doesn't peak until 7 hours into maturation (Vendrell-Flotats et al., 2020b). To my knowledge IL11's expression has not been investigated during IVM of oocytes however, its expression has been monitored throughout preovulatory follicle development (Jang et al., 2017). My interest in IL6 as an ookine was also affected by these findings and by other work suggesting that COC-dependent IL6 expression may play a role in the heat-induced acceleration of meiotic progression in cattle (Rowinski et al., 2020). A short-term COC heat shock shifted the timing of *IL6* and *IL6ST* expression 2 hours earlier than thermoneutral COCs (Rowinski et al., 2020). This led us to believe IL6 may be playing a vital role in maturation process of bovine oocytes. IL6 has been supplemented during IVM previously in mice, resulting in improved cumulus expansion (Liu et al., 2009). Additionally, LIF supplementation at IVM has been done more frequently resulting in disparate outcomes in oocyte and embryo development (see Lit review table). These previous studies of IL6 and LIF warranted us to supplement them at IVM in addition to another member of their cytokine family, IL11.

Initially, we debated removing various hormonal additives to our base IVM media prior to supplementing our cytokine treatments. For example, we discussed removing estradiol, FSH, LH, and fetal bovine serum (FBS). We were concerned if we would be able to observe any treatment effects with so many additives already in the IVM media. However, we decided to keep our typical IMV media the same. We did refrain from adding EGF to our IVM media for this study due to its role in meiotic progression.

My first experiment tested if supplementing IL6, IL11, or LIF during IVM alters the normal progression of meiotic maturation. I supplemented these cytokines respectively at the beginning of IVM and let COCs mature for 16 or 22 hours. I was interested in these two timepoints to assess if there was any difference in the number of COCs reaching metaphase II earlier than their anticipated time of 22 hours. Additionally, previous work showed that heat shocked COCs undergoing IVF 4-6 hours earlier helped improve blastocyst development due to these COCs maturing at a faster rate (Schrock et al., 2007; Rowinski et al., 2020). Because supplementation of the cytokines exhibited no significant effect on meiotic maturation, this work supports the contention that IL6, IL11, and LIF are not directly involved with the heat-induced hastening of maturation. However, the temporal changes of IL6 previously described could be a catalytic effect

of signaling that is contributing to this phenomenon. Essentially, IL6 is not the direct cause of this hastening occurrence, but it an effect of it all the same. Future work may want to produce a knockout of IL6 accompanied by the heat shock to observe if this hastening is still occurring in the absence of IL6.

My next study utilized previously validated cumulus markers of COC competency to describe whether any of the cytokines under investigation affect cumulus cell activity during IVM. The transcripts we chose are tumor necrosis factor α-induced protein 6 (*TNFAIP6),* hyaluronan synthase 2 (*HAS2),* amphiregulin *(AREG),* connexin 37 *(CX37),* and connexin 43 *(CX43).* Connexins 37 and 34 have pivotal roles in folliculogenesis and oocyte maturation, specifically aiding in granulosa communication and gap-junctional communication amongst oocyte and cumulus cells (Kordowitzki et al., 2021). *HAS2*, *AREG*, and *TNFAIP6* are each essential in cumulus expansion (Niringiyumukiza et al., 2018). Only a few of these transcript profiles were influenced by the cytokines, and changes were cytokine-specific. I noted an increased abundance of *AREG* following exposure to LIF at 4h. This coincides with previous work done in mouse and human oocytes where LIF alone in the IVM medium was able to induce cumulus expansion (De Matos et al., 2008). LH-induced GBVD requires mitogen-activated protein kinase (MAPK) activity in the cumulus cells (Su et al., 2004). Being that LIF and IL6 family members typically utilize JAK/STAT3 or Ras-MAPK pathways, De Matos et al. suggest that *in vitro*-induced cumulus expansion may be due to MAPK activity (De Matos et al., 2008). Additionally, *AREG* production in the cumulus cells is stimulated via Prostaglandin E_2 (PGE_2) binding to its receptor on cumulus cells and activating MAPK14 (Shimada et al., 2006). Further work needs to be completed to see how LIF and *AREG* work together *in vitro* to induce cumulus expansion.

I also noted that IL6 and IL11 tended to increase *TNFAIP6* abundance at 4h. *TNFAIP6* allows for extracellular matrix formation of expanded cumulus cells via the stabilization of hyaluronic acid (HA) chains (Nevoral et al., 2015). This transcript is pivotal in cumulus expansion as the phenotype of null mutations in *TNFAIP6* results in severely compromised cumulus expansion (Ochsner et al., 2003). Previous work has detailed IL6 as a paracrine factor contributing to cumulus expansion (Liu et al., 2009). IL6 binds to its soluble receptor in cells prompting cumulus expansion genes to activate (Liu et al., 2009). IL6 cumulus expansion function is regulated via tyrosine kinase A (TrkA) and MAPK activation (Liu et al., 2009; Wang et al., 2014).

Our results showing IL6 displayed a tendency to increase expression of *TNFAIP6* would coincide with these previous findings detailing IL6 as a factor in cumulus expansion.

Other groups noted IL11 not affecting cumulus expansion during the culture of COCs (Jang et al., 2017). It is worth mentioning that LIF was the sole treatment that led to a significant increase in one of the cumulus-specific competency transcripts, and LIF is the only cytokine used whose ligand-specific receptor (LIFR) can participate directly in signal transduction (Metcalfe et al., 2020). IL6 and IL11 belong to the group of cytokines that homodimerize IL6ST, while LIF belongs to the group that heterodimerizes IL6ST with the shared receptor LIF-R (Skiniotis et al., 2008). The group of "tall" cytokines which LIF belongs maintains an additional three membrane-proximal FnIII domains required for signaling that IL6 and IL11 lack (Kurth et al., 2000). IL6 and IL11 each signal via a homodimer of IL6ST while gaining specificity through their ligand-specific receptors IL6R or IL11R which do not participate in signal transduction (Garbers and Scheller, 2013). LIF can signal via both IL6ST and LIFR whereas IL6 and IL11 require IL6ST to emit any signaling. This cascade could potentially be cause for LIF significantly increasing expression of *AREG* while IL6 and IL11 merely showed a tendency to increase *TNFAIP6.*

The most notable outcome of this study was observing improvements in embryo development as a result of supplemented treatments. Based on previous studies supplementing LIF at IVM, this group decided to assess how IL6 and IL11 supplementation affected embryo development. LIF supplementation at IVM produces discrepant results on cleavage and blastocyst rates (De Matos et al., 2008; Vendrell-Flotats et al., 2020b; Tian et al., 2021). It is worth noting these studies utilized various protocols, recombinant protein preparations, concentrations, and culture conditions. We aimed to use a human recombinant version of each supplemented cytokine, purchased from a reputable company at a high level of purity. We also used a consistent concentration of 25 ng/ml based on previous work showing LIF supplementation at IVM produced the most metaphase II oocytes at a 25 ng/ml concentration (Mo et al., 2014). Our results show that LIF improved day 8 blastocyst yield. This finding is consistent with previous work describing LIF's effects on blastocyst development (Amiri et al., 2009; Dang-Nguyen et al., 2014; Vendrell-Flotats et al., 2020b; Tian et al., 2021).

Significant improvements in expanded and advanced blastocysts were observed after IL11 supplementation on day 7. Additionally, IL11 improved day 8 blastocyst yield. While IL6 failed to produce significant improvements in embryo development. IL6 has previously been

supplemented at IVM resulting in improvements in cumulus expansion (Liu et al., 2009) and increased chromosomal deterioration (Banerjee et al., 2012).

Another way we wanted to gauge oocyte competency was by quantifying fertilization rates between treatment groups as a parameter. We observed no treatment effect on cleavage rates. Previous studies have described opposing results on cleavage rates with LIF supplementation(De Matos et al., 2008; Amiri et al., 2009; Vendrell-Flotats et al., 2020a). This could be due to altering concentrations or differences in media formulations.

Although we observed significant improvements in embryo development following treatment, we questioned whether this developmental potential would continue post-cryopreservation. Previous work has shown discrepancies in post-thaw survival outcomes after supplementation of LIF at IVM (Vendrell-Flotats et al., 2020a; Stoecklein et al., 2021). IL6 and IL11's effects on embryo post-thaw survival after IVM supplementation have not been described. We observed no treatment effects for re-expansion, hatching, and overall survivability after cryopreservation. This could be attributed to the suboptimal culture conditions in SOF-based culture medium after day 8 (Ramos-Ibeas et al., 2020; Isaac and Pfeffer, 2021). Previous work from our lab described a decrease in ICM cell numbers between days 8 and 10 in non-treated blastocysts cultured in SOF medium (Wooldridge and Ealy, 2021). Extended culture work detailed embryos cultured in 5iLA, N2B27 with 5 key inhibitors, increased cell numbers at day 8 post fertilization (Brinkhof et al., 2017). Additionally, Ramos-Ibeas and colleagues found that the N2B7 medium supported complete hypoblast migration and epiblast survival *in vitro*(Ramos-Ibeas et al., 2020). Furthermore, unpublished work in our lab shows that day 12 embryos cultured in SOF have a smaller ICM than those cultured in an extended culture N2B27-based medium (Oliver et al., unpublished). The suboptimal culture conditions could have potentially impacted the post-thawing results we observed. Future work should utilize the optimal culture conditions to gain a better understanding of how these treatments affect the post-thaw survival of cryopreserved bovine embryos.

Initially, I was tasked with determining the concentration we would utilize for the cytokine treatments. We decided to use 25 ng/mL based on previous work supplementing human recombinant LIF at IVM (Mo et al., 2014). This group did a dose study supplementing LIF to bovine COCs at IVM where they found that the concentration of 25ng/ml produced significantly more MII-stage oocytes than the other concentrations (Mo et al., 2014). We used 25 ng/ml as a

conserved concentration for each cytokine treatment. We decided to use human recombinants instead of bovine recombinants due to the purity and definite composition the human company provided. However, there have been previous studies utilizing different concentrations of LIF and IL6 at IVM (Liu et al., 2009; Vendrell-Flotats et al., 2020b). Vendrell-Flotats and colleagues supplemented IL6, IL11, or LIF at IVM to assess their effects on microRNA expression (Vendrell-Flotats et al., 2020b). Each cytokine was supplemented at a different concertation in this study. Future work with these cytokines may require testing different concentrations to get a better understanding on their effects of cleavage and blastocyst development.

CONCLUSIONS

Based on these findings, we conclude that LIF and IL11 supplementation at IVM are potentially beneficial to bovine embryo development. LIF increased the abundance of *AREG*, which has a critical role in cumulus expansion. LIF also improved day 8 blastocyst yield over total zygotes. One might consider an "ookine" as a supplement added at IVM that leads to improvements in embryo development. Being that cumulus expansion is crucial in the maturation process, one can suggest that a supplement improving a key transcript in this process has the potential to improve embryo development. IL11 improved day 7 and day 8 blastocyst development. This suggests IL11 and LIF would both be beneficial supplements to IVM media in order to enhance bovine embryo development. IL6 may be required for maturation based on previous work done by others (Liu et al., 2009; Rowinski et al., 2020). Liu and colleagues concluded that IL6 alone could induce cumulus expansion in mice oocytes (Liu et al., 2009). Rowinski and colleagues' results suggested that IL6 had a role in the heat-induced hastening of oocyte maturation (Rowinski et al., 2020). However, in our supplementation study, IL6 did not show any significant improvements in oocyte maturation or blastocyst development.

Table 1. Primers used for amplification in real-time PCR

Genes	Primer Sequence	Expected Fragment size (bp)	Annealing Temperature (°C)	Reference
AREG	F: F: 5′-CTTTCGTCTCTGCCATGACCTT-3′ R: 5′-CGTTCTTCAGCGACACCTTCA-3′	100	60**°C**	(Caixeta et al., 2013)
CX37	F: 5′-GACTCATCTCCCTGGTGCTC-3′ R: 5′-GTTCTGCTCACTGGACGACA-3′	97.0	60**°C**	(Sabry et al., 2021)
CX43	F: 5′-GTCTTCGAGGTGGCCTTCTTG-3′ R: 5′-AGTCCACCTGATGTGGGCAG-3′	101.9	60**°C**	(Sabry et al., 2021)
TNFAIP6	F: 5′-GGCTTCCCAAATGAGTATGA-3′ R: 5′CCACAGTATCTTCCCACAAA-3′	185	60**°C**	(Diógenes et al., 2017)
HAS2	F: 5′-GGGTTCTTCCCTTTCTTTCT-3′ R: 5′-CCACCCAGCTTTGTTTATTG-3′	240	60**°C**	(Diógenes et al., 2017)
HPRT1	F: 5′-TGCTGAGGATTTGGAGAAGG-3′ R: 5′-CAACAGGTCGGCAAAGAACT-3′	169	60**°C**	(Goossens et al., 2005)

Table 2. Expression of IL6 family cytokine ligands in bovine cumulus and oocyte cells

	Oocyte		Cumulus	
	M	SD	*M*	SD
Ligands				
IL6	ND	ND	ND	ND
IL11	ND	ND	ND	ND
IL27	ND	ND	ND	ND
IL31	ND	ND	ND	ND
CNTF	ND	ND	ND	ND
OSM	ND	ND	ND	ND
CT-1	ND	ND	3.13	1.97
CLCF	6.08	2.02	ND	ND
LIF	ND	ND	ND	ND

Note: SD= Standard deviation, *M*= Mean, ND= Not detected
Metric: Fragments per kilobase per million reads (FPKM)
(Adapted from https://rbej.biomedcentral.com/articles/10.1186/s12958-022-00994-3)

Table 3. Expression of IL6 family cytokine receptors in bovine cumulus and oocyte cells

	Oocyte		Cumulus	
	M	SD	*M*	SD
Receptors				
IL6ST	2.57	0.43	17.37	8.37
OSMR	8.30	1.84	ND	ND
LIFR	8.00	2.41	11.89	4.10
IL11R	ND	ND	5.82	3.01
IL27R	ND	ND	19.44	7.15
IL31R	ND	ND	ND	ND
CNTFR	2.34	0.89	ND	ND
IL6R	ND	ND	9.27	5.46

Note: SD= Standard deviation, *M*= Mean, ND= Not detected
Metric: Fragments per kilobase per million reads (FPKM)
(Adapted from https://rbej.biomedcentral.com/articles/10.1186/s12958-022-00994-3)

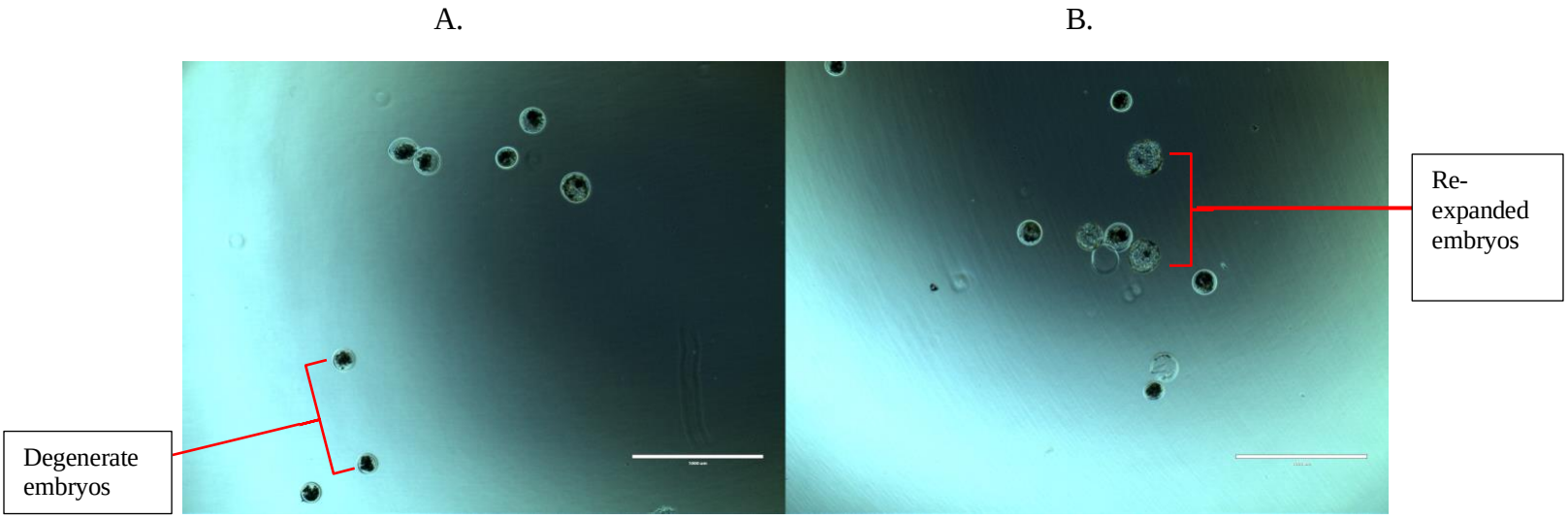


Figure 1. Post-Thawed Bovine Embryos. (A), Degenerate embryos are marked by the red bracket. (**B)**, Re-expanded embryos are marked by the red bracket.

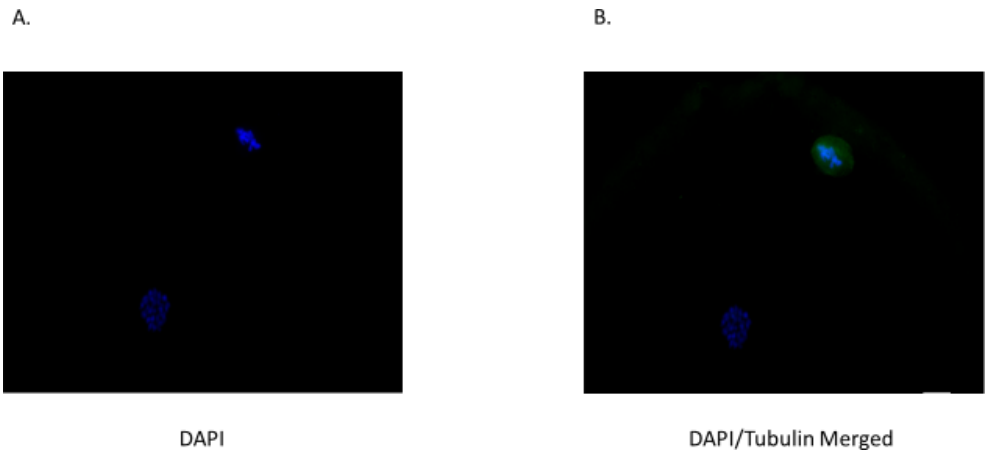


Figure 2. Nuclear staining of denuded bovine oocytes (A), DAPI (B) DAPI and Tubulin stain merged. This figure shows an oocyte at MII stage via nuclear and microtubule staining at 60x.

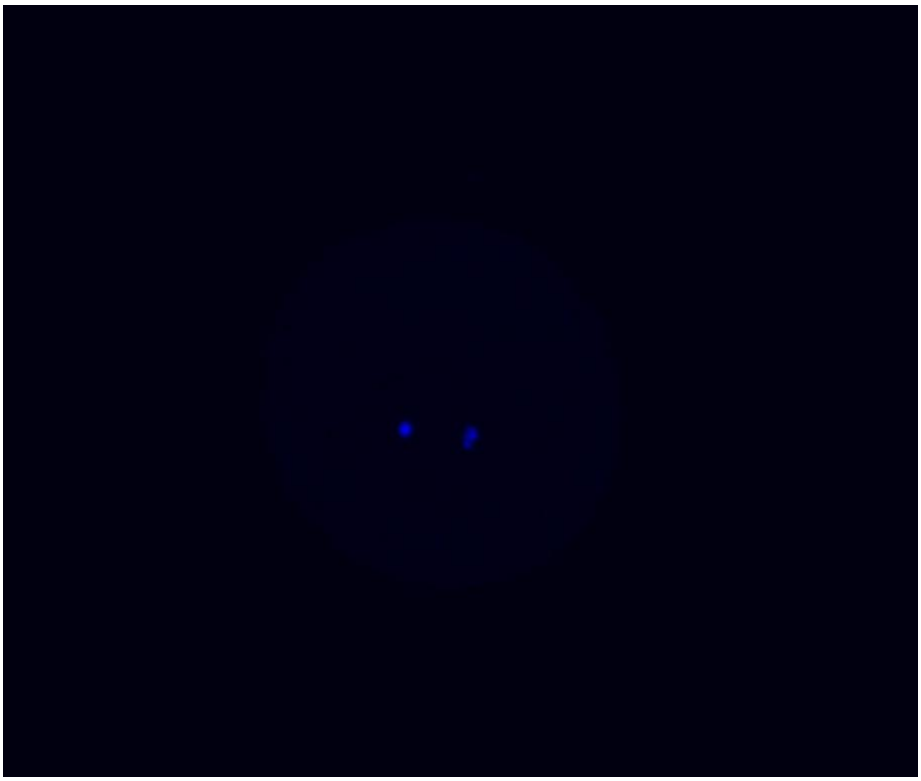


Figure 3. Nuclear staining of denuded bovine oocyte via DAPI stain This figure shows an oocyte at MII stage via nuclear staining at 20x.

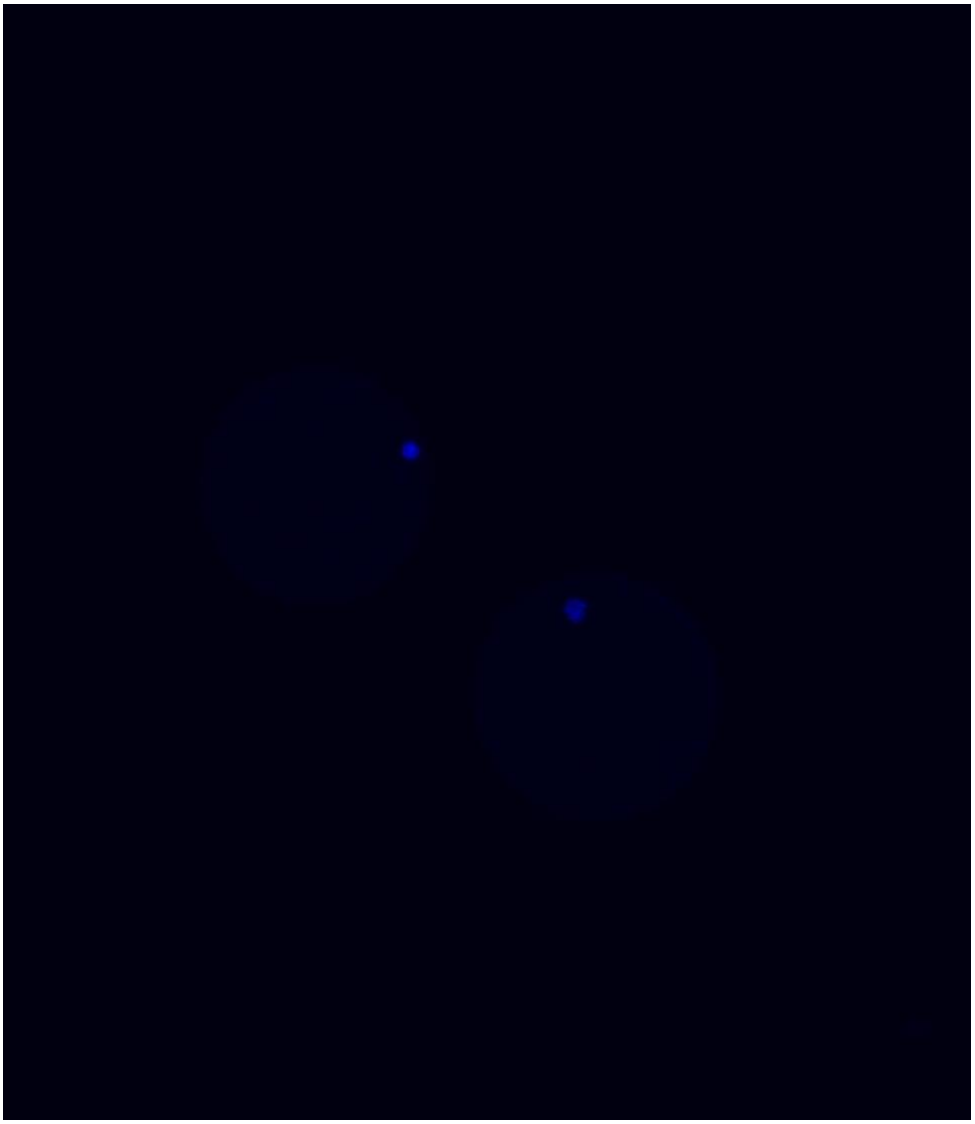


Figure 4. Nuclear staining of denuded bovine oocytes via DAPI stain, figure shows immature oocytes lacking a polar body via nuclear staining at 20x.

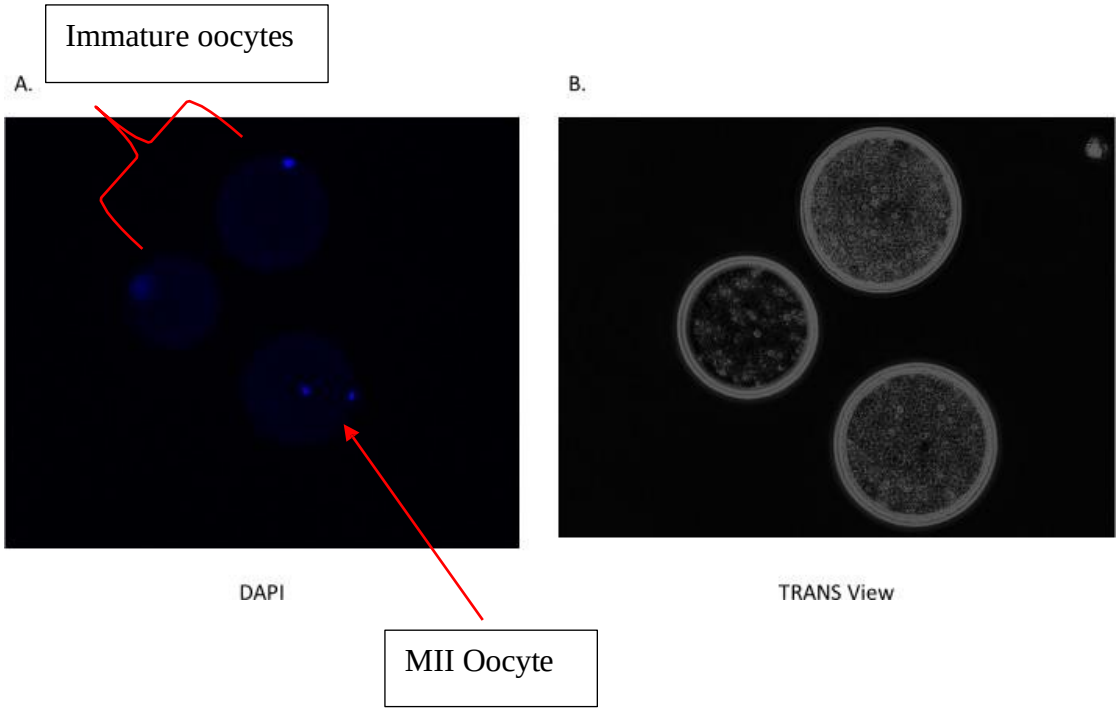


Figure 5. Nuclear staining of denuded bovine oocytes (A), DAPI stain, (B) Trans View respectively. This figure shows 3 oocytes, 1 is at the MII stage and the other 2 are immature.

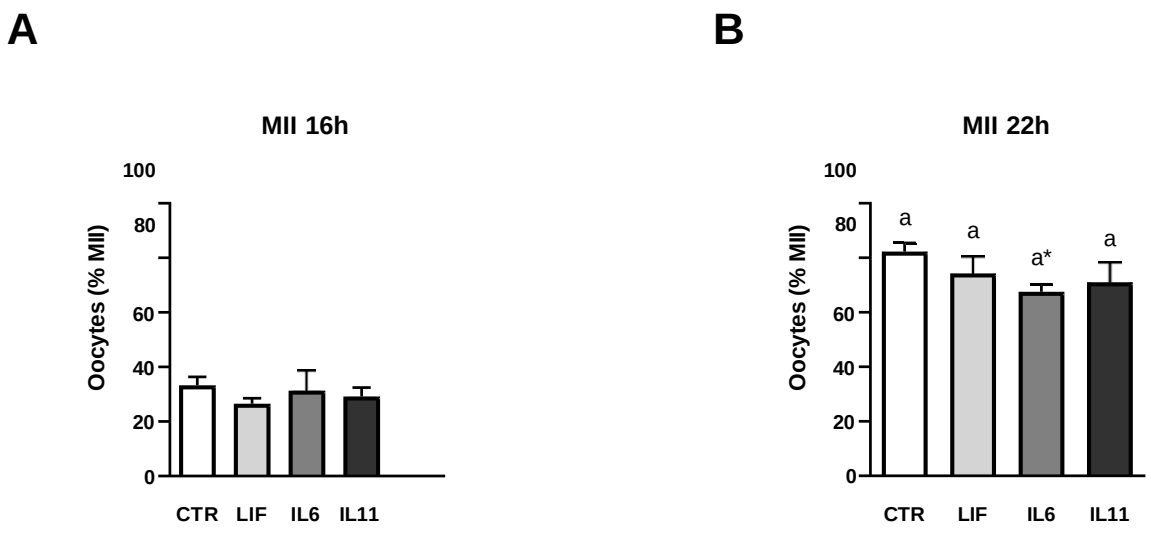


Figure 6. Percentage of oocytes that reached metaphase II at 16hrs or 22hrs. A, oocytes that reached MII at 16h. **B,** oocytes that reached MII at 22h.

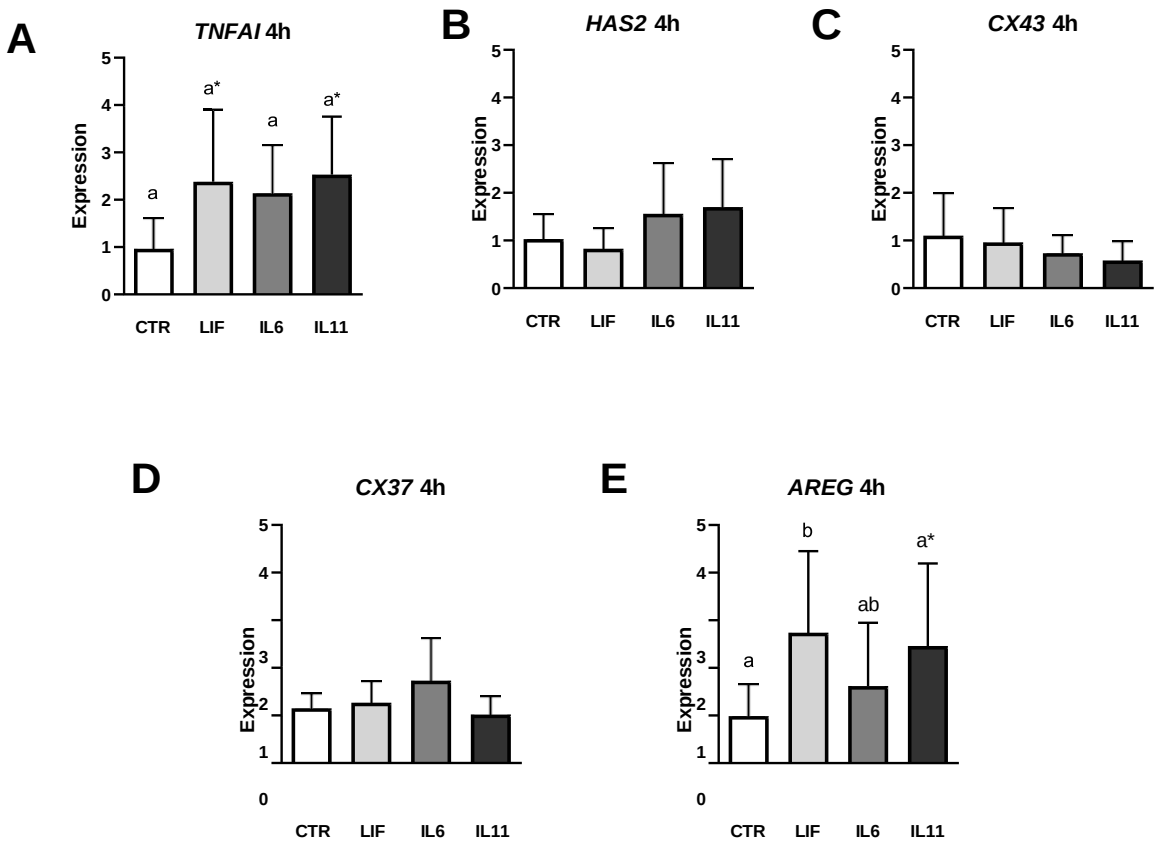


Figure 7. Effect of IL6, IL11, and LIF on *TNFAIP6, HAS2, CX43, CX37,* and *AREG* abundance in COCs after 4h of in vitro maturation. Messenger RNA abundance was measured by real-time PCR. (**A**), *TNFAIP6* expression (**B**), *HAS2* expression(**C**), *CX43* expression (**D**), *CX37* expression (**E**), *AREG* expression in bovine embryos. a-c: letters indicate statistically significant differences between treatments

(P<0.05). * Indicates tendency between treatments (0.05<P<0.1). Data are shown as mean ± SEM relative to a calibrator sample by the ΛλΛλCT method with efficiency correction.

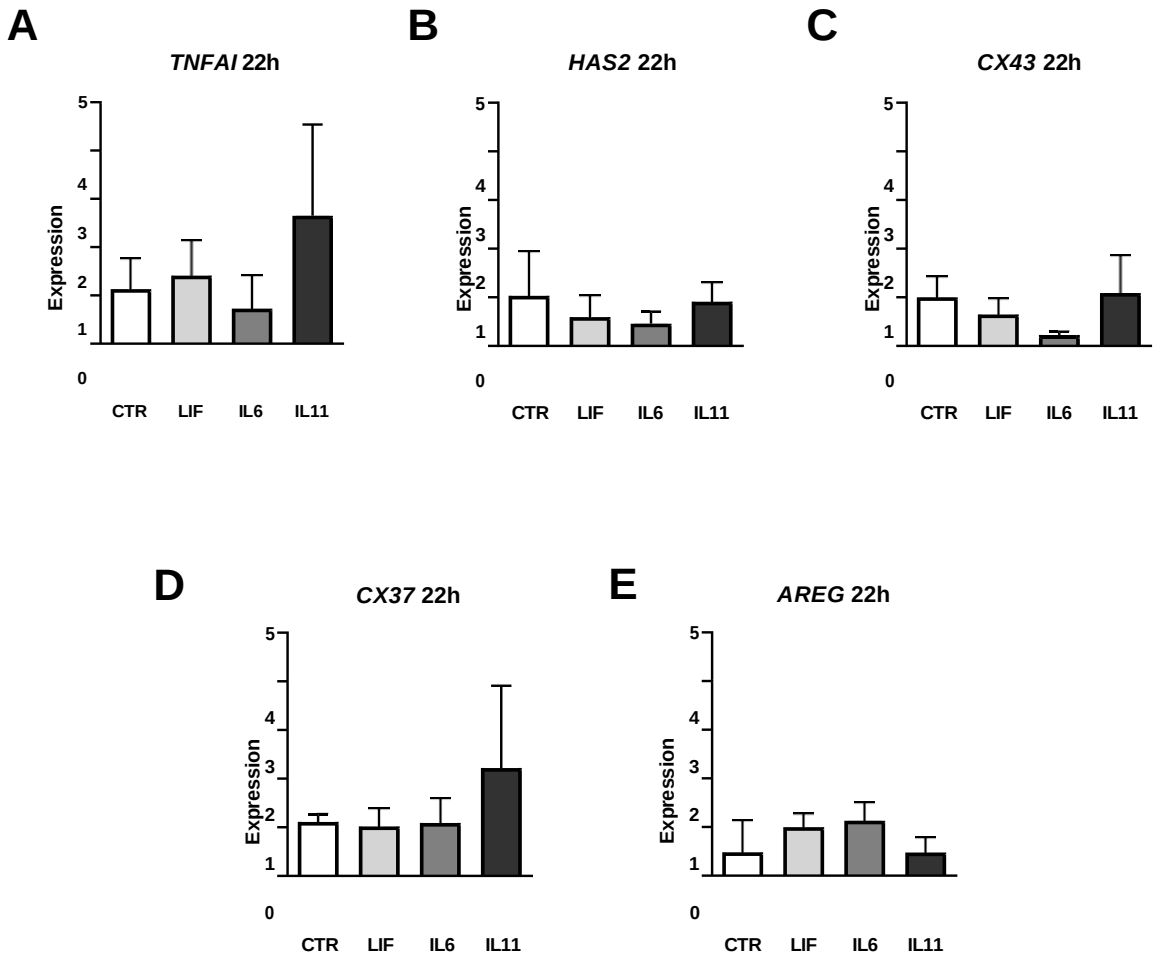


Figure 8. Effect of IL6, IL11, and LIF on *TNFAIP6, HAS2, CX43, CX37,* and *AREG* abundance in COCs after 22h of IVM. Messenger RNA abundance was measured by real-time PCR. (**A**), *TNFAIP6* expression (**B**), *HAS2* expression(**C**), *CX43* expression (**D**), *CX37* expression (**E**), *AREG* expression in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM relative to a calibrator sample by the ⦚CT method with efficiency correction.

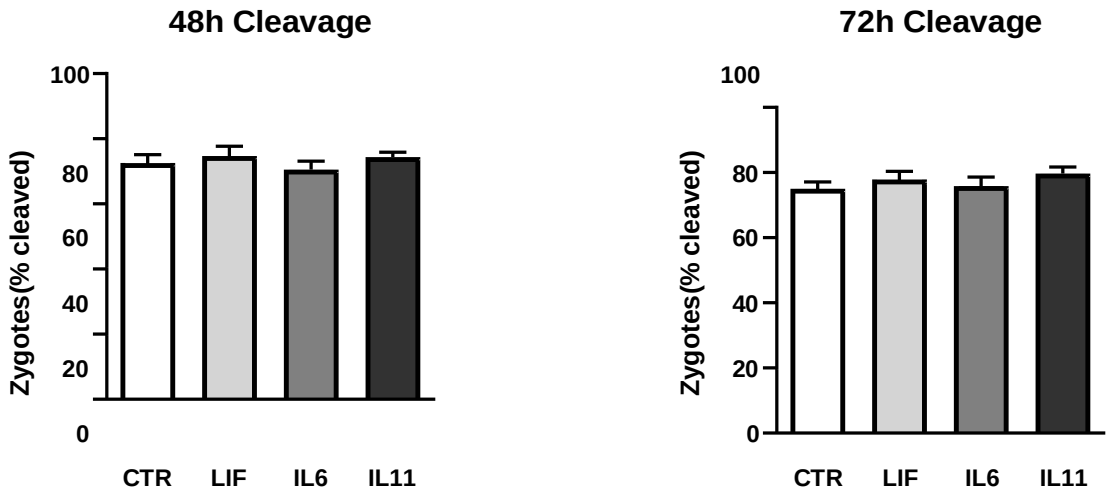


Figure 9. **Cleavage rates 48- and 72-hours post-fertilization.** Cleavage rates at 48 and 72-hours post-fertilization. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

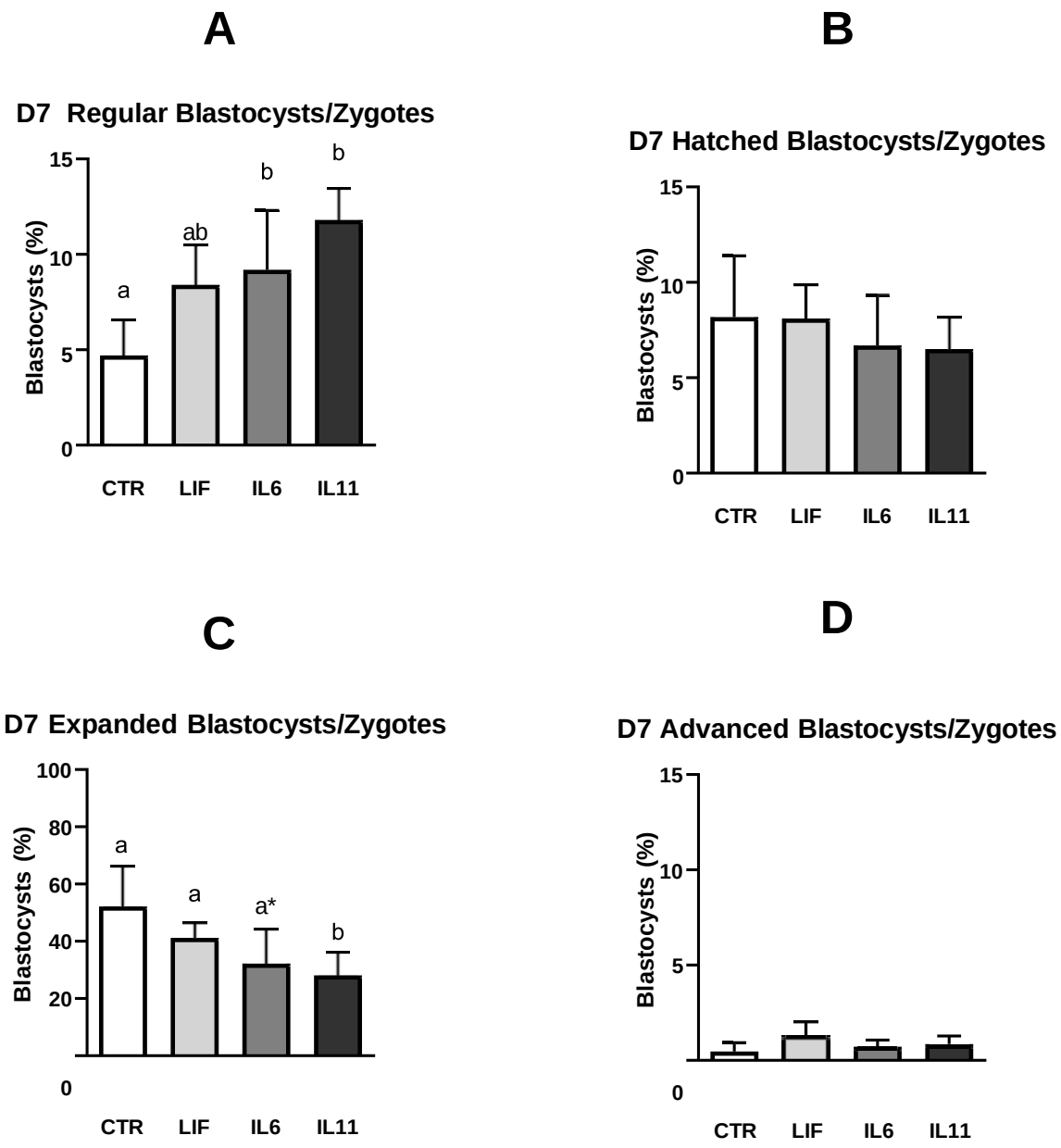


Figure 10. Day 7 regular, expanded, and hatched blastocysts out of total zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of D7 blastocysts: (**A**), D7 Regular Blastocyst (**B**), D7 Expanded Blastocyst (**C**), D7 Hatched Blastocysts (**D**), D7 Advanced Blastocysts in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments($0.05<P<0.1$). Data are shown as mean ± SEM.

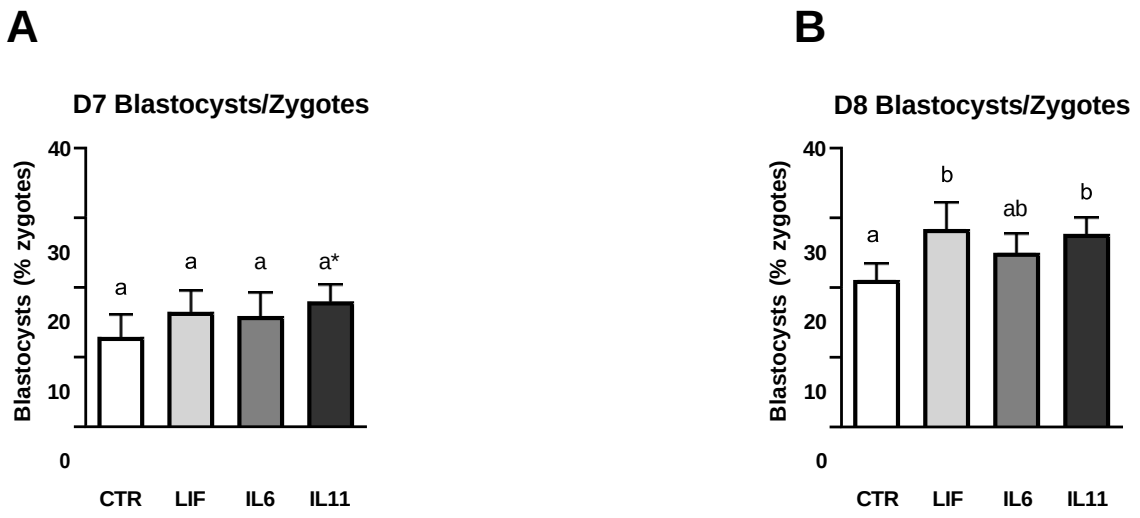


Figure 11. Day 7 and Day 8 total blastocysts out of zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Blastocyst/Zygotes (**B**), D8 Blastocyst/Zygotes in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

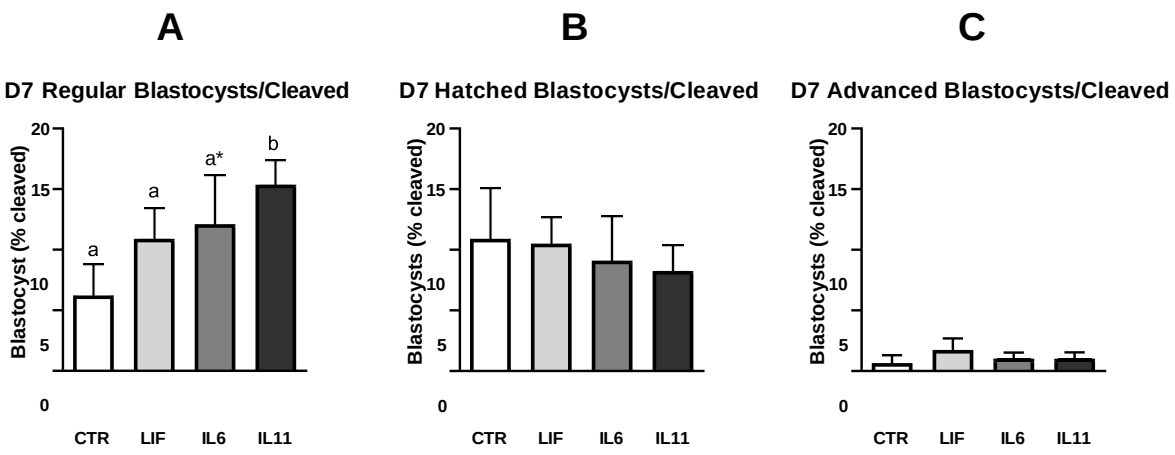


Figure 12. Day 7 Regular, Hatched, and Advanced blastocyst out of cleaved zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Regular Blastocysts/Cleaved (**B**), D7 Hatched Blastocysts/Cleaved (**C**), D7 Advanced Blastocysts/Cleaved in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

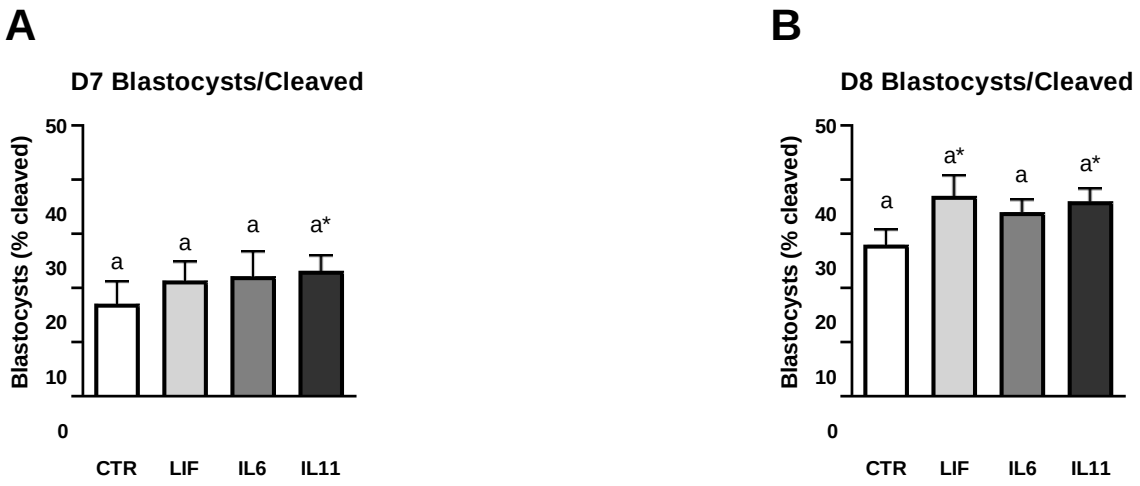


Figure 13. Day 7 and Day 8 Blastocyst yield over cleaved zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Blastocyst/Cleaved (**B**), D8 Blastocyst/Cleaved in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

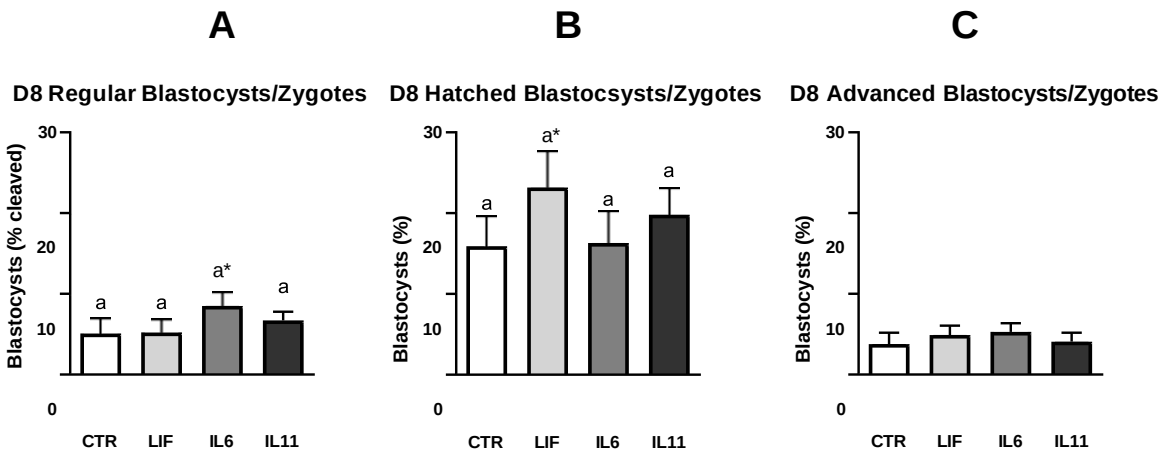


Figure 14. Day 8 Regular, Hatched, and Advanced blastocysts out of total zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Regular Blastocysts/Zygotes (**B**), D7 Hatched Blastocysts/Zygotes (**C**), D7 Advanced Blastocysts/Zygotes in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

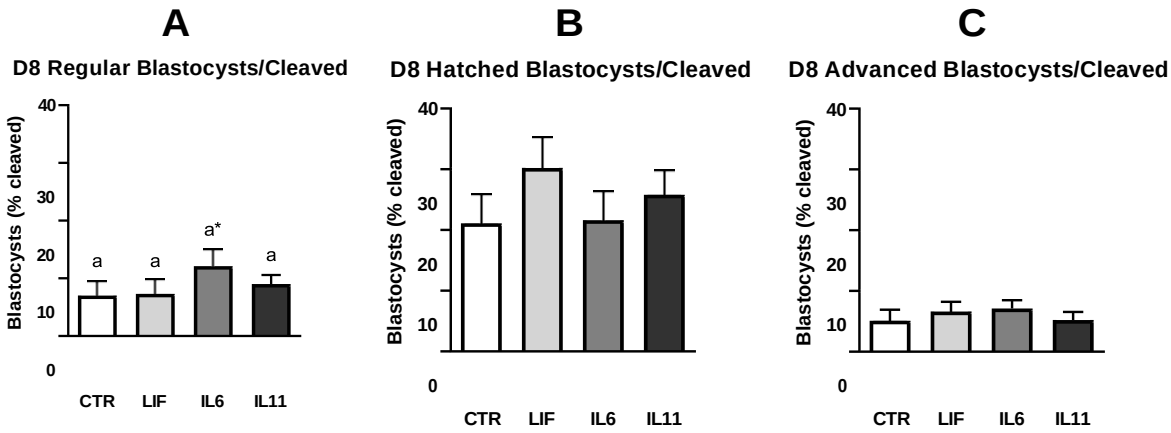


Figure 15. Day 8 Regular, Hatched, and Advanced blastocysts out of cleaved zygotes. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Regular Blastocysts/Cleaved (**B**), D7 Hatched Blastocysts/Cleaved (**C**), D7 Advanced Blastocysts/Cleaved in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

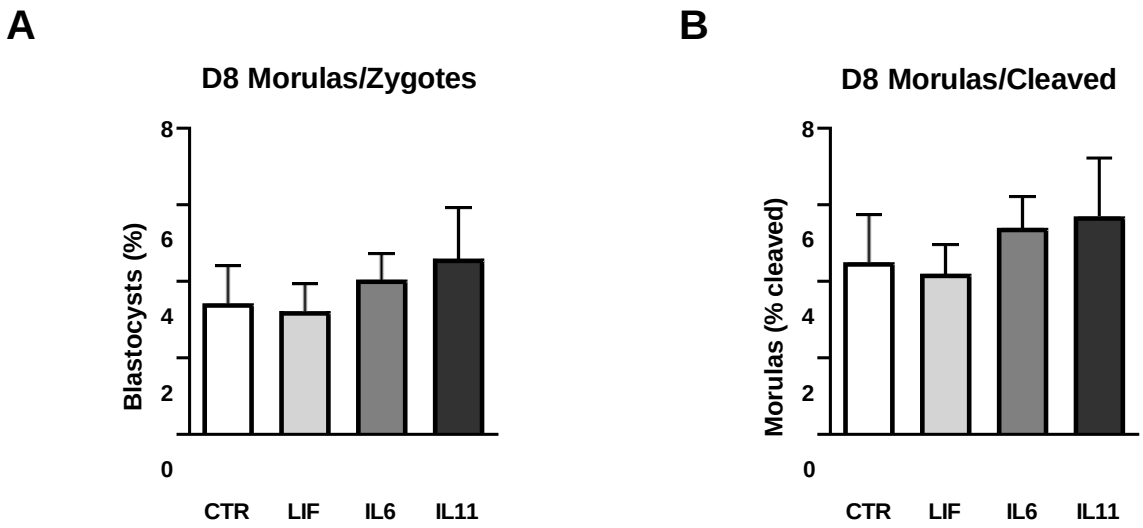


Figure 16. Effect of IL6, IL11, and LIF on D8 morula development. (**A**), D8 morulas out of total embryos plated (**B**), D8 morulas out of total cleaved zygotes. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

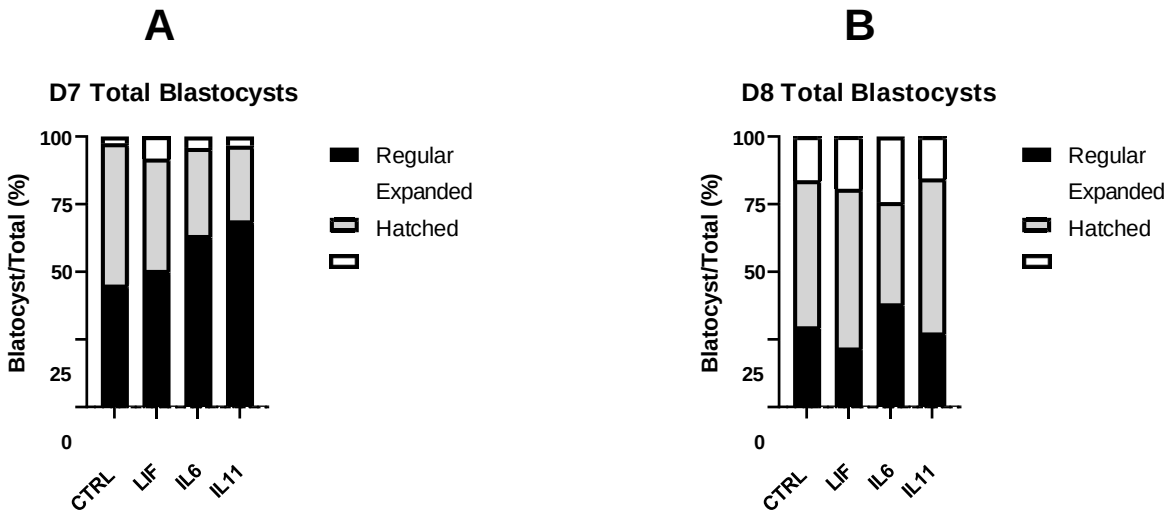
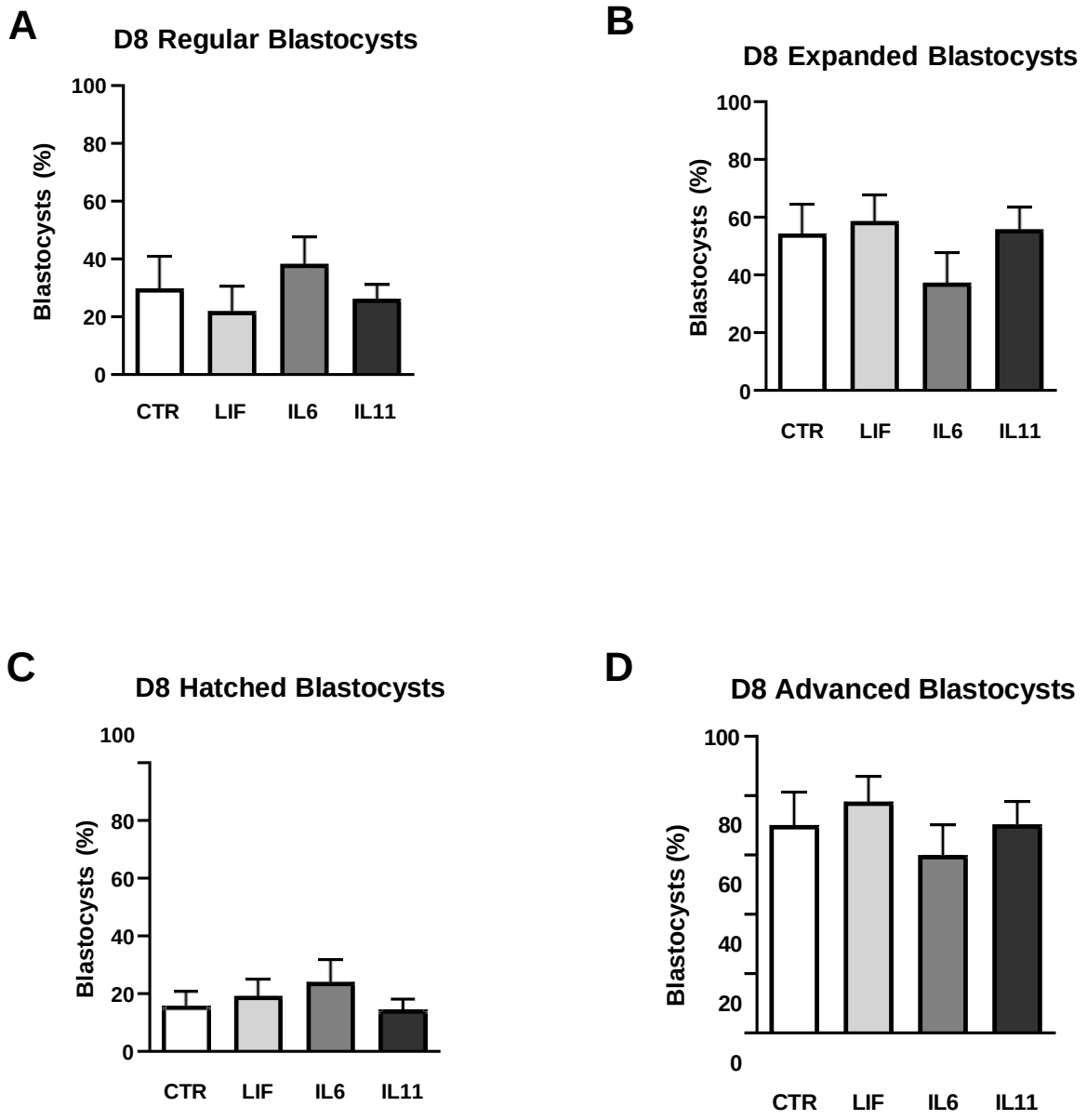
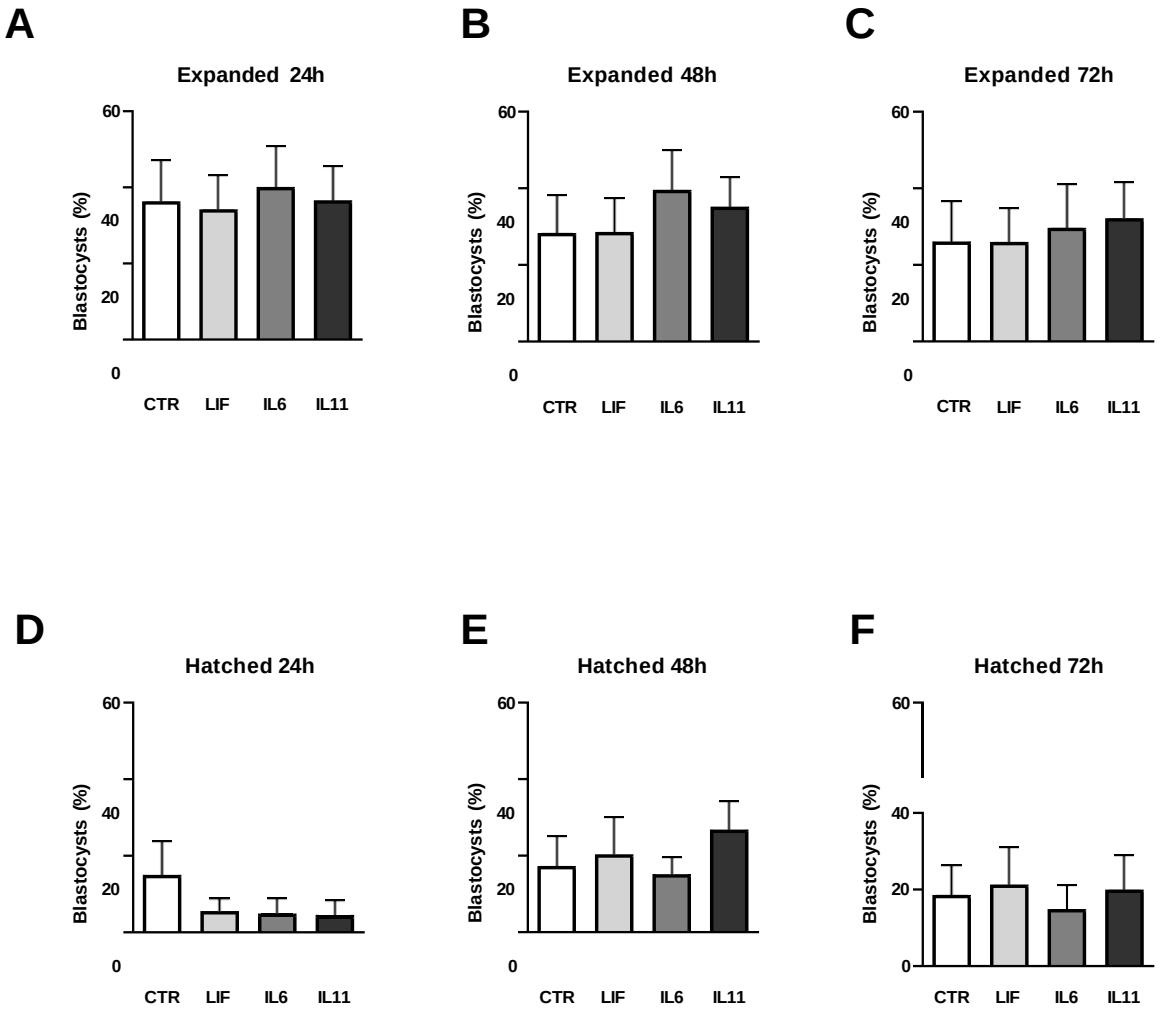


Figure 17. Effect of IL6, IL11, and LIF on Day 7 and Day 8 blastocyst development. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of blastocysts (**A**), D7 Regular, Expanded, and Hatched Blastocyst (**B**), D Regular, Expanded, and Hatched Blastocysts in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.



<u>Figure 18</u>. Day 8 blastocyst development. COCs were exposed to either 0 (CONT) or 25 ng/ml recombinant human IL6, IL11, or LIF at IVM. Percent of D8 blastocysts: (**A**), D8 Regular Blastocyst (**B**), D8 Expanded Blastocyst (**C**), D8 Hatched Blastocysts (**D**), D8 Advanced Blastocysts in bovine embryos. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.



<u>Figure 19</u>. Survival and development of slow-frozen thawed D8 bovine embryos after supplementation of IL6, IL11, and LIF at IVM. (**A**), Expanded blastocysts 24h post-thaw (**B**), Expanded Blastocyst 48h post-thaw (**C**), Expanded Blastocysts 72h post-thaw (**D**), Hatched Blastocysts 24h post-thaw (**E**), Hatched blastocysts 48h post-thaw (**F**), Hatched blastocysts 72h post-thaw. a-c: letters indicate statistically significant differences between treatments ($P<0.05$). * Indicates tendency between treatments ($0.05<P<0.1$). Data are shown as mean ± SEM.

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