All Theses   Theses

5-2024

Photobiomodulation of Bovine Oocytes During Maturation Increases ATP Content and Enhances Subsequent Embryonic Development

Kendall Richey
kdriche@clemson.edu

Recommended Citation

PHOTOBIOMODULATION OF BOVINE OOCYTES DURING MATURATION

INCREASES ATP CONTENT AND ENHANCES SUBSEQUENT EMBRYONIC

DEVELOPMENT

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Science

by
Kendall D. Richey
May 2024

Accepted by:
Dr. Celina M. Checura
Dr. Matthew J. Hersom, Committee Chair
Dr. William Bridges

ABSTRACT

Background

Bovine *in vitro* production (IVP) of embryos is a growing field for producing offspring with desirable genetics in the dairy and beef cattle industries and from prepubertal heifers, pregnant and older cows. While IVP is an effective method of embryo production, oocyte cytoplasmic maturation is compromised, and bovine oocytes matured *in vitro* have reduced metabolic activity than those matured *in vivo*. Mitochondria are the central unit of oocyte metabolism, producing ATP through oxidative phosphorylation. Photobiomodulation is a light treatment reported to improve metabolic activity. It has been proposed that mitochondria have a photoreceptor for red light in the electron transport chain. In five separate experiments, we measured the effects of photobiomodulation treatment with red LED (660-665 nm) at h 16 (L-16) and 20 (L-20) of the bovine oocyte *in vitro* maturation on subsequent embryonic development, ATP concentration, mitochondrial membrane potential, and nuclear progression through meiosis.

Methods

Bovine cumulus-oocyte complexes were aspirated from abattoir ovaries and matured following a standard protocol. In Experiments 1 and 3, bovine oocytes exposed to L-16, L-20 and Control treatment were fertilized and cultured. The cleavage- and blastocyst rates and blastocyst cell number were assessed. In Experiments 2, 4, and 5, mitochondrial activity – cytoplasmic ATP levels and mitochondrial membrane potential were measured at 24 h or over time.

Results

In Experiments 1 and 3, bovine oocytes treated with red light during maturation, L-16 and L-20, had higher embryonic development rates and cell numbers than Control. In Experiments 2, 4,

and 5, photobiomodulation treatment L-16 increased cytoplasmic ATP levels during maturation but did not affect mitochondrial membrane potential, and there was no effect of L-20 treatment on cytoplasmic ATP levels or mitochondrial membrane potential compared to its Control.

Conclusion

Treatment of bovine oocytes at h 16 and 20 of *in vitro* maturation with red LED (660-665 nm) for 10 minutes significantly improved embryonic development, blastocyst cell number and ATP production during oocyte maturation. Conversely, the treatments did not impact mitochondrial membrane potential. These results unfold a new area to study incorporating photobiomodulation treatments in the IVF industry.

DEDICATION

To my family, friends, mentors, fellow students, and community for supporting me through this journey.

Yours Lord, is the greatness, the power, the glory, the victory, and the majesty. For all that is in the heavens and on the earth is Yours. Yours is the kingdom, Lord, and You are exalted as head above all. – 1 Chronicles 29:11

ACKNOWLEDGMENTS

I want to take this time to acknowledge the many who have helped me along this path. I would like first to take the time to thank my committee members, Dr. Celina M. Checura, Dr. Matthew Hersom, and Dr. William Bridges. Their support, time, and dedication to my education and professional development have brought me to this point. I am beyond thankful for their mentorship in my education, experience, and personal life. I will do my best to represent them as a Clemson University graduate.

Fellow graduate students, undergraduates, and Hannah Culler, thank you for your constant support, encouragement and check-ups on my mental and physical state. Your words of wisdom, stories of past experiences, comments that bring joy and laughter and questions to challenge me are forever engrained in my mind. I am so thankful for all the relationships I have formed with you, and I appreciate your quirks.

To my family and soon-to-be-husband, I cannot thank you enough for your loving support of me through this journey. Thank you for listening to seminars about semen and embryos while I bake in the kitchen. Thank you for the hugs and words of encouragement when my experiments failed. Thank you for believing in me and never giving up on me, even when I wanted to. This degree is not just mine but all of yours as well.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure   Page

List of Figures (Continued)

Figure   Page

LITERATURE REVIEW

CHAPTER 1

BOVINE REPRODUCTION AND *IN VITRO* PRODUCTION OF EMBRYOS

The Cattle Industry

Since the domestication of the bovine species, bovine products have become a staple of the human diet and everyday life. Products such as milk, butter, and beef dominate the agricultural market. As the world population continuously grows, there is an increased need to produce the most efficient and healthy animals. The bovine digestive system contains a section of a four-chambered compartment. One chamber called the rumen houses millions of microbes capable of digesting cellulose and other nutrients indigestible to humans. The rumen allows the conversion of low-quality forages and indigestible nutrients into high-quality meat and milk proteins digestible for humans (Oerly et al., 2022). In order to convert indigestible nutrients into digestible nutrients through the bovine rumen, feed and production efficiency need to be maximized. At the same time, negative impacts on the environment and animal health should be reduced. Genetic selection and breeding for specific characteristics are the main points of interest in maximizing cattle production. Therefore, reproduction in the cattle industry is a significant factor in selecting the best bull and cow for a more efficient offspring. Understanding bovine reproduction, factors that affect reproduction success and how to produce more efficient offspring is increasingly essential.

Oogenesis and Folliculogenesis

Oocytes are spherical cells that are the female gamete, capable of producing offspring after fertilization by the male gamete spermatozoa, or sperm (Senger, 2006). Oocytes are formed

during fetal development, and a mammalian female will be born with the entirety of her oocytes for her lifetime. The formation of oocytes is known as the process called oogenesis. The mammalian primordial germ cell (PGC) migrates to the gonadal ridge of the fetus, and after the establishment of the female sex, the PGCs develop into oogonia. These oogonia have one complete set of homologous chromosomes, 2N, and complete mitosis to produce millions of oogonia (2N). Oogonia enter meiosis I and develop into primary oocytes (4N) that arrest in what is known as the diplotene stage of prophase I. Primary oocyte nuclear progression remains quiescent while the cytoplasm develops. Meiosis I of the primary oocyte resumes after the Luteinizing Hormone (LH) surge in post-pubertal animals (He et al., 2021).

In post-pubertal animals, gonadotropin-releasing hormone (GnRH) is released into the hypophyseal portal circulation from the hypothalamus and received by the gonadotropic cells along the anterior pituitary gland. These cells trigger the release of follicle-stimulating hormone (FSH), which acts on ovarian follicles for follicular growth and development, or folliculogenesis. Folliculogenesis, the growth and development of a follicle, and oogenesis are physiological processes where follicular cells of the ovary and immature oocytes communicate to produce a mature, fertilization-ready oocyte (Liu et al., 2015; Rimon-Dahari et al., 2016). It is hypothesized that folliculogenesis begins as early as the migration of PGCs to the embryonic genital ridge (Ginsburg et al., 1990). The PGCs with pre-granulosa cells are housed in primordial follicles, which will either remain quiescent or begin growing. Pre-granulosa cells transition into granulosa cells (GCs), and it is not until the GCs transform from their flattened morphology into cuboidal structures that primordial follicles become primary follicles (Cox & Takov, 2020). After the onset of puberty, follicular development proceeds in waves in the bovine ovary. The primary follicle becomes a secondary follicle, differentiated by multiple layers of proliferating GCs.

Secondary follicles become preantral follicles (tertiary follicles) by forming theca externa and interna cells to surround the follicle and provide androgens (hormonal substrates) for estrogen production by GCs. Preantral follicles fill with fluid to develop a collective antrum and transition into antral follicles. These follicles are characterized by mural cells lining the follicle wall surrounding the antrum. Specialized granulosa cells called cumulus cells surround the oocyte, forming the cumulus-oocyte complex (COC), and the granulosa cells that are physically in contact with the oocyte zona pellucida and form gap junctions for follicle-oocyte communication during oocyte maturation, are called corona radiata cells. Several antral follicles form, but only one dominant preovulatory follicle responds to a peak in LH known as the LH surge (Tsafriri et al., 1972; Rimon-Dahari et al., 2016). The LH surge prompts the oocyte to re-enter meiosis I, progressing to metaphase II (MII) of meiosis II (2N; Tsafriri et al., 1972). Once arrested at MII, the COC is ovulated and captured by the fimbriae in the infundibulum section of the oviduct. The COC travels to the ampullary-isthmic junction and awaits fertilization or degradation (Senger, 2006).

Bovine Reproductive Cycle

In the bovine, the reproductive cycle is known as the estrous cycle. The estrous cycle is a 21-day period in which the pubertal heifer/cow can be successfully bred. The estrous cycle is divided into four stages within the (1) follicular or (2) luteal phases. The follicular phase is only approximately 20% of the bovine estrous cycle and consists of proestrus and estrus. Proestrus is a short two-to-five-day period where progesterone levels from the previous estrus cycle are declining, and the reproductive tract prepares for estrus. The FSH levels have peaked, and after several LH pulses, an LH surge initiates the dominant preovulatory follicle to release the oocyte from meiotic arrest and complete maturation. Estrus is a 6-24 h period characterized by sexual

receptivity of the female bovine (cow or heifer) to the bull and ovulation. Immediately following ovulation, the cow/heifer begins the phase known as metestrus, beginning the luteal phase, which is approximately 80% of the estrous cycle. Metestrus lasts three to five days and is the critical period of embryonic development if fertilization has occurred. Metestrus is characterized by a corpus hemorrhagic that secretes low levels of progesterone and slowly begins to form a functional corpus luteum, a process known as luteinization. Diestrus soon follows, lasting from day 5 to day 17 after estrus. This is when the corpus luteum is fully functional and progesterone levels are high and maintained. In successfully bred cows/heifers, the corpus luteum persists in secreting progesterone for embryonic development and uterine implantation. It is not until approximately two months later that the placenta forms and takes over progesterone secretion to carry the calf to term (9 months; Reviewed in: Wiltbank et al., 2018). Without a viable embryo, prostaglandin F2α is released by the uterus, which is luteolytic (lysis of the corpus luteum), and progesterone drops. The corpus luteum regresses into a corpus albicans, diestrus ends, and proestrus begins again (Senger, 2006).

Background of Bovine Embryo In Vitro Production

Over the past 40 years, assisted reproductive technologies (ART) have rapidly developed and significantly impacted the bovine industry. Bovine embryos can be *in vitro* produced (IVP) or *in vivo* derived (IVD). *In vitro* production of embryos includes collecting bovine immature or mature oocytes from a donor cow using ultrasound-guided ovum pickup (OPU) (Callesen et al., 1987; Reviewed in: Wagtendonk-de Leeuw 2006) or from abattoir bovine ovaries. The cumulus-oocyte-complexes collected go through *in vitro* maturation (IVM) for immature COCs only, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) to the preimplantation embryo stage. The *in vivo* derived technique requires superovulation of a donor cow, fertilization via artificial

insemination (AI) or natural cover, the growth of embryo(s) within the reproductive tract of the donor cow and flushing of the embryo(s). *In vitro-produced* and *in vivo-derived* embryos are sorted and selected based on their quality. They can either be (1) cryopreserved for later use or (2) immediately implanted into a recipient cow using embryo transfer (ET). Bovine IVP and IVD embryos have enhanced the beef and dairy industries' abilities to produce offspring with desirable genetics in a shorter period. Additionally, one female can produce multiple offspring in one year when otherwise she would have only produced one. Though both IVP and IVD are effective, IVP is more efficient than IVD because embryos can be cultured from prepubertal heifers, pregnant or older cows of desired genetics (Demetrio et al., 2022). Additionally, by mimicking the environment of the ovary and oviducts through changes in the medium during the oocyte maturation, fertilization, and embryo development processes inside atmosphere and temperature-controlled incubators, IVF became a more efficient and standardized method of cattle reproduction (Reviewed in: Sirard, 2018). In 2021, 79.7% of transferable embryos globally were IVP (Viana, 2022), and this trend only seems to be growing.

 Abattoir Ovaries. One of the many discoveries that aided in optimizing IVP conditions was using COCs obtained from slaughterhouse ovaries (Leibfried-Rutledge et al., 1987; Sirard & First, 1988). Preceding aspiration of immature COCs by OPU, abattoir derived immature COCs were matured, fertilized, and cultured into embryos for transfer or cryopreservation (Reviewed in: Sirard, 2018). However, as the excised ovaries are dying tissues, COCs must be aspirated from the follicles approximately 4 h post-harvest for optimal blastocyst development (Blondin et al., 1997). Abattoir-derived COCs were initially a significant discovery that aided in optimizing media compositions and IVF protocols.

In Vitro Maturation. In 1977, it was discovered that although oocyte maturation relies on the follicle's growth and development, oocyte maturation is possible outside the follicle. The *in vitro* maturation of bovine oocytes is achievable by adding specific gonadotropins, amino acids, carbohydrates, antioxidants, and antibiotics to the in vitro maturation (IVM) media (Thibault, 1977). However, since the introduction of *in vitro* maturation into the process, the composition of the media has not changed much over the past 30 years (Reviewed in: Sirard, 2018).

One of the most critical aspects of IVP of embryos is the quality of the COC throughout maturation. Oocyte competence is defined as the ability of the oocyte to develop into a preimplantation embryo. Upon removal of immature COCs from the follicle, meiosis I resumes and progresses to metaphase II (MII) for fertilization. While nuclear maturation is spontaneous and likely to occur, cytoplasmic maturation depends on the culture conditions and COC quality. Oocyte cytoplasmic homogeneity during maturation determines preimplantation embryo development and quality (Reviewed in: Sirard, 2018). The correct spindle fiber arrangement, organelle distribution, and energy metabolism all contribute to successful, competent embryonic development, and the *in vitro* maturation of bovine oocytes yields lower blastocyst quality than COCs that are *in vivo* matured or derived (Liebfried-Rutledge et al., 1987; Dieleman et al., 2002; Barfield, 2015).

Factors Affecting Oocyte Maturation. There are several factors affecting donor collected or abattoir oocyte *in vitro* maturation, such as composition and type of media, pH of the media, temperature of the media and incubator, humidity within the incubator, atmospheric conditions of the incubator, and handling of the oocytes after the onset of maturation (Demetrio et al. 2022). Temperature, pH, and atmospheric gas fluctuations can disrupt regular cellular metabolic activity, leading to increased production of reactive oxygen species (ROS) and pro-apoptotic

6

factors (Reviewed in: Hardy et al., 2021). In addition, many characteristics of the donor cow/heifer (abattoir ovaries included) can affect the quality of the oocyte and subsequent embryonic development, such as the age of the cow/heifer, reproductive status, metabolic disease, the surrounding environment and general physiological health of the animal (Mermillod et al., 2008; Mietkiewska et al., 2022). When collecting oocytes from donor cows for *in vitro* maturation, fertilization, and culture, the ovary size and age of the donor establish the quality and number of oocytes obtained. Additionally, the IVP of embryos from prepubertal heifers is a new approach that allows calves as young as two months with select genetics to produce offspring before they reach sexual maturity. However, oocytes recovered from prepubertal calves have decreased cleavage and blastocyst rates when compared to adult cows (Demetrio et al., 2022; Currin et al., 2021; Warzych et al., 2017; Palma et al., 2008).

Another influential factor is follicle size. Follicle size can affect oocyte quality and competence. However, it does not appear to affect nuclear maturation. The follicles undergo a phase of growth, plateau, and atresia, and the quality of the oocyte varies from stage to stage (Sirard, 2019). Follicles that are small (<3mm) or large (>9mm) yield lower blastocyst rates compared to those that are between 3-9mm in diameter (Luciano & Sirard, 2018; Bezerra et al., 2021). Follicle-stimulating hormone administration in the cow can contribute to follicular growth and subsequent *in vitro* oocyte nuclear progression. Luciano and Sirard (2018) reported that recovery of oocytes after FSH stimulation and a 48 h FSH withdrawal period before OPU delivered the most competent oocytes.

In Vitro Fertilization. Bovine oocytes reach maturity within 24 h after the onset of maturation (0 h); however, studies determined that fertilization at 22 h of -maturation yields higher blastocyst rates 168-192 h post-insemination (PI; Holm et al., 1998; Ward et al., 2002;

Park et al., 2005; Agung et al., 2006; Koyama et al., 2014). Oocytes are not ready to be fertilized before their arrest in metaphase II, and over-matured COCs (>24 h) typically begin degeneration and lead to decreased embryonic development (Hyttel et al. 1986; Reviewed in: Sirard, 2018).

In-vitro fertilization of bovine mature COCs commonly takes place in one of two ways: (1) co-culture of spermatozoa and oocytes or (2) intracytoplasmic sperm injection (ICSI) of individual oocytes. Intracytoplasmic sperm injection is not widely used in the bovine industry. Bull semen is cryopreserved and used as needed (Reviewed in Bahwan, 2022; Arif et al., 2022). In most instances, frozen bull spermatozoa are thawed, washed from cryoprotectants, evaluated for normal morphology and motility, and either diluted for co-culture (at $1x10^6$ or $2x10^6$ motile sperm per mL of media) or sorted for a single sperm to use for ICSI. Semen can be sorted, sexed, and genetically tested to reduce the likelihood of producing the unwanted sex or offspring with less desirable genetics. Both methods have their advantages and disadvantages. One primary disadvantage of the co-culture of mature COCs and bull spermatozoa is the increased probability of polyspermy. Polyspermy can occur when the oocyte does not correctly respond to initial fertilization by a single sperm penetration, and other sperm are capable of penetrating the oocyte. Reduced mitochondrial activity, delayed calcium release, and failure of cortical granule activity (zona hardening) are a few main reasons why polyspermy occurs. Inadequate oocyte cytoplasmic maturation is the prominent cause of polyspermy (Hyttel et al., 1986; Parrish, 2014).

In Vitro Culture. Post-insemination, presumptive zygotes, one cell made of two gamete's genes, are placed into culture, typically denuded of their surrounding cumulus cells. Meiosis II will progress upon successful fertilization, followed by the extrusion of the second polar body (1N of maternal DNA), and two pronuclei will form, one with the male DNA and the other with the female DNA. Syngamy, the fusion of the pronuclei, is brief, and the zygote begins the

ongoing process of mitosis, producing two identical daughter cells called blastomeres (Reviewed in: Unnikrishnan et al., 2021). This first division is called cleavage and is where the cleavage furrow is formed between the daughter cells, and the two cells complete a full division. This first complete cleavage identifies a "cleaved" embryo from an "uncleaved" (likely an unfertilized oocyte). Early embryonic development is usually assessed between 48- and 72 h PI. Based on individual IVF laboratory standards', early embryos are considered competent at ≥4 cells or ≥8 cells at 72 h PI (Oliveira et al., 2019). At the morula stage (≥32 cells), outer blastomeres differentiate into a trophectoderm (outer layer), which will be the placental cells and inner blastomeres become an inner cell mass (ICM), which will become the fetus. A blastocoel cavity forms between the trophectoderm and ICM, distinguishing a blastocyst from a morula (Van Soom et al., 1997; Van Leeuwen et al., 2020). In cattle, blastocyst development occurs ~168 h PI, and in some cases, blastocysts are carried out *in vitro* to 192 h PI.

In bovine IVP, average blastocyst rates are lower than their respective cleavage rate and oocyte nuclear maturation rates, with only 40% of blastocysts developing from an average of 70% cleaved embryos and 90% matured COCs (Ferre et al., 2020). Reduced embryonic development rates limit the number of potential pregnancies. The selection of bovine embryos for transfer is based on the morphology of the blastocyst, which makes the process very subjective. There are many efforts to create standards determining embryonic quality for transfer based on the preimplantation embryos' physiological, genetic, and metabolic characteristics. These preimplantation embryonic characteristics can then be associated with an increased or decreased pregnancy rate and specific calf characteristics (Wang et al., 2022). Techniques used to check the quality of a blastocyst include counting blastocyst cell number and timing of embryonic development. The timing of the progression from morula to blastocyst can determine

9

embryonic quality, as Mateusen et al. (2005) found that embryonic development and blastocyst quality could be determined by the time it took for embryos to reach the 5-cell and morula stages. Early embryos that reached the 5-cell stage at 77 h PI and embryos that reached the morula stage before 102 h PI yielded higher blastocyst rates than those that did not reach these stages by 77- or 102 h PI. Although genetic testing and selection through embryo biopsies (removal of several blastomeres through a minute hole in the zona) are available, as previously mentioned, most embryo quality evaluations are subjective opinions of the lab technician.

Other Topics in In Vitro Production (IVP) of Embryos

Pregnancy Loss & Calf Characteristics. The IVP of bovine embryos is preferred over the IVD method in the bovine industry because of its efficiency; however, when comparing the quality of IVP oocytes and blastocysts to those that are IVD, researchers have found that there are many extruded cells and other visible physiological differences that could be attributed with lower overall embryonic development, success in ET, and eventual pregnancy rates (Barfield, 2015). For transferred IVP embryos, pregnancy rates tend to be lower, abortion rates are higher, and risks of large-calf syndrome increase compared to transferred IVD embryos (Young et al., 1998; Hasler, 2000; Rivera et al., 2022). Decreased pregnancy rates, increased pregnancy loss and large calf syndrome are attributed to poor oocyte and embryo quality. Alterations in genetics and metabolism due to culture conditions seem to be the root cause of these issues.

Cryopreservation. Cryopreservation is preserving biological material below -196°C (Reviewed in: Jang et al., 2017). Two primary cryopreservation techniques exist: (1) slow cooling and (2) vitrification. Slow cooling and vitrification are used for the preservation of female oocytes, embryos, or germplasm, in addition to the cryopreservation of semen (Phillips &

10

Lardy, 1940; Polge et al., 1949; Reviewed in: Yanez-Ortiz et al., 2022) and other tissues (Reviewed in: Chen et al., 2023). These techniques open opportunities for creating gamete banks of female calves, heifers, or cows with desirable genetics, removing the need for estrous synchronization between donor and recipient cows and allowing for long-distance transport of the oocytes or embryos. While embryos are likely to return to their typical structure and function, oocytes that are cryopreserved by either method have an elevated risk of cryoinjuries or damage to spindles, organelles, and DNA compared to oocytes that were not cryopreserved (Hwang & Hochi, 2014). Some attempts at bovine mature oocyte cryopreservation have been successful, with 15-25% of vitrified, warmed, and inseminated oocytes developing into blastocysts, and can be comparable to the developmental rates of non-treated (non-cryopreserved) mature oocytes (Checura & Siedel, 2007; Reviewed in: Hochi, 2022).

Additionally, IVP embryos are more challenging to cryopreserve than IVD embryos (Fair et al., 2001). Decreased IVP embryo cryopreservation tolerance is due to reduced competence of *in vitro* matured oocytes (Stoecklein et al., 2021; Fair et al., 2001; Rizos et al., 2002) and adverse effects of *in vitro* embryo culture environment (Crosier et al., 2000; Stockelein et al., 2021).

The significant areas of research for mitigating the effects of cryoinjury are improving oocyte metabolism and removing lipid droplets pre-cryopreservation, and recovering oocyte spindle function and metabolism post-thaw (Reviewed in: Zhang et al., 2012; Brambillasca et al., 2013). Removing lipid droplets is particularly critical for the successful cryopreservation of IVP oocytes and embryos. Lipid droplets are spherical vesicles made of a phospholipid bilayer that encases triglycerides used in fatty acid β-oxidation (Reviewed in: Quinn, 1985). Though necessary for the oocyte's metabolism, lipid droplets create a challenge in cryopreservation because of their sensitivity to shifts in physiological temperatures, and they cause

11

oocytes/embryos to shatter during cryopreservation or warming/thawing (Reviewed in: Dunning et al., 2014). Studies have found some success by including supplements in the warming or maturation media or changing the concentrations of cryoprotective additives (Abe et al., 2002; Hwang & Hochi, 2014; Zolini et al., 2019) to improve oocyte/embryo cryotolerance.

Supplementation of Media. Supplementation of maturation and culture media is a growing trend to improve the culture system. Many studies have been conducted to improve IVP blastocyst rates and quality by supplementing the media with some nutrient or additive, such as hormones, antioxidants, or metabolic enhancers.

Reactive oxygen species are a target for improving the IVP embryo culture system. Due to altered metabolism or adverse culture conditions, ROS accumulates within the blastomeres of early embryos or blastocysts. Buildup and improper dissociation of ROS can lead to apoptosis of the blastomeres, thereby reducing the total blastomere number (Reviewed in: Deluao et al., 2022). Several studies have added or increased antioxidant concentrations, like melatonin (Majidi et al., 2021) and cysteine (Ali et al., 2003; Sun et al., 2021), to the culture media to aid the embryo in the dissociation of ROS.

Bovine oocytes have a dark cytoplasm or oolemma due to a high lipid content. Reduced lipid metabolism because of adverse culture conditions *in vitro* is a proposed cause of reduced oocyte competence (Gad et al., 2012; Ghanem et al., 2014). L-carnitine is a naturally occurring derivative of the amino acid carnitine, which plays a vital role in transporting fatty acids to the mitochondria for fatty acid β oxidation (Reviewed in: Bremer, 1983). Several studies have concluded that supplementing the maturation or culture media with L-carnitine has been shown to improve maturation-, fertilization-, and blastocyst rates (Yamada et al., 2006; Phongnimitr et al., 2013; Knitlova et al., 2017).

CHAPTER TWO

OOCYTE METABOLISM AND MATURATION

Nuclear Maturation

In the bovine, it is not until after puberty and the LH surge that primary oocytes, arrested as germinal vesicles (GV), spontaneously resume meiosis I. Prophase I transitions from the diplotene stage into its 5th stage, diakinesis (Tsafriri et al., 1972). This is where the nuclear envelope begins to disappear, and the condensed homologous chromosomes are now visible, also known as germinal vesicle breakdown (GVBD). The critical step of GVBD occurs during the first 6-8 h of bovine oocyte maturation, both *in vivo* and *in vitro* (Hyttel et al., 1986; Sirard et al., 1989). The oocyte has two complete sets of homologous chromosomes (4N), received from mitosis of the "parent" oogonium (2N), and the DNA does not replicate further. Instead, spindle fibers form and anchor to centromeres of the homologous sister chromosomes for chromosomal movement during meiosis I (Reviewed in: Barr & Amon, 2009). The chromosomes are moved to the center around an imaginary axis in metaphase I (MI; 4N) nuclear stage. Chromosomal rearrangement occurs during this stage, and instead of progressing through cytokinesis to create two daughter cells, half of the DNA is condensed and extruded in a cytoplasmic vesicle known as the first polar body (PB) at the end of anaphase I (AI; 2N). The oocyte progresses to meiosis II through prophase II and arrest in metaphase II (MII; 2N). Bovine oocytes have been found to complete nuclear maturation, with the extrusion of the first polar body and formation of the metaphase plate between 18 h and 21 h of maturation (Hyttel et al., 1986; Sirard et al., 1989). Metaphase I is distinguishable from metaphase II, as sister chromosomes (4N) characterize MI,

while MII consists of sister chromatids (2N) aligned around an equator or metaphase plate (Trebichalska et al., 2021).

The oocyte arrests as an MII until fertilization. Upon successful fertilization, one set of the oocyte's sister chromatids is condensed and extruded from the now zygote as the second polar body. With half of the female (N) and half the male (N) DNA, two pronuclei are formed and undergo syngamy, or the fusion and recombination of the maternal and paternal DNA (Reviewed in: Unnikrishnan et al., 2021). Meiosis II progresses and begins the genetic and epigenetic modifications for embryonic development (Reviewed in: He et al., 2021).

The Role of Cyclic Adenosine 3',5' Monophosphate (cAMP). As previously mentioned, primary oocytes are arrested in the 4th stage of prophase I, the diplotene stage (4N). The chiasma is visible in this stage as the synaptonemal complex, formed in the zygotene stage (2nd), has begun to disappear. Large volumes of cyclic adenosine 3',5' monophosphate (cAMP) maintain the diplotene stage (Tsafriri et al., 1972). Cyclic AMP acts on cyclin B and CDK1 by phosphorylation to inhibit maturation-promoting factor (MPF), a critical player in the nuclear progression of meiosis I and II (Mitra & Schultz, 1996; Reviewed in: Sirard et al., 1997; Wu et al., 1997). MPF is a cyclin-CDK complex that is activated by removing phosphatase at the end of the growth phase 2 (G2) cycle of prophase I. For further development, MPF must be active for the first polar body extrusion and metaphase plate formation (Reviewed in: Fan & Sun et al., 2019). High levels of cAMP initiate phosphorylation/activation of protein kinase A (PKA), which phosphorylates/activates WEE1 for phosphorylation/inactivation of CDK1 (Mitra & Schultz, 1996; Reviewed in: Cohen, 2018). Additionally, PKA suppresses CDC25B, the molecule responsible for the dephosphorylation of CDK1 for initiating the resumption of meiosis I.

The cAMP is produced by the surrounding granulosa cells, and it is not until the LH surge that phosphodiesterase 3A (PDE3A) inhibits granulosa cAMP-promoting factors, and PKA activity ceases, MPF is activated, and meiosis I resumes (Conti et al., 1998; Conti et al., 2002). Tsafriri et al. in 1972 first demonstrated in the mouse model that oocytes enclosed in their intact follicle did not mature *in vitro* without adding LH to the media; however, Aktas et al. in 2003 concluded that granulosa and cumulus-intact oocytes cultured in serum-free media resumed meiosis. Additionally, cAMP derivatives also maintain oocyte arrest at the diplotene stage, as meiosis did not resume when oocytes were exposed to dibutyryl cAMP, a cAMP analog and phosphodiesterases (PDEs), which help to inhibit cAMP activity (Tsafrri et al., 1996; Reviewed in: Conti et al., 1998). Therefore, granulosa-made cAMP contributes to meiotic arrest.

Cytoplasmic Maturation

In vitro maturation of oocytes yields decreased preimplantation embryo developmental potential compared to those that are matured *in vivo* (Greve et al., 1987; Hawk & Wall, 1994; Reviewed in: Rizos et al., 2008), and a primary culprit is insufficient cytoplasmic maturation (Hyttel et al., 1986; Reviewed: Hyttel et al., 1997; Kirillova et al., 2021). As previously mentioned, the oocyte undergoes meiosis I and an essential component of cytoplasmic maturation is organelle rearrangement and distribution for optimal oocyte metabolism and chromosomal movement. A comprehensive study by Trebichalska et al. 2021 examined the ultrastructure of human oocytes during maturation from GV to metaphase II. Their study revealed that GVs do not have homogenous organelle distributions. Instead, they were primarily arranged around the nucleus. Metaphase I and II oocytes differed because they had homogeneously distributed organelles, which were only devoid in areas where spindle formation occurred. In the bovine, several studies have determined that oocytes in the GV stage have

peripherally distributed organelles, whereas MII-arrested oocytes have a more homogenous distribution, sometimes with specific organelles in aggregates around the spindle fibers (Hyttel et al., 1986; Trebichalska et al., 2021; Reviewed in: Ferreira et al., 2009).

Mitochondria. Mitochondria are the most abundant organelle in the oocyte, followed by the endoplasmic reticulum (ER) and Golgi apparatus (GA) (Trebichalska et al., 2021). The mitochondria are the central organelle in metabolic activity. The mitochondria control aerobic ATP production, intracellular signaling, calcium homeostasis, iron metabolism, biosynthesis of organic compounds and apoptosis (Stojkovic et al., 2001; Dumollard et al., 2006; Reviewed in: Van Berklom, 2004). Mitochondria are imperative for successful oocyte nuclear and cytoplasmic maturation, and any impairment can lead to decreased oocyte competence, early embryonic loss, or reduced embryo quality (Jeseta et al., 2014; Reviewed in: Cummins, 2004).

Mitochondrial DNA (mtDNA) in the Oocyte. Mitochondria are uniquely set apart because they are the only organelle with their copy of DNA --- mtDNA. Mitochondrial DNA, like nuclear DNA, is double-stranded but circular, with just over 16,000 base pairs in mammals (Anderson et al., 1981; Anderson et al., 1982). It encodes for proteins involved in adenosine triphosphate (ATP) production, cell signaling, and apoptosis. It replicates and mutates at different rates depending on the cellular environment and metabolic requirements (Mikhaylova et al., 2021; Reviewed in: Kirillova et al., 2021).

Crosstalk activity between the mitochondria and nucleus is essential to control metabolic activity, intracellular communication, and apoptosis/homeostasis. Oocytes have over 200,000 mtDNA copies, with an estimated two copies per mitochondria (Michaels et al., 1982). Moreover, though mtDNA copy number duplication is not fully understood and was initially

thought to not change during maturation (Wai et al., 2010), Read et al. (2021) found that bovine oocytes arrested at the GV stage had fewer copies of mtDNA than those arrested at the MII stage. This supports Zeng et al.'s (2009) assumption that the increase in ATP content observed during mouse COC maturation in the antral follicle was due to increased mitochondrial number rather than increased ATP production of each mitochondrion. While mtDNA seems to be the basis of mitochondrial function, recent studies have shown that blastocysts can still form when mtDNA is reduced in oocytes (Wei et al., 2008); however, reduced amounts of the mitochondria organelle and mtDNA decrease early embryonic development and blastocyst quality (Adhikari et al., 2022).

A distinguishable characteristic of mtDNA is that it is highly susceptible to creating heteroplasmy or differences between the individual mitochondria in one cell. Heteroplasmy is due in part to the highly mutable nature of mtDNA (~70%), which can lead to the inheritance of mutant-type mtDNA encoded for metabolic diseases or other cellular dysfunctions (Cree et al., 2008; Stewart & Chinnery, 2021).

Mitochondrial Morphology & Maturation in the Oocyte. Mammalian immature oocytes have been found to have mitochondria that are morphologically different from those found in somatic cells; they are round with very few cristae inside of a dense mitochondrial matrix (Trebichalská et al., 2021). It is not until early embryonic development that the mitochondria shift from immature structures to a more elongated shape with cristae and an evenly dispersed matrix (Tilly & Sinclair, 2013). It is suggested that this prolonged state of immature mitochondria in the oocyte and early embryo may be desirable to reduce damage from ROS, an unavoidable output of mitochondrial metabolism (Leese et al., 2007; Dumollard et al., 2007). Oocytes contain immense amounts of immature mitochondria, ~200,000, which produce

17

adequate amounts of ATP for maturation and early embryonic development yet potentially avoid ROS damage because of their immaturity and participation in fission and fusion. Several studies have presented the concepts of mitochondrial fusion and fission (Matinez-Diez et al., 2006; Liu et al., 2020) occurring during mitochondrial maturation in oocytes and somatic cells. Fusion is the merging of two mitochondria to form one large organelle, typically seen in an environment of limited nutrients where the fused mitochondria can work together to produce more ATP and increase bioenergetic efficiency (Liu et al., 2020). Fission is the division of a mitochondria to form two separate organelles. Under certain conditions, such as high concentrations of ROS and nutrient overload, fission may be a preventative measure for mitigating the detrimental effects of oxidative stress (Twig et al., 2008; Youle & van der Bliek, 2012).

  Mitochondria – Smooth Endoplasmic Reticulum. Mitochondrial fission and fusion rely on the formation of mitochondrial-endoplasmic reticulum complexes. In *in vivo* maturation of oocytes, mitochondria, and smooth endoplasmic reticulum form mitochondrial-smooth endoplasmic reticulum complex structures (Coticchio et al., 2015). However, under *in vitro* culture conditions, mitochondria form larger structures with vesicles known as mitochondrial vesicles. Mitochondrial-smooth endoplasmic reticulum complexes are thought to be a precursor of mitochondrial vesicles, and the quick movement from mitochondrial-smooth endoplasmic reticulum complexes to mitochondrial vesicles or direct conjugation of mitochondrial vesicles could be a sign of reduced cytoplasmic maturity (Motta et al., 1988; Motta et al., 2000; Coticchio et al., 2015). The endoplasmic reticulum is the central organelle responsible for housing calcium (Ca^{2+}) in preparation for sperm penetration and inducing shifts in ATP production (Rossi et al., 2018). These mitochondrial vesicles are purposed to create quick calcium transport from the smooth endoplasmic reticulum to the mitochondria for cellular signaling post-fertilization.

18

Mitochondrial Migration during Oocyte Maturation. Oocytes undergo complex rearrangements of spindle fibers for nuclear maturation; this rearrangement requires ATP to be readily available. A comprehensive study conducted by Dalton and Carroll (2013) on the mitochondrial-spindle superstructure found that over 40% of mitochondria in the mouse oocyte cytoplasm are concentrated around the spindle fibers during the transition from GV to MII and are homogeneously distributed throughout the cytoplasm by the MII stage with a polar body extrusion. This movement can also be in conjunction with COC cumulus morphology. In porcine and equine oocytes, the peripheral to centralized or clustered mitochondrial pattern correlates with the cumulus expansion throughout maturation (Torner et al., 2004). Mitochondria are proposed to be distributed in a peripheral position during early maturation events to quickly obtain substrates from cumulus cells via gap junctions (Plante & King, 1994; Tarazona et al., 2006; Reviewed in: Kirillova et al., 2021). Additionally, mitochondrial migration is heavily dependent on motor proteins dynein and kinesin-1 and clusters of mitochondria around microtubule-organizing centers provide energy during organelle and spindle rearrangements (Dalton & Carrol, 2013; Sanchez et al., 2015).

Mitochondrial Membranes & Oxidative Phosphorylation. The mitochondria comprise an outer membrane, intermembrane space, inner mitochondrial membrane (IMM), and matrix. The IMM has a series of folds called cristae, which enclose the matrix. The outer and inner mitochondrial membranes contain several phospholipids like phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Schenkel & Bakovic, 2014). Only found in the mitochondrial membrane, cardiolipin is vital in the beginning of apoptosis and mitochondrial metabolism (Yin & Zhu, 2011). Cardiolipin is a stabilizing molecule within the

inner membrane for the transmembrane proteins involved in oxidative phosphorylation (Pfeiffer et al., 2003).

Along the IMM, there are five major transmembrane protein complexes involved in oxidative phosphorylation: NADH-CoQ reductase (complex I), succinate dehydrogenase (complex II), cytochrome bc1 (complex III), cytochrome oxidase (complex IV) and ATP synthase (complex V). Complexes I and II are responsible for the oxidation of NADH and $FADH_2$ to release high-energy electrons that are transported to complex III, CoenzymeQ, via electron transport (Reviewed in: Pallotti et al., 2022). All complexes except complex II consist of proton pumps that move free hydrogen molecules (H_2 released from NADH and $FADH_2$) from the matrix to the intermembrane space. Electrons at complex III bind to the cytochrome b molecule and are transferred to the peripheral membrane protein, cytochrome c. Reduced cytochrome c, moving one electron at a time until four electrons are transported, binds with complex IV to create an active cytochrome c oxidase transmembrane protein (Sharma et al., 2013). Under aerobic conditions, cytochrome c oxidase transfers the electrons to oxygen (O_2), the terminal electron acceptor, which forms water molecules (H_2O) with hydrogen ions in the mitochondrial matrix. Cytochrome c oxidase also pushes hydrogen ions across the IMM into the intermembrane space, increasing the electrochemical gradient and potential energy. The increased hydrogen ion concentration formed in the intermembrane space pushes hydrogen ions back into the matrix through complex V, ATP synthase. The energy released from the electrochemical proton gradient across the IMM, also known as the process of chemiosmosis, activates ATP synthase. Active ATP synthase phosphorylates adenosine diphosphate (ADP) molecules to form ATP molecules (Reviewed in: Zorova et al., 2018; Mitchell, 1966).

Mitochondrial Membrane Potential (ΔΨm). The mitochondrial membrane is an essential physiological barrier for ATP production and cellular signaling. The IMM continuously has H^+ ions flowing across during oxidative phosphorylation and cellular signaling. This flux of H^+ ions and other molecules across the membrane generates an electrochemical gradient across the IMM, also known as mitochondrial membrane potential (MMP or ΔΨm; Mitchell, 1966). The rotation of ATP synthase is driven by the ΔΨm, as the polarization of the membrane determines if ATP is produced or hydrolyzed (Walker, 2013). The ΔΨm is based on the polarization of the IMM, in which the inner membrane is polarized (negative) in its normal state and depolarized (positive) under unfavorable conditions (Reviewed in: Zorova et al., 2018). The inner mitochondrial membrane is constantly negatively charged (polarized), and as H^+ ions flow across the membrane to generate ATP, the ΔΨm becomes less and less polarized. In a normally functioning mitochondrial respiratory chain, depolarization is prevented by the flow of H^+ ions back into the intermembrane space by Complexes I-IV. However, under stressful conditions, such as fluctuations in pH, temperature, atmospheric gases, and culture contaminants, the ΔΨm cannot be adequately generated by normal mitochondrial oxidative phosphorylation and switches to ATP hydrolysis in the presence of the ATPase enzyme (Reviewed in: Zorova et al., 2018). Complex V, ATP synthase, reverses direction (counterclockwise) to send H^+ ions up their gradient to hydrolyze ATP into Adenosine diphosphate (ADP) plus one phosphate molecule (Pi). The mitochondria's hydrolysis of ATP to maintain the ΔΨm is known as a "selfish" behavior, and it induces the homeostatic response of ATPase inhibitory factor 1 (IF1), which prevents ATPase activity and keeps the ΔΨm depolarized to send ATP out of the mitochondria for cellular activities. (Gledhill et al., 2007).

Because the oocyte is heavily dependent on mitochondrial oxidative phosphorylation and ATP production during maturation, the MMP has been heavily investigated to see if it is affected by *in vitro* culture conditions. A comprehensive study published by Al-Zubaidi et al. (2019) determined that in the mouse model, MMP gradually increased throughout maturation. They concluded that changes in $\Delta\Psi$m were associated with meiotic events as oocytes that remained arrested in the GV stage had little-to-no changes in $\Delta\Psi$m. Additionally, $\Delta\Psi$m was higher in the perinuclear region compared to the rest of the cytoplasm during oocyte maturation.

Energy Sources for Oocyte Metabolic Activity During Maturation

Oocyte maturation in the follicle is different from oocyte maturation *in vitro*, and *in vitro* maturation of bovine oocytes was achievable through the addition of specific gonadotropins, amino acids, carbohydrates, and other molecules to the IVM media (Thibault, 1977). However, the oocytes' metabolic activity heavily influences oocyte maturation. Whether or not gonadotropins and other necessary nutrients are present to initiate maturation, the oocyte cannot proceed without sufficient energy to make nuclear and cytoplasmic changes.

Adenosine Triphosphate (ATP). Adenosine triphosphate is the most abundant and primary energy source for cellular metabolism. Pyruvate is the primary substance used to generate ATP (Johnson et al., 2007) and activation of metabolic activities leads to ADP phosphorylation to ATP. Oocytes undergoing maturation depend on ATP for proper nuclear and cytoplasmic maturation. Spindle fiber formation for chromosomal, microtubule, and organelle rearrangement requires large amounts of ATP. Oocytes that have compromised mitochondria due to oxidative stress or are denuded of their surrounding cumulus cells during maturation have been found to have reduced ATP concentrations and decreased developmental competence (Stojkovic et al.,

2001). Dalton et al. (2013) determined a decrease in ATP levels over time by denuding the oocytes before maturation. Prior to GVBD, ATP levels were comparable between denuded and cumulus-intact oocytes. However, after GVBD, ATP levels in denuded oocytes drastically decreased compared to cumulus-intact oocytes.

ATP production is also proposed to oscillate during maturation depending on the oocytes' metabolic needs. Several studies have confirmed that ATP levels fluctuate as maturation progresses, and the arrangement of DNA during meiosis in the oocyte is easily associated with specific ATP levels (Stojkovic et al., 2001; Read et al., 2021). Dalton et. Al (2013) found in the mouse model that COCs, MI, and MII oocytes produced more ATP than COCs arrested at the GV stage. ATP levels are also higher just after the onset of meiosis I at GVBD, and they peak at the extrusion of the first PB (Dalton et al., 2013; Zhao & Li, 2012). Similarly, Read et al.'s (2021) evaluation of ATP levels in bovine oocytes determined that MII-arrested oocytes had greater ATP levels than GV-arrested oocytes. Higher ATP levels at MII have been associated with increased fertilization rates and preimplantation embryo rates (Zhao & Li, 2012).

The Role of Cumulus Cells. Cumulus-oocyte complexes are oocytes surrounded by cumulus cells. In IVP, oocytes are typically selected for maturation if they have two or three layers of tightly surrounding cumulus cells. As maturation progresses, the tightly adhered cumulus cells undergo expansion and are seen to be wholly expanded at h 18 of maturation, where the cumulus is maintained until insemination or degeneration occurs, and the zygote progresses absent of the cumulus cells (Hyttel et al., 1986). Although the amount of cumulus cells surrounding oocytes during *in vitro* maturation seems minimal, they are critical in oocyte maturation and competence because oocytes do not have active transporters or a mature cytoplasm to uptake glucose and other metabolites from their surrounding environment

(Reviewed in: Richani et al., 2020). Therefore, the corona radiata cells in direct contact with the oocyte play an essential role in oocyte maturation. Crosstalk communication occurs between the surrounding cells and oocyte through cytoplasmic extensions and gap junctions that transverse the zona pellucida and perivitelline space, reaching into the oolemma (Reviewed in: Winterhager & Kidder, 2015). This crosstalk initiates the highly glycolytic corona radiata cells to increase glycolysis to provide glycolytic ATP and metabolic substrates such as pyruvate, acetyl CoA, NADH, CO_2, and FADH for oocyte metabolic activity and maturation (Suigura et al., 2005; Reviewed in: Richani et al., 2020). Oocytes that have very little or no surrounding corona and cumulus cells have minimal developmental potential (Reviewed in: Kirillova et al., 2021). King et al. (1986), and later reaffirmed by Aktas et al. (2003), demonstrated that oocytes removed from their cumulus (denuded) had little to no developmental competence, even when cultured with hormones. Several other studies have seen a reduction in oocyte metabolism when matured in vitro without their surrounding cumulus (Dalton et al., 2013; Reviewed in: Richani et al., 2020), concluding that oocyte maturation and competence depend on the COC.

Lipids & Fatty Acid β-Oxidation. Lipids are macronutrients with the highest energy density, and in the bovine oocyte, they are abundant in cells as lipid droplets. Lipid droplets are clusters of fatty acids used for membrane formation, intra- and extracellular signaling, and energy storage (Reviewed in: Houten et al., 2016). Lipid droplets are made of a phospholipid bilayer held together by integral and peripheral proteins encasing a cluster of lipids. These lipids are used in cellular starvation to provide energy for homeostasis and can be used to repair or form phospholipid membranes during high cellular activity. Lipids, commonly stored as triglycerides, are freed from lipid droplets during metabolic need and are broken down via lipolysis into monomeric forms of fatty acids and glycerol in the cytoplasm (Reviewed in:

Olzmann & Carvalho, 2019). Free fatty acids are bound to acyl-coenzyme A (CoA) esters and then transported into the mitochondria by the carrier protein L-carnitine. The β-oxidation process consists of acyl-CoA-bound fatty acids by undergoing a series of degradation processes in the mitochondria to form isolated acyl-CoA. The degrading processes also reduce FAD^+ and NAD^+ for respiratory chain activities (Reviewed in: Houten et al., 2016).

In the oocyte, lipid droplets are a significant source of energy storage and are readily used during maturation (Cetica et al., 2002). In the mouse model, Dunning et al. (2010) determined that oocytes matured *in vitro* with β-oxidation inhibitors had reduced subsequent embryonic development. Meanwhile, oocytes matured with L-carnitine supplementation had improved embryonic development even under glucose deficits. Additionally, a study on the relationship between lipid droplets and cumulus cells discovered that denuded oocytes had reduced lipid droplets and fatty acid synthase proteins along with varying gene expression compared to their cumulus-intact counterparts (Auclair et al., 2013). Lipid droplets are a critical constituent in cumulus cells utilized to provide energy to the oocyte for maturation.

Mitochondrial Dysfunction & The Effects on Oocyte Metabolism & Maturation

Oxidative Stress. Oxidative stress is the imbalance or overproduction of oxidants in a cell (Sies, 2019). The first sign of oxidative stress is an increase in ROS. Reactive oxygen species are oxygen-bound molecules with incomplete and unstable bonds, leaving a negatively charged molecule that can freely move throughout the cell. This ROS molecule damages lipids, proteins, and nucleic acids by bonding with the macromolecule to stabilize its free electron. ROS binding shifts protein conformation and, therefore, their functions, destabilizes lipid membranes and breaks the bonds of nucleic acids in DNA. Common ROS include superoxide (O_2^-), hydroxyl

(HO$^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxide ion (OH$^-$; Reviewed in: Shields et al., 2021).

Mitochondria have naturally occurring antioxidants that dissociate ROS molecules such as

antioxidant enzymes glutathione peroxide and thiol-disulfide oxidoreductases, and antioxidant

low-molecular-weight molecules ubiquinol and α-tocopherol (Reviewed in: Apostolova & Victor,

2015). Although mitochondria are equipped with antioxidants, several factors can contribute to

oxidative stress, including increased oxidative phosphorylation, adverse atmospheric conditions,

metallic cations, metabolic inhibitors, and visible light (Reviewed in: Guerin et al., 2001).

Reactive oxygen species impair embryonic development due to oxidative stress

(Reviewed in: Hardy et al., 2021). Studies have found that mammalian embryos cultured in high

O$_2$ had reduced development to the blastocyst stage (Li et al., 2016; Reviewed in: Guerin et al.,

2001). Additionally, an *in vitro* study of oocyte maturation conducted in the mouse model found

that mouse oocytes that underwent oxidative stress through exposure to various doses of H$_2$O$_2$ in

culture resulted in meiotic spindle disassembly, reducing maturation, fertilization, and blastocyst

rates (Zhang et al., 2006). Though ROS are associated with normal oocyte and embryonic

metabolic functions such as fertilization (Lopes et al., 2010), excess ROS is an ongoing issue

with *in vitro* maturation and culture of embryos. *In vivo,* surrounding follicular and oviductal

fluid provide antioxidants to the oocyte/embryo to reduce ROS (Carbone et al., 2003; Reviewed

in: Deluao et al., 2022), and while there are antioxidants present in *in vitro* culture media, *in vitro*

oocytes/embryos are still negatively impacted (Reviewed in: Guerin et al., 2001 & Deluao et al.,

2022).

Mitochondrial Dysfunction. Mitochondria are the central unit for cellular metabolic

activity, and impairing normal function in mitochondria can have severe consequences

(Reviewed in: Grindler & Moley, 2013). Dysfunctional mitochondria are suggested to be one of

the leading causes of reduced fertility (Stojkovic et al., 2001; Zhao & Li, 2012). Several factors contribute to mitochondrial dysfunction, such as aging, obesity, and metabolic diseases of the cow (Reviewed in: Adhikari et al., 2022).

A definitive cause of reduced mitochondrial activity in the oocyte is age. The age of the female can affect mtDNA copy number and ATP content (May-Panloup et al., 2005; Iwata et al., 2011). Several studies have determined that in older mice, cows, and humans, the oocyte has reduced maturation, fertilization, embryonic development and pregnancy rates (mouse & human: Al-Zubaidi et al., 2021; cow: Itami et al., 2014 & Malhi et al., 2007). Malhi et al. (2007) examined the number of oocytes recovered, embryonic development, and pregnancy rates in old cows (>13 years old) and their young daughters (1-3 years old). Malhi et al. (2007) determined that older cows had higher oocyte recovery but lower embryonic development than their young daughters. Additionally, there was a tendency for higher pregnancy loss at day 45 of gestation for the older cows than younger cows whose embryos were transferred into an unrelated recipient group.

A theory for the reduced capabilities of aged oocytes is the mitochondrial free radical theory (MFRTA). This theory states that increased cellular and organismal aging is due to the oxidative damage caused by mitochondrial ROS generated during mitochondrial activity (Sanz & Stefantos, 2008). Specifically in the *in vitro* production of embryos, several studies have looked further into treating maturation and culture media with antioxidants to reduce ROS (Reviewed in: Iwata, 2016). Al-Zubaidi et al. (2019) used mitochondrial-targeted therapeutics, MitoQ and BGP-15, which scavenge and dissociate ROS in young and aged mice. They found that spindle reassembly in the aged mice oocytes matured with BGP-15 was improved compared to those matured without BGP-15.

Overnutrition and undernutrition also contribute to mitochondrial dysfunction (Reviewed in: Marcovina et al., 2012). As previously mentioned, lipids, in the form of fatty acids, are essential nutrients in the production of ATP in oocytes (Cetica et al., 2002). However, increased lipid accumulation in the oocyte can reduce metabolic activity (Reviewed in: Purcell & Moley, 2011). In obese women and female animals, there is a significant decrease in fertility and conception rate, with increased risks of miscarriage and congenital disease in their children (Reviewed in: Purcell & Moley, 2011). There is also an association of polycystic ovarian syndrome in obese women (Reviewed in: Jungheim & Moley, 2013). A study published in 2020 monitoring the mitochondrial function of mouse oocytes derived from mice fed a high-fat diet had increased MMP, mitochondrial and ultrastructure abnormalities, and ROS concentrations (Marei et al., 2020). Additionally, bovine oocytes cultured with high leptin concentrations, the hormone secreted by adipocytes (specialized cells for fat storage), had reduced embryonic development rates (Arias-Alvarez et al., 2011).

LITERATURE REVIEW

CHAPTER 3

PHOTOBIOMODULATION

A Brief History of Photobiomodulation

Photobiomodulation (PBM), once known as Low-Level Laser (Light) Therapy (LLLT), was initially discovered and reported by Endre Mester in 1967. Mester attempted to replicate an experiment that used a ruby laser to treat rats with their implanted tumors. Mester, however, built a ruby laser that was much less powerful than the one he was attempting to replicate. When applied to the rats' tumor implant wound, Mester did not observe the remission of the tumors, but he did observe faster wound healing and hair growth at the incision site (Mester et al., 1968). After his initial discovery of photostimulation using a red (600-1000 nm) laser, Mester continued his work looking at bone repair, revascularization of wounds, and collagen production in fibroblasts (Reviewed in: Gaspar, 2009). Mesters' accidental discovery opened a new avenue of light therapy using low-powered light sources for wound healing, cell regeneration, and metabolic activation (Reviewed in: Hamblin, 2016).

Lasers, or light amplification by stimulated emission of radiation, were patented by Charles Townes and Arthur Schawlow and built by Theodore Maiman in 1958 (Reviewed in: Chodos, 2003). Lasers were, and still are, photobiomodulation therapy's primary light source due to their monochromatic beams and high-power densities, and though effective, lasers require greater precision and safety training for use compared to LEDs or UV lamps (Heiskanen & Hamblin, 2018). Additionally, lasers can be manufactured with varying power, and no one knew Mester's definition of a "low-level" laser. It was not until much later that "low-level" lasers were

classified as having an output power of 500 mW or less (Dompe et al., 2020). Since then, light-emitting diodes (LEDs) have proven to be an equally effective light source compared to low-powered lasers. Light emitting diodes make sufficient substitutes for lasers, though not as coherent; they are more accessible and safer to use, have a more extensive range of coverage and are more affordable (Heiskanen & Hamblin, 2018). Thus, the term "laser" became controversial in light therapy. As a result, photobiomodulation or "light, life, modification," became the term for this new field of science. However, much controversy still stands over this field, and many years have passed, overlooking photobiomodulation as a technology to use in medical and research practices due to numerous unsuccessful published studies testing various wavelengths, devices, treatment protocols, and power densities (Tuner & Hode, 1998; Reviewed in: Huang et al., 2009).

Underlying Mechanisms

Photobiology & Photochemistry. Photobiology and photochemistry are the founding principles for understanding the mechanisms of photobiomodulation treatments and parameters. Photobiology is the study of interactions between visible and UV light on living organisms. Photochemistry is the study of chemical and physical changes in non-biological systems due to non-ionizing radiation (Smith, 2014; Reusch, 2013). Photochemistry is broken down into two significant laws: (1) light must be absorbed by the system, and (2) each photon of light only activates one molecule (Smith, 2014).

The Physics of Light. In the late 17th and early 18th century, mathematicians and physicists Christiaan Huygens and Sir Issac Newton debated whether light was a wave, Huygens' wave theory, or made of corpuscles (small particles or photons), Newton's corpuscular theory. It is now understood to be some mix of both. In 1905, theoretical physicist Albert Einstein and

physicist Niels Bohr re-explored and affirmed the related corpuscular theory, stating that these

photons act on receptors that transfer the corpuscular or light energy to molecules. Their

confirmation of this fundamental principle of photochemistry was partly due to Planck's theory,

which was established in 1900 by theoretical physicist Max Planck. The total energy of a photon

of light can be determined using Planck's equation: $E = hc/\lambda$ or $E = hv$, where h is Planck's

constant (6.625×10^{-34}), c is velocity, λ is wavelength and v is frequency (Reviewed in: Born &

Wolf, 1980). Light's velocity directly results from frequency and wavelength: $c = \lambda \times v$ (Tanner,

1985). Wavelength is defined as the distance from one crest or trough to the next crest or trough

(Smith, 2014). Long wavelengths have lower amounts of energy, and shorter wavelengths have

higher amounts of energy. The frequency of light, measured in hertz (Hz), is the number of

waves that move through a medium (gas, liquid, solid or plasma) per second (Tanner, 1985).

These concepts merge to form and understand the world of photochemistry and photobiology.

In direct correlation with Einstein and Bohr's findings, photobiomodulation treatment is

an example of corpuscular energy transfer. It relies on photons' specific wavelengths' interaction

with photoreceptors, or chromophores, within the cell with varying excitation levels. The first

law of thermodynamics can further explain this concept: energy must be conserved (Zohuri,

2018). This law in correlation with photobiomodulation energy transfer leads to one of three

pathways: (1) internal conversion, where the excited electron transitions back down to its

original spin and the energy is given off in the form of vibrations or heat; (2) the molecule

absorbs the photon and re-emits a photon with a longer wavelength creating fluorescence; and

(3) and oxidation, reduction or dissociation of a molecule or complex which transfer the energy

permanently (Reviewed in: Hamblin, 2008). The first pathway is the most common in biological

systems. These photoreceptors generally are in a ground (S_0) energy state, where electrons are

located within their respective spin, producing the lowest energy possible. Then, when the photoreceptor absorbs a photon of light, an electron may shift from its original spin to the opposite, shifting the molecule from the ground state to excited (Mroginski et al., 2021). The energy's pathway depends entirely on the wavelength and frequency of the light. Most commonly, photobiomodulation treatment is noted to be investigated in the 400-1100 nm wavelength visible light- to near-infrared- spectrum, which can activate all three pathways depending on the molecule and light parameters (Reviewed in: Hamblin, 2006).

Photoreceptors & Chromophores

Photoreceptors, or chromophores, are generally known as the specialized neurons of the eye that send signals to the brain and induce a response (Lamkin-Kennard & Popovic, 2019). However, photoreceptors are not specific to the eye or neurons alone; photoreceptors can also be defined as light transducers or energy converters (Braslavsky, 2003). They are typically found as conjugated pi (**π**) electron systems, or double-bonds, and metal complexes (Huang et al., 2009). These light transducers have an absorption and action spectrum (Passarella & Karu, 2014). The absorption spectrum is the wavelengths that the molecule absorbs, and the action spectrum is the wavelengths that cause an energy emission from the molecule to another molecule, creating a cascade of energy (corpuscular theory and energy pathway).

The mitochondria participate in cellular crosstalk with the nucleus, initiating the activation of genes involved in cell proliferation, differentiation, apoptosis, and pro- or anti-inflammatory events (Reviewed in: Shteinfer-Kuzmine et al., 2020; Reviewed in: Leyane et al., 2021). Therefore, the mitochondria, the organelle in nearly all eukaryotic cells, is one of the main targets of photobiomodulation treatment. The mitochondria are considered the "powerhouse" of

the cell by generating ATP through oxidative phosphorylation, maintaining homeostasis, regulating apoptosis, and sending intracellular signals to the rest of the cell via reactive oxygen species (ROS), calcium (Ca^{2+}), and other molecules. Most photobiomodulation studies are centered around mitochondria, not only because of their imperative role in cellular function but because they appear to house almost all photoreceptors (Karu, 2008). A few examples are: (1) cytochrome c oxidase, excited by red light (600-850 nm); (2) flavins and porphyrins, excited by blue light (400-450 nm); (3) light-gated ion channels, excited by violet-to-blue (390-425 nm) and near-infrared (>980 nm); and (4) opsins are excited by blue or green light (380-550 nm) (Hamblin, 2018; Serrage et al., 2019; Sharma et al., 2023).

Cytochrome c Oxidase. Passarella and Karu used a 665 nm absorption band to characterize if cytochrome c oxidase (COX) was oxidized or reduced by irradiation. They concluded that irradiation reduced the molecule. Therefore, when treated by photobiomodulation therapy, the more oxidized the steady-state molecule is, the more oxygen reduction is achieved in the electron transport chain, driving ATP synthase. The transport molecule, cytochrome c, carries only one electron and activates Complex IV by binding four electrons that are then moved to oxygen to produce two water molecules (Reviewed in: Hamblin, 2018). Oxygen's electron acceptance pushes four more hydrogen ions (protons) into the intermembrane space to drive ATP synthase. Karu et al. 2005 determined that the photoreceptor in cytochrome c oxidase was likely the copper A (Cu_A) molecule at the center of the transport protein complex. The hemeα group (iron or $Fe_{II\&III}$) accepted the higher energy from Cu_A and transferred it to hemeα3 for two final electron movements from Cu_B to the final electron acceptor, O_2. When COX is oxidized completely, the copper molecules are in the Cu_{II} oxidation state, and the iron molecules are in the Fe_{III} oxidation state. In contrast, when COX is reduced, Cu_I and Fe_{II} are present (Hamblin, 2008).

However, because there are four metal molecules within COX, they can be oxidized and reduced in some combination at any time. This can explain why COX has action spectra of 610-625 nm, 660-685 nm, 750-775 nm and 810-850 nm (Karu & Kolyakov, 2005).

Flavins & Porphyrins. Flavins are riboflavin derivatives involved in many cellular processes, specifically the electron transport chain in the mitochondria. Flavin dinucleotide (FAD) and flavin mononucleotide (FMN) are cofactors that assist oxidoreductase flavoproteins in enzymatic and metabolic processes (Toogood & Scrutton, 2020). There are specific groups of flavins called cryptochromes that absorb blue light (400-450 nm) to affect the flavin redox state (Bouly, 2007). Cytochrome FAD is a notable molecule found in Complex II of the electron transport chain, and blue light could play a role in activating this complex. Cofactor FMN is a helper of catalytic enzymes capable of reducing free oxygen (O_2) to the reactive oxygen species (ROS) and superoxide (O_2^-). Therefore, activation of FMN with blue light could induce a toxic effect on the cell (Yang et al., 2017).

Porphyrins are water-soluble pigment rings attached to proteins (Marengo-Rowe, 2006). When porphyrin is exposed to blue light (400-415 nm), an electron jumps from one orbital ring to the next (Serrage et al., 2019). Porphyrin is another molecule found in Complex IV of the electron transport chain, and studies have shown that blue light treatment increased Complex IV and overall mitochondrial activity (Buravlev et al., 2015; Ankri et al., 2010).

Light-Gated Ion Channels & Opsins. Light-gated ion channels, transient receptor potential (TRP) channels, are a relatively newly discovered sensory perceptive molecule. Though thought to be isolated in insects only (Minke, 2010), TRPs are found in many organisms' tissues and cells (Reviewed in: Hamblin, 2018). TRPs are cellular sensors that respond to temperature,

34

pressure, smell and taste changes. Their primary function is to create a non-selective permeable membrane to calcium, sodium and magnesium ions (Montell, 2011). Stimulation of TRPs via light is aided by the function of and interaction with opsins. Opsins are the primary photoreceptor of most species. This class of molecules is responsible for nearly all visual systems, and their role as a photoreceptor includes creating a signal cascade and physiological response due to their activation by absorption of a photon of light (Shichida & Matsuyama, 2009). These visual proteins, opsins, function through photoisomerization or the creation of a molecular isomer using light (Reviewed in: Iyer, 2020), which leads to phototransduction or induction of an electrical response due to light (Remington, 2012). Some common opsins are rhodopsin (OPN1) and OPN2-5. The opsin's function and response to stimuli depends on which G-coupled protein they are in conjunction with.

Clinical Applications

Factors Affecting Treatment. There are many factors to consider before applying photobiomodulation treatment. The treatment depends on the addressed pathology: wound healing, inflammation, cancer, arthritis, and more. Huang et al. state that photobiomodulation treatment has specific parameters of wavelength, irradiance (Power (W)/Area (cm^2)), pulse structure, coherence, and polarization. Additionally, a treatment protocol of the light energy (J), energy density (J/cm^2), time (s) and treatment interval (hours, days, weeks) are essential factors when considering photobiomodulation treatment in clinical applications. Some other parameters that must be considered are that photons can be absorbed or scattered, and those that are scattered will escape the tissue or be absorbed by other tissues. (Reviewed in: Hamblin, 2008). For some pathologies, photon scattering may not be an issue, but for others, it can reduce the effectiveness or even be detrimental to surrounding tissues.

As photobiomodulation is a relatively new form of light therapy, studies show that it can stimulate beneficial cellular signals and functions or induce damaging and toxic effects on the subject. The Arndt-Schultz Law is a principle coined to describe a biphasic dose-response, meaning that with weak stimuli, there is an increase in physiological response; as the incentives increase, the response eventually plateaus until heightened stimuli push physiological activity to the point of damage (Huang et al., 2009). Therefore, one of the main focuses of photobiomodulation is small doses to enhance physiological responses and improve treatment outcomes. Though the visible light spectrum covers an array of wavelengths, most literature cites successful tissue healing and anti-inflammatory responses with treatment in the red to near-infrared spectrum: ~600-1100 nm (Foley et al., 2016; Chow et al., 2009).

Cellular Responses to Photobiomodulation. Since Mesters' discovery, countless studies have been conducted to determine light's effects on cellular function and the underlying mechanisms of how it helps or hurts. As previously mentioned, light stimulates mitochondrial activity, specifically ATP synthesis; there is a cascade effect where the drive-in ATP synthesis increases the activity of antiporters (Na^+/H^+ and Ca^+ and Na^+, pumps and carrier ions). Energy molecule ATP also controls second messengers, cyclic AMP and Ca^+ levels. Calcium is a significant player in many bodily functions, such as muscle contraction and signal transduction, to name a few (Reviewed in: Hamblin, 2018). A study in 1979 showed laser-induced photostimulation (532nm) of the mitochondria, specifically cytochrome c oxidase, increased the beat rate of rat pacemaker cells. COX was determined to be the photoreceptor because, when inhibited, the cells ceased beating (Salet et al., 1979). Another mitochondrial reaction to photostimulation is a shift in mitochondrial membrane potential (MMP or $\Delta\Psi m$). Mitochondrial membrane potential is an electrochemical gradient comprising an electrochemical

transmembrane gradient and a proton gradient (Karu, 2008). The change in the electrochemical gradient is what drives ATP synthesis. Irradiation of rat liver cells with a He-Ne laser was one of the first studies to find that the mitochondrial membrane potential is a direct contributor to ATP synthesis; additionally, irradiation with a He-Ne laser produces more significant amounts of ATP than when not treated (Passarella et al., 1984). Reactive oxygen species are also a result of increased mitochondrial activity, and wavelengths of 550 nm or less seem to contribute to increased ROS production (Buravlev et al., 2015; Oh et al., 2007). While ROS is usually considered a negative result of photobiomodulation treatment, it can be used in some instances to kill cancer cells, blood vessels, or bacteria (Reviewed in: Hamblin, 2018). Lastly, photobiomodulation can also induce upregulation of genes. One study examined gene regulation in human fibroblasts treated with a 628 nm laser (Zhang et al., 2003). Zhang and colleagues' analysis revealed that over 100 genes were upregulated, most for cell proliferation with a few for transcription factors and inflammation.

Photobiomodulation of Embryos. Photobiomodulation treatments are applied to various tissues and cell types to induce a positive or negative response; and it has been proposed as a potential mechanism for improving *in vitro* embryo culture systems. A study published by Oh et al. (2007) measured the effect different types of illuminance (measured in lux or lumens per square meter) and frequencies had on Golden hamster embryonic development *in vitro*. Zygotes were surgically recovered from the female hamsters and placed into culture. The first experiment determined that embryos exposed to 200 lux visible ray had the highest blastocyst rates at 82-96 h post-insemination compared to 500- and 900 lux. Different frequencies were isolated using the 200-lux visible ray, and their preimplantation embryo rates were compared. They found a significant difference between the visible light (390-750 nm) and blue light (450-500 nm), with

visible light having a higher morula and blastocyst rate; 97%, 85%, 65% and 49%, respectively. However, the red light (620-750 nm) outperformed them all with an 84% blastocyst rate (p<0.05). The study also looked at several other factors, such as the Hsp70 gene (heat-stress) activity, ROS and cell numbers of blastocysts. The blue light increased *Hsp70* and ROS presence in the embryos compared to visible light (p<0.05), and red light mitigated the effects of *Hsp70* and ROS production. Likely due to overexpression of *Hsp70* and higher ROS concentrations, blue light had reduced cell numbers while visible and red light was no different (p>0.05). A similar study was conducted in 2008 on mouse zygotes using a xenon lamp (150 W), considered artificial sunlight, and an orange-red photoluminophore screen (626 nm). Their study showed that the luminescent component yielded higher blastocyst rates than the control and the artificial sunlight: 96.2%, 67.1% and 75.5%, respectively. The orange-red light also increased the hatching rate of the blastocysts compared to the control and the artificial sunlight: 80.8%, 28.8% and 45.3%, respectively (Sviridova-Chailakhyan et al., 2008).

Photobiomodulation treatment is an entirely unexplored and tested method of medicine. Its primary target is the mitochondria and the photoreceptors housed in its membranes. Photostimulation of mitochondria has the potential to improve many areas of medical research and advance healing and curing processes of metabolic diseases, cancers, and inflammatory responses. Additionally, it is a potential candidate for improving *in vitro* culture systems of oocytes and embryos of varying species.

CHAPTER 4

EFFECTS OF PHOTOBIOMODULATION ON BOVINE OOCYTE MATURATION,

MITOCHONDRIAL ACTIVITY AND SUBSEQUENT EMBRYONIC DEVELOPMENT

INTRODUCTION

The bovine industry is a staple of agriculture all over the world. Since the birth of the first *in vitro-produced* (IVP) calf in 1982, IVP of embryos has been a growing system in the bovine industry (Brackett et al., 1982). Offspring can be produced from prepubertal heifers, lactating, pregnant, and reproductively compromised cows with desirable genetics (Demetrio et al., 2022; Palma et al., 2008; Reviewed in: Sirard, 2018). Since 2016, IVP of bovine embryos has surpassed *in vivo* derived (IVD) production of embryos, with more than 70% of the world's transferable bovine embryos being IVP. Advances in other technologies like embryo biopsy, genetic testing, sexed semen, and embryo sexing have hurdled IVP toward success (Demetrio et al., 2022). However, the one major issue still impacting the practice is the reduced competence of *in vitro* matured oocytes. Most studies show that oocytes matured *in vivo* yield higher blastocyst rates and better blastocyst quality than their *in vitro* matured counterparts (Barfield, 2015; Aguila et al., 2020; Ferre et al., 2020).

The cumulus-oocyte complex (COC) metabolism is critical to oocyte maturation. Adenosine triphosphate (ATP) is the primary energy source for oocyte maturation and is generated in the mitochondrial electron transport chain (ETC) by oxidative phosphorylation. Glycolysis is limited in the immature oocyte (Suigura et al., 2005; Adhikari et al., 2022), and though mitochondria are immature in the oocyte, they are abundant, with hundreds of thousands per oocyte (Blerkom, 2011) producing adequate ATP for metabolic efficiency and oocyte maturation. Surrounding corona cells, attached to the oocyte by gap junctions, provide substrates

for catabolic ATP metabolism and communicate with the oocyte to optimize maturation (Richani et al., 2020). The *in vitro* maturation system of bovine COCs is a limiting factor in successful embryonic development, and one of the primary sources of this reduction in oocyte competence a sub-optimal COC metabolism *in vitro* (Stojkovic et al., 2001; Reviewed in: Sirard, 2018)

Photobiomodulation is the application of specific wavelengths of light (400-1000 nm) to tissues and systems for improved metabolic efficiency (Hamblin, 2006). Photobiomodulation treatment, specifically in the red to near-infrared spectrum, 600-1000 nm, is reported to reduce the time of tissue repair (Mester et al., 1968; Foley et al., 2016), have anti-inflammatory properties (Chow et al., 2009), and even improve mouse blastocyst rates when applied to presumptive zygotes (Sviridova-Chailakhyan et al., 2008). Discoveries of photoreceptors of blue (400-500 nm) and red (600-700 nm) light in the mitochondria have piqued interest in medical treatments of metabolic disease, cancer, and injuries (Karu, 2008; Reviewed in: Hamblin, 2018). Brief exposure to red light (600-700 nm) can enhance COC metabolism and improve oocyte maturation for successful subsequent embryonic development.

Hytell et al. (1986) observed bovine oocyte nuclear maturation *in vitro* and determined that the first polar body is extruded between 18 and 21 h of maturation. Their study, and many others, have observed mitochondrial redistribution from a peripheral location to a more homogenous distribution between 12 and 18 h of oocyte maturation (Dalton and Carroll, 2013; Reviewed in: Kirillova et al., 2021). In preliminary studies, treatment with red light (660-665 nm) at 18 to 20 h of bovine oocyte maturation yielded inconsistent improved blastocyst development at 168 h post-insemination compared to an untreated Control (unpublished data). Therefore, for this thesis work, experiments were designed to administer photobiomodulation treatment at two separate time points of bovine oocyte maturation aiming to increase ATP

availability at two critical stages for subsequent embryo development: (1) 16 h, before extrusion of the polar body, and (2) 20 h, before fertilization. We hypothesize that photobiomodulation treatment with red light (660-665 nm) at 16 h and 20 h of bovine oocyte maturation will improve subsequent embryonic development and oocyte mitochondrial activity.

MATERIALS AND METHODS

Media and Chemicals

All culture media was obtained from IVF Limited T/A IVF BioScience (Cornwall, TR11 4TA, UK) and chemicals from ThermoFisher Scientific Inc. (Waltham, M.A., USA) or Sigma-Aldrich Inc. (St. Louis, MO, USA) unless noted otherwise.

Experiment One

Objective

Experiment 1 was designed to determine the effects of photobiomodulation treatments at 16 h (L-16) and 20 h (L-20) of maturation compared to Control (no light) on subsequent embryonic development rates and blastocyst quality.

Experimental design

Cumulus-oocyte complexes (50 COCs/treatment) were matured in separated four-well dishes, treated with light (L-16 or L-20) or no light (Control), fertilized, and cultured. The cleavage and blastocyst rates were assessed, and the cell number of the blastocysts at 168 h PI was determined as a measure of blastocyst quality. The experiment was replicated six times; blastocyst cell number was determined in four of the replicates.

Procedures

Oocyte Collection, Selection, and In Vitro Maturation (IVM)

Abattoir bovine ovaries were obtained locally and transported in Dulbecco's Phosphate

Buffered Solution (D-PBS; Life Technologies Limited, Paisley, UK, 21600-051) or 0.09% saline

solution (EMD Millipore Corporation, Burlington, M.A., USA, 01803) at ~25°C. Cumulus-

oocyte complexes were aspirated from follicles with a diameter of 2-6mm. A silicone or rubber

stopper was set up with two portals, an 18-gauge needle was mounted on one portal, and a hose

connected to a vacuum pump (Thomas Industries Inc., Sheboygan, WI, USA, Model:

907CA118-430-2) to the other portal. The stopper was placed on a 50 mL tube (Falcon®,

Corning, Glendale, AZ, USA, 352098) containing 5mL of ovum pick-up solution (OPU; IVF

BioScience) or 5mL of D-PBS + 5% Fetal Bovine Serum (FBS; F2442) + 1% Penicillin

Streptomycin (Pen Strep; Gibco™, Lift Technologies Corp, Grand Island, NY, USA, 15140-122)

+ 95 USP sodium heparin (BD Vacutainer®, Franklin Lakes, NJ, USA, 367878) for collection of

COCs. The collected follicular fluid was allowed to sediment, and 3mL of the pellet was

resuspended into 7mL of OPU or D-PBS + 5% FBS + 1% Pen Strep + 95 USP sodium heparin in

a 100mm x 15mm plastic petri dish (Fisherbrand™, Pittsburg, PA, USA, 08-7577-13). Cumulus-

oocyte complexes were identified under a stereomicroscope at 400X and moved into a 35mm

tissue culture dish (ThermoFisher Scientific, Rochester, NY, USA, 130180) with 2.5mL of D-

PBS + 5% FBS + 1% Pen Strep. Cumulus-oocyte complexes with homogeneous cytoplasm and

3-5 tight surrounding layers of cumulus were selected and divided into equal groups (50

COCs/treatment). Each group was placed in 500µL of pre-equilibrated BO-IVM™ media (0 h)

in a four-well dish (Fisherbrand™, Pittsburg, PA, USA, FB012926) at 38.5 ºC in 5.5% CO_2 in

air, with high humidity.

Photobiomodulation Treatments

A light source was built with three Super-Bright LEDs at a wavelength of 660-665 nm (NTE Electronics Inc., Bloomfield, NJ, USA, NTE30041) assembled in a Styrofoam stage, arranged to illuminate a single well of a four-well dish. The light intensity of the three Super-Bright LEDs, measured with a power meter (Thorlabs Inc., Newton, NJ, Power meter PM320E), was 450μW in a single well of the four-well dish with 500μL of media.

Photobiomodulation treatments were applied by placing the designated four-well dish onto the Styrofoam stage with the culture well directly above the Super-Bright LEDs inside the incubator. The dish was covered with an opaque box to avoid light reflection coming back to the dish. Photobiomodulation treatments were as follows: exposure to the Super-Bright LEDs for 10 minutes at 16 h maturation (L-16), at 20 h maturation (L-20), or no light exposure (Control).

Sperm Preparation and In Vitro Fertilization (IVF)

Semen from one bull was used. A straw was thawed by immersion in a water bath at 38.5 ºC for 60 seconds, and its contents were placed on top of 2 ml of BO-SemenPrep™ and washed twice by centrifugation at 330g for 5 minutes. Sperm concentration was adjusted to 10×10^6 motile sperm/mL.

At 22 h maturation, each group of COCs was placed in 450 µL of BO-IVF™, and 50 µL of sperm preparation was added to each well (final sperm concentration: 1×10^6 motile sperm/mL). Fertilization was at 38.5 ºC in 5.5% CO_2 in air, with high humidity.

In Vitro Culture (IVC)

At 18 h PI, presumptive zygotes were placed in 50μL of 1.4IU/μL hyaluronidase (H3884) in D-PBS, incubated for 5 minutes at 38.5ºC, vortexed for 105 seconds (Vortex Genie, Scientific Industries Inc., Bohemia, NY), then moved through 50μL droplets of Wash solution (IVF BioScience) to remove excess cumulus cells, and placed into 500μL BO-IVC™ in a four-well

dish. Presumptive zygotes were cultured at 38.5°C in 5.5% O_2 and 5.5% CO_2, with high humidity for 54 h.

Embryonic Development Assessment and Blastocyst Cell Number

Cleavage rates were assessed at 72 h PI. Early embryos of four or more cells were considered viable and placed into a new four-well dish of 500µL pre-equilibrated BO-IVC™ at 38.5°C in 5.5% O_2 and 5.5% CO_2, with high humidity for 120 h. Blastocyst rates were assessed at 168- and 192 h PI.

At 168 h, blastocysts were removed from culture and fixed in 5% formalin D-PBS + 5% FBS (F2442) and kept at 4°C for ~18 h. Embryos were washed in 50 µL droplets of D-PBS + 5% FBS + 1% Pen Strep and then placed into a 1µg/mL Hoechst 33342 (Invitrogen™, Thermo-Fisher Scientific, Waltham, M.A., H21492) solution for 15 minutes. Blastocysts were rinsed from the stain in droplets of D-PBS + 5% FBS + 1% Pen Strep and mounted onto a microscope slide (12-550D) based on the procedure established by Moreira et al. (2001) and Pursel et al. (1985). The mounted blastocysts were imaged on a Cytation-1 cell-imaging instrument (BioTek® Instruments Inc., Winooski, VT) with Bright-Field and 377/447 nm filters (autoexposure settings) with a 10X objective. Cell numbers were determined by manually counting the stained blastomeres identified using the ImageJ cell counter software (National Institute of Health, Bethesda, MD), as previously described (Labno, 2020).

Statistical Analysis

Statistical analysis of embryonic development rates (cleavage and blastocyst rates) was performed using a Mixed Model procedure of repeated measures over time. Each well was considered the experimental unit; the main effects were treatment, time, and treatment-by-time interaction with a block by replicate; significance was set at $p < 0.05$. Embryos with 70 cells or

less at 168 h PI were considered early embryos, not blastocysts, and were removed from developmental rates and average blastocyst cell counts. Blastocyst cell number at 168 h PI was analyzed using a Mixed Model of least square means with the well considered as an experimental unit and main effect of treatment. The model assumption of independent residuals was met by including random blocking factors. The model assumptions of equal residuals and variance were evaluated with a combination of residual plots and tests, such as the Shapiro-Wilk. (SAS 9.4, SAS Institute Inc. Cary, NC).

Experiment Two

Objective

This experiment was designed to determine the effects of photobiomodulation treatments during maturation on oocyte cytoplasmic ATP levels and mitochondrial membrane potential (MMP) at 24 h of maturation.

Experimental Design

Cumulus-oocyte complexes (50 COCs/treatment) were placed under maturation conditions; photobiomodulation treatments were light at 16 h (L-16) and 20 h (L-20) of maturation and Control (no light). At 24 h of maturation, COCs were vortexed to remove cumulus cells and stained to determine ATP concentration or mitochondrial membrane potential.

Procedures

Oocyte collection, selection and maturation, and photobiomodulation treatments were conducted as previously described in Experiment 1.

Cumulus Removal

45

Cumulus-oocyte complexes (20-50 COCs/treatment) were removed from maturation at 24 h. COCs were incubated in 2.1IU/µL hyaluronidase diluted in D-PBS for 5 minutes at 38.5℃, vortexed for 105 seconds, then moved through 5-50µL drops of D-PBS + 5% FBS + 1% Pen Strep to remove any remaining cumulus cells.

Fluorescence Staining for Mitochondrial Activity in Bovine Oocytes

The denuded oocytes, for each treatment, were subjected to one of two fluorescence staining procedures: (1) for ATP determination: oocytes were incubated in 5µM BioTracker™ ATP-Red Live Cell Dye (EMD Millipore Corp., Temecula, CA, USA, SCT045) suspended in D-PBS + 5% FBS for 15 minutes at 38.5℃ in a four-well dish (15-20 denuded oocytes/treatment); or (2) for MMP determination: oocytes were incubated in 200nM MitoTracker™ Red CMXRos Dye (Invitrogen™, Thermo-Fisher Scientific, Waltham, M.A., M7512) suspended in the used BO-IVM™ for 30 minutes at 38.5℃ and 5.5% CO_2 (15-20 denuded oocytes/treatment), then fixed in 5% formalin diluted in D-PBS + 5% FBS + 1% Pen Strep, and stored at 4℃ for 18 h until imaging. For both fluorescence staining procedures, a Hoechst 33342 co-stain of 1µg/mL was added for the duration of the stain incubation period.

Imaging Settings

Oocytes or blastocysts were then rinsed in droplets of D-PBS + 5% FBS to remove excess solution and placed in a BD Falcon™ Microtest™ 96-well Assay Plate, Optilux™ (BD Biosciences, Franklin Lakes, NJ, 353293) at 15-20 oocytes per well. Oocytes were imaged using the Cytation-1 cell-imaging instrument, set to incubate at 38℃ for ATP or at room temperature for MMP. Images were captured in Bright-Field (settings of integration time 100s, gain at 0, and LED intensity at 5), with 377/447 nm excitation and emission filters for Hoechst (setting on autoexposure), and (1) 531/593 nm excitation and emission filters for cytoplasmic ATP levels

(settings on auto exposure) or (2) 586/647 nm excitation and emission filters (settings on auto exposure) for MMP at 10X objective. A Z-Stack imaging protocol was used with the Z-top and Z-bottom established between 3300 ± 100 μm and 3100 ± 100 μm, respectively. Z-Stack images comprised approximately 10 ± 5 slices of the oocytes. Images were saved for later image analysis.

Image Analysis of Z-Stacks

Three-dimensional (3D) Z-Stack images of each oocyte were converted to a two-dimensional (2D) Z-projection using the Gen5™ software (BioTek® Instruments Inc., Winooski, VT, USA, version 3.08). Each Z-Projection consisted of 10 ± 5 images (slices) every 20 µm and were then analyzed using the Image Statistics application in Gen5™. A plug tool was shaped to the cytoplasm of each oocyte to reduce any background autofluorescence and analyze individual oocytes. Each oocyte's mean fluorescence in relative fluorescence units (RFU) was recorded. A minimum threshold of ~17000 RFU was established to eliminate degenerating or dead oocytes, and these oocytes were excluded from statistical analysis.

Statistical Analysis

The average fluorescence per treatment per replicate for the ATP (six replicates) and MMP (three replicates) was calculated for each group: L-16, L-20, and Control. A Mixed Model procedure of least square means was used to analyze each (1) cytoplasmic ATP levels and (2) mitochondrial membrane potential at 24 h of maturation. Each well was considered an experimental unit with the main effect of treatment and a replicate block; significance was set at $p < 0.05$. The model assumption of independent residuals was met by including random blocking factors. The model assumptions of equal residuals and variance were evaluated with a combination of residual plots and tests, such as the Shapiro-Wilk. (SAS 9.4, SAS Institute Inc.).

Experiment Three

Objective

The purpose of this experiment was to evaluate the effects of photobiomodulation treatments (L-16, L-20, and Control) during oocyte maturation on ATP levels at the blastocyst stage.

Experimental Design

Cumulus-oocyte complexes (50/treatment) were matured, treated as for experiments 1 and 2, fertilized, and cultured to the blastocyst stage. At 188 h PI, blastocyst ATP levels and cell numbers were determined. The experiment was replicated six times.

Procedures

Oocyte collection, selection and *in vitro* maturation, photobiomodulation treatments, cumulus cell removal, *in vitro* culture, and cleavage rate assessment were conducted as described in Experiment 1. Semen preparation and *in vitro* fertilization were as previously described, except two bulls were used alternately per replicate. Blastocyst rates were assessed at 168 h PI and 188 h PI. Blastocyst cell number was assessed at 188 h PI.

Fluorescence Staining for Mitochondrial Activity in Bovine Oocytes

At 188 h PI, blastocysts were immediately placed into a co-stain solution of 5μM BioTracker™ ATP-Red Live Cell Dye and 1μg/mL Hoechst 33342 diluted in D-PBS + 5% FBS for 15 minutes at 38.5°C in a four-well dish (15-25 blastocysts/treatment).

Imaging and image analysis were conducted as described in Experiment 2, except blastocysts were imaged as 15 ± 5 slices per blastocyst.

Statistical Analysis

48

Statistical analysis of the embryonic development rates (cleavage and blastocyst rates) was as described in Experiment 1, except that main effects of bull and bull-by-treatment interaction were added to the model for embryonic development (cleavage and blastocyst rates) and blastocyst cell number.

Cytoplasmic ATP content was analyzed as described in Experiment 2 (for cytoplasmic ATP content) with added main effects of bull and bull-by-treatment interaction. Raw ATP data was not normally distributed as per the Shapiro-Wilk, Kolmogorov-Smirnov, and the residual plot normality tests; therefore, the data was log-transformed for statistical analysis. The model assumption of independent residuals was met by including random blocking factors. The model assumptions of equal residuals and variance were evaluated with a combination of residual plots and tests, such as the Shapiro-Wilk. (SAS Institute Inc.).

Experiment Four

Objective

This experiment was designed to determine the cytoplasmic ATP levels over time of bovine oocytes exposed to L-16, L-20, or Control during maturation.

Experimental Design

Oocyte ATP levels were determined before treatment and every two hours after treatment, up to 24 hours of maturation. For practical purposes, the experiment was split into two smaller trials, one per light treatment (L-16 vs. Control and L-20 vs. Control). Each trial was replicated six times.

Procedures

49

Oocyte collection, selection and maturation, and photobiomodulation treatments were conducted as previously described in Experiment 1.

Cumulus-Oocyte Complex Redistribution After Photobiomodulation Treatment

Immediately after photobiomodulation treatments in each trial, L-16 vs. Control and L-20 vs. Control, COCs were removed from their original IVM well and placed into a new four-well dish with 250µL pre-equilibrated unused IVM and 250µL of the original IVM for 20 COCs/treatment/h. The four-well dishes were separated into two separate incubators with a four-hour difference in the COCs/treatment/h (i.e., L-16 vs. Control: the 18 h and 22 h imaging groups were placed in incubator A and the 20 h and 24 h imaging groups were placed in incubator B). The incubators used for each hour grouping were rotated for each replicate.

Cumulus Cell Removal During Maturation

In the first trial, L-16 vs. Control, at 16 h of maturation, before photobiomodulation treatment, and at h 18, 20, 22 and 24 of maturation, 20 COCs/treatment were removed from maturation and vortexed to remove cumulus cells. In the second trial, L-20 vs. Control, at 20 h of maturation, before photobiomodulation treatment, and at h 22 and 24 of maturation, 20 COCs/treatment were removed from maturation and vortexed to remove cumulus cells.

Fluorescence Staining and Imaging for Cytoplasmic ATP Levels

For L-16 vs. Control, at h 16 (pre-treatment), 18, 20, 22, and 24 of maturation, denuded oocytes (15-20/treatment/h) were stained for cytoplasmic ATP levels, imaged, and the images analyzed as described for experiment 2, except that 1 µg/mL Hoechst was added to the stain solution. For L-20 vs. Control, at h 20 (pre-treatment), 22, and 24 of maturation, denuded oocytes (15-20/treatment/h) were stained for cytoplasmic ATP levels, imaged, and the images

50

analyzed as described for experiment 2, except that 1 µg/mL Hoechst was added to the stain solution.

Nuclear Stage Progression Analysis

For both L-16 vs. Control and L-20 vs. Control, the Z-Stack images obtained for Hoechst were used to identify the stained DNA in each oocyte. Oocytes were categorized as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII) or degenerated as described by Hyttel et al. (1986) and Roth and Hansen (2005).

Statistical Analysis

Each trial, L-16 vs. Control and L-20 vs. Control was analyzed independently using a Mixed Model procedure of repeated measures over time. For cytoplasmic ATP, oocytes below the designated 17,000 RFU threshold were considered non-viable and excluded from the statistical analysis. Each well was considered an experimental unit with the main effects of treatment, time, and time-by-treatment interaction blocked by replicate; significance set at $p < 0.05$. The model assumption of independent residuals was met by including random blocking factors. The model assumptions of equal residuals and variance were evaluated with a combination of residual plots and tests, such as the Shapiro-Wilk. (Mixed Procedure, SAS 9.4 Institute Inc.).

For nuclear progression, oocytes considered degenerated or identified in the GV or GVBD stages were excluded from the analyses. The average percentage of metaphase II and metaphase I oocytes was determined, and only the percentage of MII oocytes over time was used for statistical analysis. Each well was considered an experimental unit, and a Mixed Model of repeated measures over time was used with the main effects of treatment, time, and treatment-by-time interaction blocked by replicate; significance set at $p < 0.05$. The data for L-20 vs. Control

51

for MII percentage was not normally distributed as per the Shapiro-Wilk and Kolmogorov-Smirnov tests and the residuals plot for normality. The residual plot identified two extreme outliers in the data, and the outliers were removed from the analysis after a residual outlier test. After that, the data was normally distributed. The model assumption of independent residuals was met by including random blocking factors. The model assumptions of equal residuals and variance were evaluated with a combination of residual plots and tests, such as the Shapiro-Wilk. (SAS 9.4. Institute Inc.).

Experiment Five

Objective

This experiment was designed to determine the mitochondrial membrane potential of bovine oocytes exposed to L-16, L-20, or Control during maturation.

Experimental Design

Mitochondrial membrane potentials were determined before treatment and every two hours after treatment, up to 24 h of maturation. For practical purposes, the experiment was split into two smaller trials, one per light treatment (L-16 vs. Control and L-20 vs. Control). Each trial was replicated six times.

Procedures

Oocyte collection, selection, maturation, and photobiomodulation treatments were conducted as previously described in Experiment 1. Oocyte redistribution post-treatment and cumulus cell removal at each timepoint were carried out as described in Experiment 4. Fluorescence staining, image settings and image analysis were performed as described in Experiment 2, except the 200nM MitoTracker Red CMXRos and 1 µg/mL Hoechst 33342 co-

stain was diluted in D-PBS + 5% FBS + 1% PenStrep. Statistical analysis was conducted as described in Experiment 4.

RESULTS

Experiment One

Embryonic development rates (cleavage and blastocyst rates) were higher in the treatment groups, L-16 and L-20 than in the Control group ($p < 0.05$). There was an effect of time ($p < 0.05$), with cleavage rates being higher than blastocyst rates for all groups (**Table 4.1**). Blastocyst cell numbers 168 h PI did not differ ($p > 0.05$) between groups in the four replicates; however, there was a tendency ($p = 0.09$) for higher cell numbers for the L-20 treatment group compared to the L-16 treatment group. There was no effect of treatment-by-time interaction ($p > 0.05$) on embryonic development rates.

Experiment Two

At 24 h of maturation (**Figure 4.1**), cytoplasmic ATP levels were different among treatments ($p < 0.05$). Oocytes exposed to L-16 had higher levels of ATP than those exposed to the L-20 treatment ($p < 0.05$). There was a tendency ($p = 0.08$) for higher cytoplasmic ATP levels in L-16 than in Control. There was no significant difference ($p > 0.05$) in MMP among the groups (**Figure 4.2**).

Experiment Three

Overall embryo development rates (**Table 4.3**) were different among treatments ($p < 0.05$); both treatments had higher overall embryonic development rates than Control ($p < 0.05$), but L-16 and L-20 did not differ ($p > 0.05$). There was an effect of time ($p < 0.05$) and bull ($p < 0.05$) on embryonic development, as well as a tendency for treatment-by-bull interaction ($p =$

53

0.07). The blastocyst cell numbers were different among treatments ($p < 0.05$), being higher in L-20 than in the Control group ($p < 0.05$); L-16 was intermediate (**Table 4.4)**. Additionally, there was an effect of bull on cell numbers ($p < 0.05$).

The cytoplasmic ATP levels of blastocysts at 188 h PI (**Figure 4.3**) were not different among groups ($p > 0.05$).

Experiment Four

Cytoplasmic ATP Levels Over Time – L-16 vs. Control and L-20 vs. Control. For L-16 treatment (**Figure 4.4**), cytoplasmic ATP levels over time were significantly higher than the Control ($p < 0.05$). There was no effect of time or treatment-by-time interaction. There was no treatment effect ($p > 0.05$) for L-20 compared to the Control (**Figure 4.5**). Cytoplasmic ATP levels did not differ across time ($p > 0.05$), nor was there an effect of treatment-by-time interaction ($p > 0.05$).

Nuclear Progression Over Time – L-16 vs. Control. There was no treatment effect ($p > 0.05$) for L-16 compared to the Control; however, there was an overall effect of time ($p < 0.05$) on nuclear maturation (**Figure 4.6**). At h 16 of maturation, before treatment, 45.0% ± 8.0% (Mean ± Standard Error; SE) of oocytes were arrested in metaphase II. The percentages of metaphase II oocytes at h 18, 20, 22, and 24 were 78.1% ± 3.0%, 83.3% ± 2.0%, 77.1% ± 6.0%, 85.0% ± 3.0% for L-16, and 73.6% ± 4.0%, 82.0% ± 3.0%, 78.5% ± 5.0%, and 84.5% ± 3.0% for Control, respectively. There was no time-by-treatment interaction ($p > 0.05$) on the nuclear progression through meiosis.

Nuclear Progression Over Time – L-20 vs. Control. There was no treatment effect ($p > 0.05$) for L-20 compared to the Control, however, there was an overall effect of time ($p < 0.05$) on nuclear maturation (**Figure 4.7**). At h 20 of maturation, there was a higher percentage of MII

oocytes than at h 22 ($p < 0.05$), and there was a tendency ($p = 0.07$) for a significant difference between h 20 and 24. There was no time-by-treatment interaction ($p > 0.05$).

Experiment 5

Mitochondrial Membrane Potential Over Time – L-16 vs. Control and L-20 vs. Control. There was no significant difference ($p > 0.05$) between the groups for treatment, time, or treatment-by-time interaction for either L-16 vs. Control (**Figure 4.8**) or L-20 vs. Control **Figure 4.9**).

DISCUSSION

Photobiomodulation, specifically in the 600-1000 nm range, has proven to be effective in accelerating tissue repair, reducing inflammation and increasing metabolic activity (Mester et al., 1968; Foley et al., 2016; Chow et al., 2009; Gordon & Surrey, 1960; Passarella et al., 1984; Review: Hamblin, 2018). We used photobiomodulation as a treatment aimed at improving oocyte metabolic activity and developmental competence. With very few studies testing light treatments on bovine embryonic development and the knowledge that the final stages of nuclear and cytoplasmic maturation take place between 12- and 22 h of oocyte maturation (Hyttel et al., 1986; Kruip et al., 1983; Sirard et al., 1989; Trebichalska et al., 2021), we established the treatment times at h 16 (L-16) and 20 (L-20) of *in vitro* maturation.

Effects on Embryonic Development. To first observe L-16 and L-20 effects on embryonic development, we monitored cleavage and blastocyst rates of COCs inseminated post-photobiomodulation treatments. Embryonic development rates of the photobiomodulation-treated groups were significantly higher than the Control (**Table 4.1**). The results from Experiment 3 supported the findings from Experiment 1, with the treatment groups having higher embryonic

rates than the Control (**Table 4.3**). Neither experiments 1 or 3 indicated differences between L-16

and L-20 embryonic development rates. Oh et al. (2007) showed that golden hamster embryonic

development was significantly higher after 10 min of exposure to red light (620-750 nm) at the

2-cell embryo group stage. The red light out-performed visible (390-750 nm), blue (445-500

nm), green (500-575 nm), and yellow (575-585 nm) lights with 100% of red-light-exposed 2-

cell-embryos progressing to the morula stage and 84% of them progressing to the blastocyst

stage. A similar study exposing mouse zygotes to a solar light with an orange-red luminescent

component (626 nm_{max}) for 15 min after recovery from the mice oviducts increased blastocyst

development (72 h and 96 h post-fertilization) and blastocyst hatching rates compared to the

Control (Sviridova-Chailakhyan et al., 2008). Additionally, an evaluation of the effects of red

light (625 nm) for microscope-embryo work, specifically in the EmbryoScope time-lapse system,

determined that the red light does not negatively affect mouse or porcine embryonic development

(Li et al., 2014). These studies support our hypothesis that red light, specifically in the 620-700

nm range, aids in mammalian *in vitro* embryonic development.

Effects on Blastocyst Cell Numbers. A trained person can quickly identify a blastocyst

with the optics of a stereomicroscope. Though a prominent blastocoel cavity, inner cell mass and

trophectoderm are visible, the quality of the blastocyst could be compromised. One of the most

common forms of evaluating blastocyst quality in embryonic research studies is to count the

number of blastomeres present at the appointed blastocyst check time for bovine blastocysts --

typically between 172- and 196 h PI (Enright et al., 2000). Therefore, in Experiments 1 and 3, we

counted the blastomeres in blastocysts derived from photobiomodulation treated, L-16 or L-20,

or Control oocytes. In Experiment 1, there was a tendency for improved cell numbers for the L-

20 treatment group compared to the Control (**Table 4.2**) and a significant difference between the

L-20 and L-16 treatment groups (**Table 4.2**). In Experiment 3, a significant effect of higher blastocyst cell numbers for L-20 treatment than for Control was confirmed (**Table 4.4**). In support, Sviridova-Chailakhyan et al. (2008) evaluation of blastocyst cell number determined that the embryos exposed to "solar" light plus the red-orange luminescent filter (626 nm$_{max}$) yielded the highest cell numbers compared to the "solar" only exposed and Control embryos. In conjunction with the results from the study of Sviridova-Chailakhyan et al. (2008), our results reveal a potential improvement in blastocyst quality by photobiomodulation treatment *in vitro*.

An important difference between Experiment 1 and Experiment 3 is that only one bull was used in Experiment 1, and two bulls were used in Experiment 3. In Experiment 3, there was an effect of bull on blastocyst cell numbers. Differences in *in vitro* embryonic development rates and quality between bulls are not uncommon when conditions are standardized for research and not necessarily optimized for each animal (Seidel, 2022). Interestingly, there was a tendency for an effect of bull-by-treatment interaction on embryonic development rates, even though photobiomodulation treatment was administered before fertilization. It can be hypothesized that an altered COC metabolism could have been carried over to fertilization and possibly affected gene activation (Reviewed in: Wu & Sirard, 2018). Additional research is needed to explain this effect.

Effect on Mitochondrial Activity. Under sub-optimal culture conditions such as fluctuations in temperature, atmospheric gases, and pH, *in vitro* oocyte/embryo metabolic function can be negatively impacted (Hawk& Wall, 1994). A single oocyte houses over 200,000 immature mitochondria (Micheals et al., 1982), and understanding that the mitochondria are the central unit of ATP production for the oocyte due to a block on glycolysis up until late embryonic development (mouse: Biggers et al., 1967; bovine: Sutton-McDowell et al., 2010), makes it a

target for treatment to increase oocyte metabolism (Adhikari et al., 2022; Review: Van Blerkom, 2011; Reviewed in: Spinelli & Haigis, 2018).

Because nearly all of an oocyte's ATP is mitochondrially produced by the oocyte or shuttled in from the attached corona cells, measuring ATP content quickly evaluates if the metabolic activity of the oocyte or its surrounding cells is altered. Stojkovic et al. in 2001 determined that oocytes of grade 1, those that were surrounded with tight layers of cumulus cells and had homogenous cytoplasm, contained higher ATP levels than those that were grade 2-4, with some defect in the surrounding cumulus cells or cytoplasm.

Following the improved embryonic development after L-16 and L-20 treatments that were observed in Experiment 1, we investigated if light exposure would alter oocyte metabolic function. In Experiment 2, the mitochondrial activity was measured as cytoplasmic ATP levels and MMP at 24 h of maturation, post-photobiomodulation treatment (L-16 and L-20), or no treatment (Control). In Experiment 2, we found a significant increase in ATP levels in the L-16 photobiomodulation treatment compared to the L-20 and a tendency for an increased treatment effect of L-16 compared to the Control (**Figure 4.2**), but no effect of light treatment on $\Delta\Psi m$ (**Figure 4.3**).Because bovine oocyte ATP levels were affected by L-16 treatment at 24 h of maturation and there was improved embryonic development, in Experiment 3, we aimed to determine if this increase in cytoplasmic ATP carried over into the blastocyst stage. In Experiment 3, we measured ATP levels of blastocysts at 188 h PI; however, no differences were found among the groups. We concluded that L-16 and L-20 treatments induced an increased level of ATP within the oocytes, but this effect was not carried over to the blastocyst stage. Therefore, based on our studies thus far, we hypothesize that our photobiomodulation treatments did not permanently alter ATP production in the embryo.

To our knowledge, there are no previous studies monitoring the behavior of cytoplasmic ATP in bovine oocytes during *in vitro* maturation. Therefore, we studied the effect of the photobiomodulation treatments on ATP levels over time during late maturation. In Experiment 4, we measured ATP before and every two hours after photobiomodulation treatments until the end of oocyte maturation (24 h). Unfortunately, an issue we faced in Experiment 4 was that L-16 and L-20 treatments and their time point assessments could not be done in the same replicate. Doing L-16 and L-20 treatments in the same replicate could have exposed the oocytes to fluctuations in the incubator environment because of opening/closing or prolonged the oocytes' time spent in media unfit for oocyte maturation. Therefore, statistical analysis was not performed directly comparing L-16 to L-20, but each to their respective Control group. Additionally, redistribution of the COCs into separate four-well plates with 50% equilibrated and unused IVM and 50% used equilibrated IVM was required for Experiment 4. Cumulus cells are necessary for oocyte cytoplasmic maturation and metabolic activities (Donahue and Stern, 1968; Jamnongjit & Hammes, 2005), so separate groups of COCs had to be taken at each set hour instead of having one homogeneous group of oocytes denuded immediately following treatment and then monitored over the time period. This repeated manipulation could have affected the health of the oocytes or increased the variability of the data collected. In future projects in this line of work, the procedures for treating, redistributing, denuding, and imaging must be optimized to minimize the effects that extensive handling of the COCs has on the results.

Despite these challenges, the overall ATP level (RFU) over time for L-16 was significantly higher than its respective Control (**Figure 4.4**). This outcome was expected since in Experiment 2, L-16 had the highest ATP levels at h 24 of maturation, leading to the conclusion that photobiomodulation treatment at h 16 of *in vitro* maturation increases oocyte cytoplasmic

ATP levels. Photobiomodulation treatment at h 20 of bovine oocyte maturation did not yield a treatment effect on ATP levels over time compared to its Control. Dalton et al. (2013) evaluated ATP levels in mouse oocytes in correlation with their nuclear maturation and determined that the ATP levels gradually rose after GVBD and peaked at maturation when the first polar body was extruded. According to several studies previously published, by h 20 of bovine oocyte maturation, the polar body is already extruded (Hyttel et al., 1986; Ward et al., 2002; Review in: Aguila et al., 2020). Thus, if ATP levels peaked before treatment and steadily declined after the polar body extrusion, the L-20 treatment may not have had an immediate or noticeable effect by h 24 of maturation.

As ATP synthesis and $\Delta\Psi$m are inversely related, the increase in cytoplasmic ATP levels over time in Experiment 4 led us to expect a change in MMP in Experiment 5. However, in Experiment 5 for L-16 and L-20 (**Figures 4.8** and **4.9**), the MMPs are not different from their respective Control groups. The first and best explanation for this outcome is an inadequate technique for measuring MMP. Many studies use other fluorescence stains, such as the red/green fluorescence probe JC-1 (Lin et al., 2018; Kim et al., 2023), while others use new approaches to image analysis like ratiometric imaging and image analysis (Al-Zubaidi et al., 2019). A more sensitive fluorescent probe and an imaging and image analysis system that standardizes the fluorescent intensity of each pixel to a set value could provide different outcomes than observed in Experiment 5.

A second potential explanation for a lack of change in MMP, but a difference in cytoplasmic ATP, could be that the photobiomodulation treatment enhanced the surrounding corona and cumulus cells' metabolic activity during *in vitro* maturation. As the ATP levels of the corona radiata cells rose, more ATP molecules could have been shuttled into the oocyte via gap

junctions, increasing the ATP levels in the oocytes. Oocytes have very immature mitochondria compared to somatic cells (Trebichalska et al., 2021), and there is a possibility that they did not respond to the photobiomodulation treatment. Independently of the source of ooplasmic ATP, from the corona cells, the oocyte itself, or both, the treatment did increase ooplasmic ATP and improved subsequent embryonic development.

Though no previous studies prove this hypothesis, the metabolic activity of mouse COC has been studied and revealed that granulosa cells are more metabolically active just before maturation and ovulation (Takahashi et al., 2016; Reviewed in: Zhang et al., 2023). Most mammalian oocytes are incapable of glucose uptake and conversion to metabolic substrates like pyruvate and lactate due to a block on glycolysis until late embryonic development. Corona radiata cells communicate with the oocyte via gap junctions and paracrine signaling to break down glucose and lipids into pyruvate and lactate for oxidative phosphorylation and produce glycolytic ATP for the oocyte (Donahue and Stern, 1968; Jamnongjit & Hammes, 2005; Reviewed in: Richani et al., 2020; Kirillova et al., 2021). Without surrounding corona/cumulus cells, the oocyte's metabolism is compromised. For example, Dalton et al. (2013) established that denuded oocytes in the mouse model rapidly decreased in ATP after the GVBD and were significantly lower in ATP levels compared to their cumulus-intact oocyte counterparts.

Effects on Nuclear Maturation. Testing the efficacy of L-16 and L-20 photobiomodulation treatments on nuclear maturation is essential. Our step in this direction was to determine if there were any alterations in the timing or advancement through meiosis I and arrest at the MII nuclear stage. The L-16 pre-treatment had less than 50% MII oocytes and more than 80% MII oocytes from h 18 to 24 of maturation (**Figure 4.5**). The L-20 group had approximately 90% MII oocytes at pre-treatment and each succeeding time point (**Figure 4.6**).

Hyttel et al. (1986) first monitored the nuclear and cytoplasmic maturation in the bovine oocyte and determined that the polar body was extruded by h 18 of maturation. This shows that our photobiomodulation treatments did not impact oocyte nuclear progression through meiosis I up to metaphase II of meiosis II.

However, an interesting finding was that, for the L-20 treatment group, more oocytes were arrested at MII at h 20 than at h 22 of *in vitro* maturation. While each hour had its own group of oocytes, nuclear maturation should not have been so different between h 20 and 22. The likely explanation lies in the handling and manipulation of the h 22 group during the re-distribution of COCs post-treatment. Oocytes that were still progressing to the MII stage could have been hindered by the change in environment (IVM media and incubator conditions).

Limitations. The biggest challenge related to the photobiomodulation treatments, L-16 and L-20, is the brief exposure of COCs during maturation to altered environmental conditions within the incubator. To maintain the incubator's airtight seal, the light source had to remain outside the incubator in a warming oven and only placed inside the incubator during treatment times. The stage and span of the light source (three LEDs) also limited the light exposure to only one well at a time, requiring that all COCs being exposed be in the same well at that time, even if they were not initially placed in the IVM well at h 0 of maturation.

Future Directions. Many more factors must be tested to determine the safety and efficacy of photobiomodulation treatments to positively affect oocyte maturation and subsequent embryonic development. While the embryonic development rates increased for the treatment groups compared to the Control, and L-16 yielded increased ATP levels during maturation compared to the Control, this photobiomodulation treatment could affect many other cellular components. To grasp what the L-16 and L-20 photobiomodulation treatments are doing to

bovine COCs and subsequent embryos, further studies are needed to examine the effects on granulosa cells, denuded oocytes, mtDNA, nuclear gene expression, DNA methylation, ROS, pregnancy rates, and calf characteristics, to mention a few.

CONCLUSION

The bovine industry relies heavily on the IVP of embryos to create offspring with select genetics and improve the dairy and beef production systems (Demetrio et al., 2022). The IVP of bovine embryos is efficient and advanced with the use of sexed semen and genomic testing. However, IVP still lags in producing good-quality embryos for successful pregnancy and parturition compared to embryos produced or derived *in vivo* (Liebfried-Rutledge et al., 1987; Dieleman et al., 2002; Barfield, 2015). The IVP reduced embryonic development is largely due to inadequate oocyte cytoplasmic maturation and metabolism (Review: Sirard, 2018). We, therefore, aimed to determine if photobiomodulation treatment in the red- to near-infrared range could improve bovine embryonic development and oocyte metabolism *in vitro*. Our treatment of bovine oocytes at h 16 and 20 of *in vitro* maturation with red LED (660-665 nm) significantly improved embryonic development, blastocyst cell number, and ATP production during oocyte maturation, and the treatments did not impact mitochondrial membrane potential, nuclear maturation, and ATP at the blastocyst stage. Further research is needed to determine if this photobiomodulation treatment impacted other aspects of embryonic development, like gene expression or epigenetic remodeling. This body of work shows that photobiomodulation treatment is a promising candidate for improving the bovine *in vitro* maturation system.

TABLES

Table 4.1. Experiment 1: Embryonic development rates of photobiomodulation treated (L-16 and L-20) vs. untreated (Control) bovine oocytes.

Treatment (Mean ± SE)	Cleavage Rate 72 h PI	Blastocyst Rate 192 h PI	
L-16	88.5 ± 2.4	49.3 ± 2.9	A
L-20	87.3 ± 1.9	51.6 ± 1.9	
Control	81.3 ± 3.8	49.1 ± 3.0	B

[A,B]superscripts indicate a significant difference (p < 0.05) in overall embryonic development comprised of cleavage and blastocyst rates
Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation

Table 4.2. Experiment 1: Blastocyst cell number of photobiomodulation treated (L-16 and L-20) vs. untreated (Control) bovine oocytes.

Treatment (Mean ± SE)	Cell Number 168 h PI
L-16	149.3 ± 5.4[a]
L-20	164.0 ± 6.4[b]
Control	159.9 ± 9.4[ab]

[a,b]superscripts indicate a tendency for differences (p = 0.09) in cell number
Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation

Table 4.3 Experiment 3: Embryonic development rates of photobiomodulation treated (L-16 and L-20) vs. untreated (Control) bovine oocytes.

Treatment (Mean ± SE)	Cleavage Rate 72 h PI	Blastocyst Rate 192 h PI	
L-16	88.5 ± 2.4	44.0 ± 5.5	A
L-20	89.0 ± 2.5	44.3 ± 5.5	
Control	85.0 ± 1.1	35.3 ± 4.6	B

[A,B]superscripts indicate a significant difference ($p < 0.05$) in overall embryonic development, comprised of cleavage and blastocyst rates
Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation

Table 4.4 Experiment 3: Blastocyst cell number of photobiomodulation treated (L-16 and L-20) vs. untreated (Control) bovine oocytes.

Treatment (Mean ± SE)	Cell Number 188 h PI
L-16	145.8 ± 10.7[ab]
L-20	153.9 ± 12.4[a]
Control	142.5 ± 12.0[b]

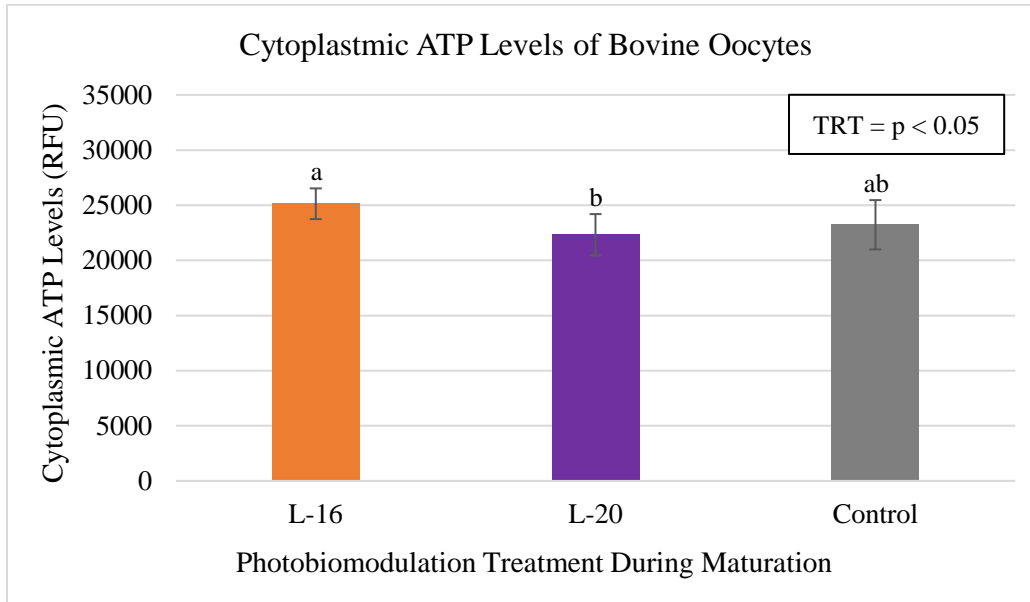[a,b]superscripts indicate a tendency for differences ($p = 0.09$) in cell number
Least Square Means (LSM) ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation

Figure 4.1 Experiment 2: Cytoplasmic ATP Levels in relative fluorescent units (RFU) of bovine oocytes at 24 h of maturation exposed to L-16 and L-20 photobiomodulation treatments.

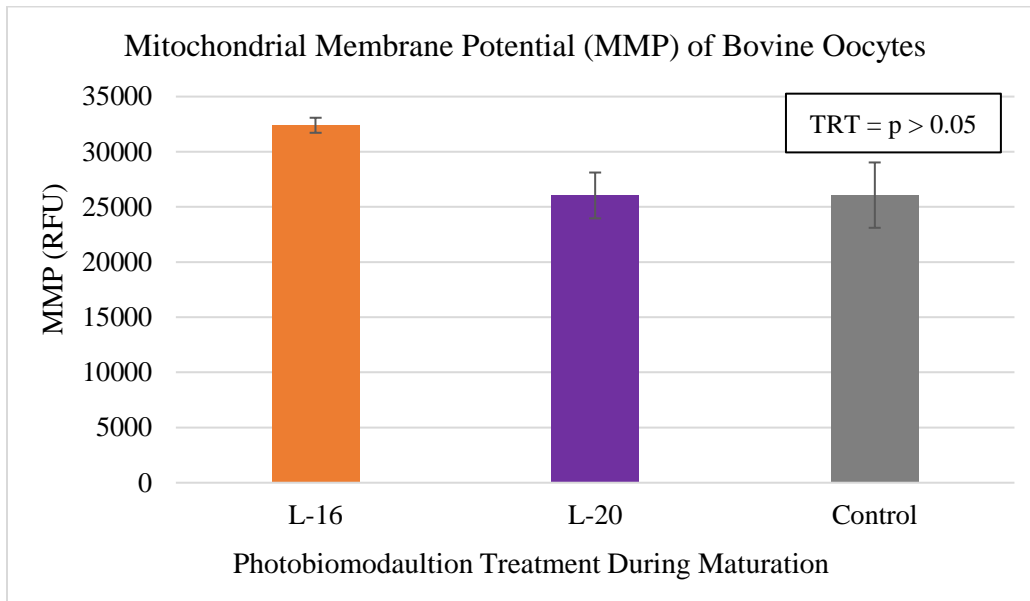[a,b] superscripts indicate significant differences ($p < 0.05$)
Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment

Figure 4.2 Experiment 2: Mitochondrial Membrane Potential (MMP) in relative fluorescence units (RFU) of bovine oocytes at 24 h of maturation exposed to L-16 and L-20 photobiomodulation treatments.
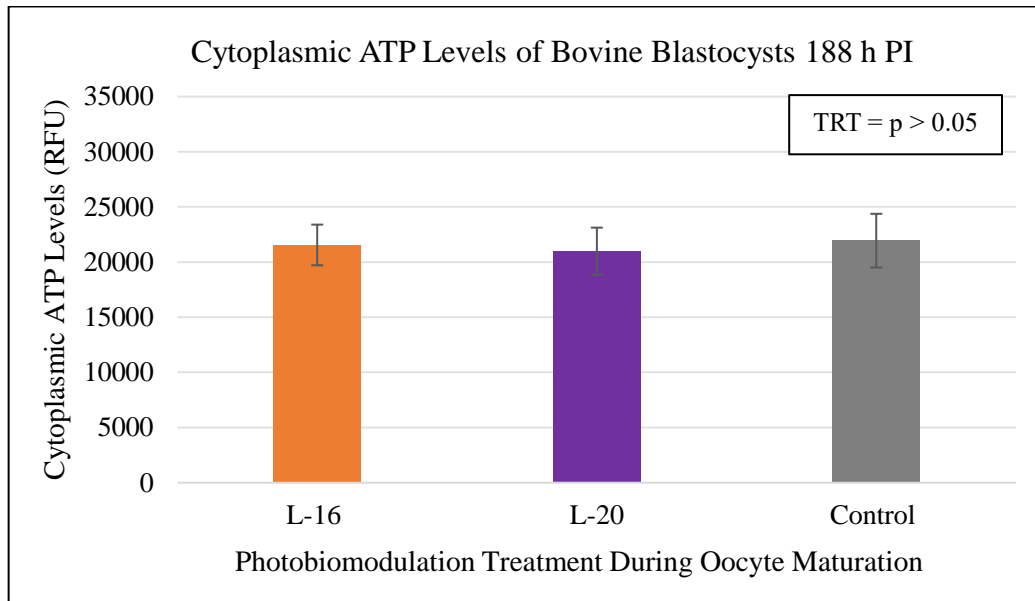

Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment

Figure 4.3 Experiment 3: Cytoplasmic ATP Levels in relative fluorescence units (RFU) of blastocysts (188 h Post-Insemination) derived from oocytes exposed to L-16 and L-20 photobiomodulation treatments.
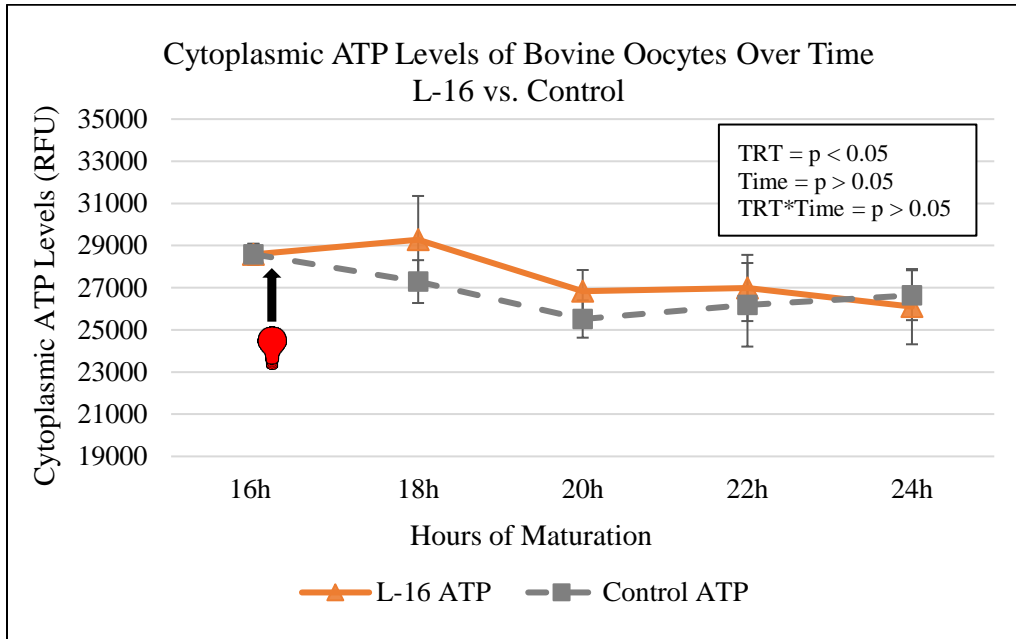

Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment

Figure 4.4 Experiment 4: Cytoplasmic ATP levels, in relative fluorescence units (RFU), over time of bovine oocytes exposed to L-16 photobiomodulation treatment.
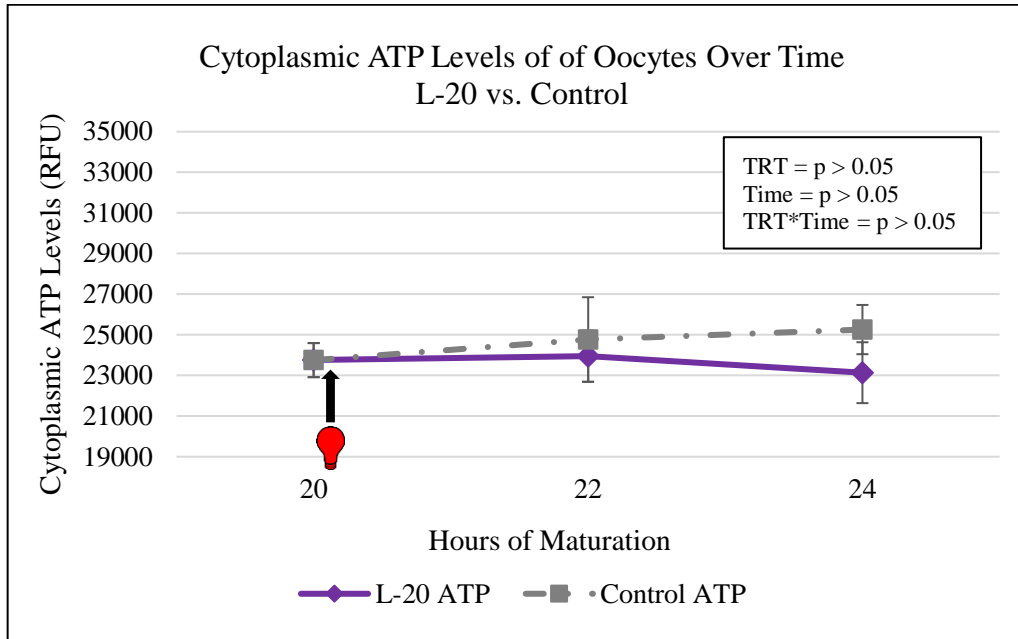

Means ± Standard Error (SE)

L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation

TRT = treatment

Red light bulb + arrow = L-16 treatment after removal of pre-treatment group from maturation

Figure 4.5 Experiment 4: Cytoplasmic ATP levels, in relative fluorescence levels (RFU), over time of bovine oocytes exposed to L-20 photobiomodulation treatment.
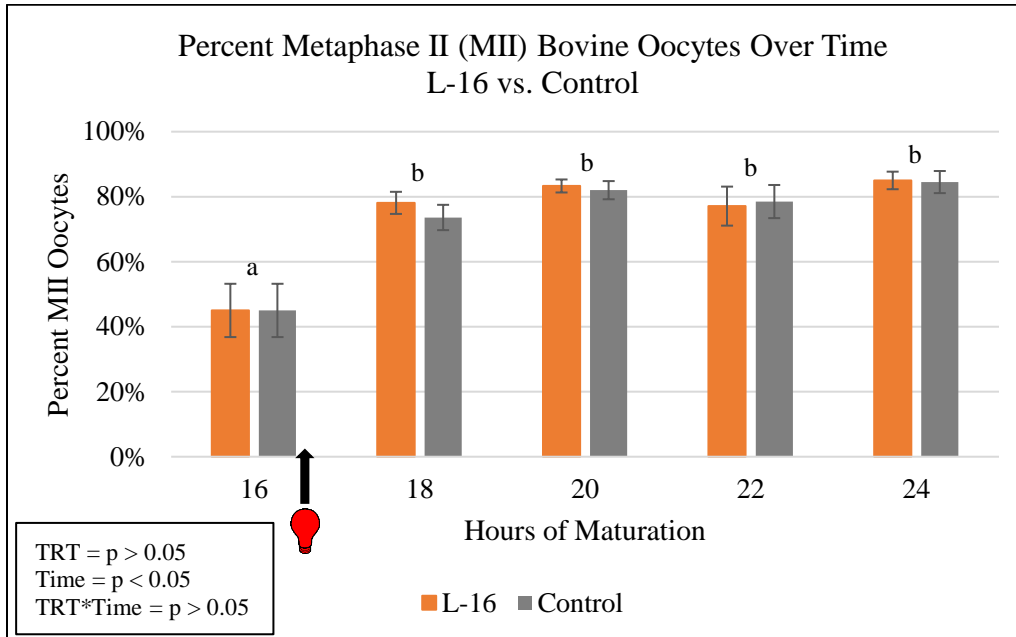

Means ± Standard Error (SE)
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment
Red light bulb + arrow = L-20 treatment after removal of pre-treatment group from maturation.

Figure 4.6 Experiment 4: Percentage of metaphase II (MII) oocytes over time after L-16 photobiomodulation treatment.

a,bsuperscripts indicate significant differences among time points ($p < 0.05$)
Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
TRT = treatment
Red light bulb + arrow = L-16 treatment after removal of pre-treatment group from maturation.
Treatment bars are shown individually for illustrative purposes only; there was no difference ($p > 0.05$) between treatments.

Figure 4.7 Experiment 4: Percentage of metaphase II (MII) oocytes over time after L-20 photobiomodulation treatment.

[a,b]superscripts indicate significant differences among time points ($p < 0.05$)
Means ± Standard Error (SE)
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment
Red light bulb + arrow = L-20 treatment after removal of pre-treatment group from maturation.
Treatment bars are shown individually for illustrative purposes only; there was no difference ($p > 0.05$) between treatments.

Figure 4.8 Experiment 5: Mitochondrial membrane potential (MMP), in relative fluorescence units (RFU), over time of bovine oocytes exposed to L-16 photobiomodulation treatment.
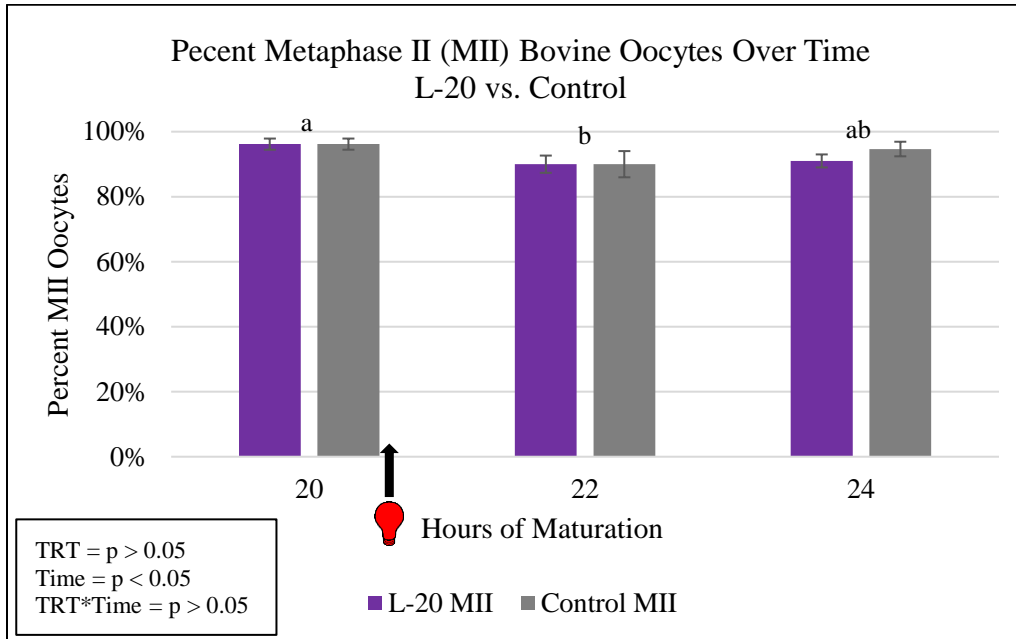

Means ± Standard Error (SE)
L-16 = Photobiomodulation treatment at 16 h of *in vitro* maturation
TRT = treatment
Red light bulb + arrow = L-16 treatment after removal of pre-treatment group from maturation.
Illustration over time separated by treatment for a visualization of the fluctuation in MMP of bovine oocytes in *in vitro* maturation

Figure 4.9 Experiment 5: Mitochondrial membrane potential (MMP), in relative fluorescence units (RFU), over time of bovine oocytes exposed to L-20 photobiomodulation treatment.
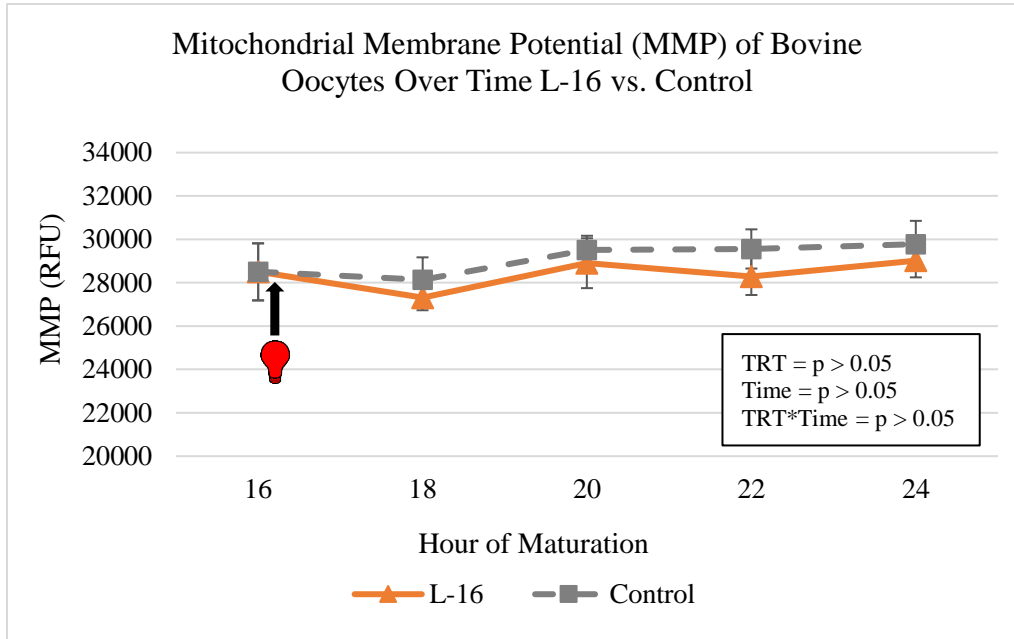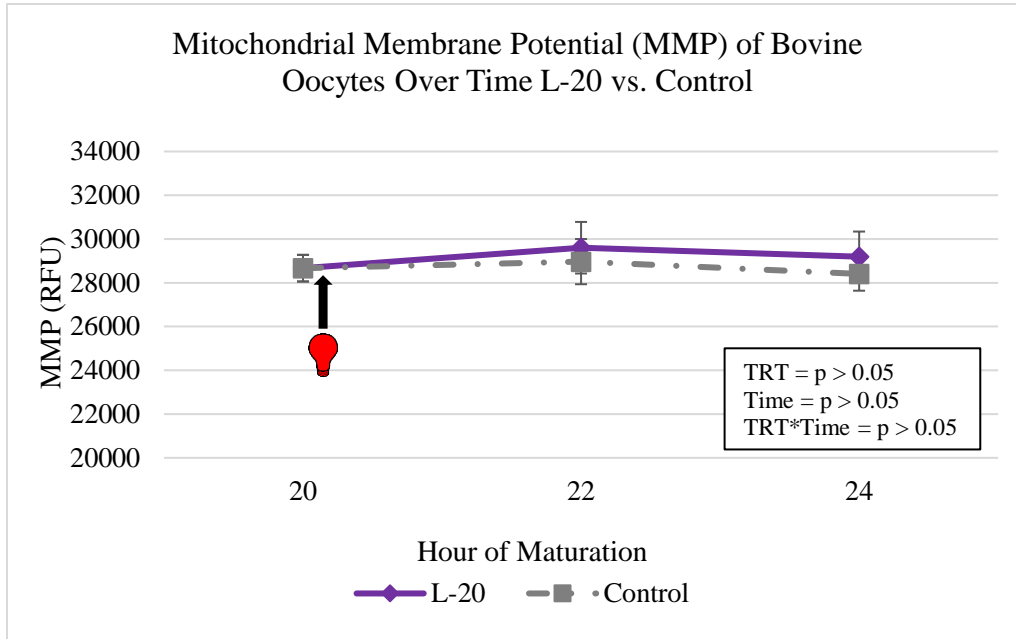

Means ± Standard Error (SE)
L-20 = Photobiomodulation treatment at 20 h of *in vitro* maturation
TRT = treatment
Red light bulb + arrow = L-20 treatment after removal of pre-treatment group from maturation.
Illustration over time separated by treatment for a visualization of the fluctuation in MMP of bovine oocytes in *in vitro* maturation

REFERENCES

Abe, H., Yamashita, S., Satoh, T. & Hoshi, H. (2002). Accumulation of Cytoplasmic Lipid Droplets in Bovine Embryos and Cryotolerance of Embryos Developed in Different Culture Systems Using Serum-Free or Serum-Containing Media. *Mol. Reprod. & Dev.*; 61: 57–66.

Adhikari, D., Lee, I-W., Yuen, W.S. & Carroll, J. (2022). Oocyte Mitochondria -- Key Regulators of Oocyte Function and Potential Therapeutic Targets for Improving Fertility. *Biol Reprod.*; 106(2): 366-377.

Aguila, L., Treulen, F., Therrien, J., Felmer, R., Valdivia, M. & Smith, L.C. (2020). Oocyte Selection for In Vitro Production in Bovine Species: Noninvasive Approaches for New Challenges of Oocyte Competence. *Animals (Basal)*; 10(12): 2196.

Agung, B., Otoi, T., Wongsrikeao, P., Taniguchi, M., Shimizu, R., Watari, H. & Nagai, T. (2006). Effect of Maturation Culture Period of Oocytes on the Sex Ratio of In Vitro Fertilized Bovine Embryos. *J Reprod. Dev.*; 52(1): 123-127.

Aktas, H., Leibfried-Rutledge, M.L, & First, N. (2003). Meiotic State of Bovine Oocytes is Regualted by Interactions Between cAMP, Cumulus and Granulosa. *Mol. Reprod. & Dev.*; 65: 336–343.

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G. & Gilchrist, R.B. (2010). Simulated Physiological Oocyte Maturation (SPOM): A Novel *In Vitro* Maturation System that Substantially Improves Embryo Yield and Pregnancy Outcomes. *Hum. Reprod.*; 25(12): 299-3011.

Ali., A.A., Bilodeau, J.F. & Sirard, M.A. (2003). Antioxidant Requirement for Bovine Oocytes Varies During In Vitro Maturation, Fertilization and Development. *Theriogen.*; 59(3): 939-949.

Al-Zubaidi, U., Liu, J., Robker, R.L., Adhikari, D. & Carroll, J. (2019). The Spatio-Temporal Dynamics of Mitochondrial Membrane Potential During Oocyte Maturation. *Mol. Hum. Reprod.*; 25(11): 695-705.

Al-Zubaidi, U., Adhikari, D., Cinar, O. Zhang, Q.H., Yuen, W.S., Murphy, M.P., Rombauts, L., Robker, R.L. & Carroll, J. (2021). Mitochondria-Targeted Therapeutics, MitoQ and BGP-15, Reverse Aging-Associated Meiotic Spindle Defects in Mouse and Human Oocytes. *Hum. Reprod.*; 36(3): 771-784.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nerlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., smith, A.J.H., Staden, R. & Young, I.G. (1981). Sequence and Organization of the Human Mitochondrial Genome. *Nature*; 290: 457-465.

Anderson, S., de Bruijn, M.H.L, Coulson, A.R., Eperon, I.C., Sanger, F. & Young, I.G. (1982). Complete Sequence of Bovine Mitochondrial DNA Conserved Features of the Mammalian Mitochondrial Genome. *Mol. Biol.*; 156: 683-717.

Ankri, R., Lubart, R. & Taitelbaum, H. (2010). Estimation of the Optimal Wavelength for Laser-Induced Wound Healing. *Lasers Surg. and Med*.; 42: 760-764.

Apostolova, N. & Victor, V.M. (2015). Molecular Strategies for Targeting Antioxidants to Mitochondria: Therapeutic Implications. *Antioxid. Redox Signal.*; 22(8): 686-729.

Arias-Alvarez, M., Bermejo-Alvarez, P., Gutierrez-Adan, A., Rizos, D., Lorenzo, P. & Lonergan, P. (2011). Effect of Leptin Supplementation During In Vitro Oocyte Maturation and Embryo Culture on Bovine Embryo Development and Gene Expression Patterns. *Theriogen.*; 75(5): 887-896.

Arif, A.A., Maulana, T. Kaiin, E.M., Purwantara, B. & Arifiantini, R.I. (2022). The Quality of Frozen Semen of Limousin Bull in Various Semen Diluents. *Trop. Ani. Sci.*; 45(3): 284-290.

Auclair, S., Uzbekov, R., Elis, S., Sanchez, L., Kireev, I., Lardic, L., Dalbies-Tran, R. & Ubekova, S. (2013). Absence of Cumulus Cells During *In Vitro* Maturation Affects Lipid Metabolism in Bovine Oocytes. *Am. J. of Physiol.-Endo. & Metab.*; 304(6): E599-E613.

Barfield, J. (2015). Evaluation of *In-Vitro* Produced Bovine Embryos. Proceedings of the CETA/ACTE & AETA Joint Convention.

Bensasson, R.V., Salet, C., Sloper, R.W., Truscott, T.G. & Land, E.J. (1979). Molecular Mechanisms of Phototherapy. *British J. Radiol.*; 52(619): 602

Bernard, C., Valet, J.P., Beland, R. & Lambert, R.D. (1983). Prediction of Bovine Ovulation by Rapid Radioimmunoassay for Plasma LH. *Reprod.*; 68(2): 425-430.

Bezerra, F.T.G., Dau, A.M.P., Van den Hurk, R. & Silva, J.R.V. (2021). Molecular Characteristics of Oocyte and Somatic Cells of Follicles at Different Sizes that Influence *In Vitro* Oocyte Maturation and Embryo Production. *Dom. Anim. Endocrin.*; 74: 106485.

Bhawan K. Minimum Standards for Production of Bovine Frozen Semen. New Delhi: Department of Animal Husbandry & Dairying; 2022: 1–56.

Blerkom, J.V. (2011). Mitochondrial Function in the Human Oocyte and Embryo and Their Role in Developmental Competence. *Mitochon.*; 11: 797-813.

Blondin, P., Coenen, K., Guilbault, L.A. & Sirard, M.A. (1997). In Vitro Production of Bovine Embryos: Developmental Competence is Acquired Before Maturation. *Theriogenology*; 47(5): 061-1075.

Brambillasaca, F., Guglielmo, M.C., Coticchio, G., Renzini, M.M., Dal Canto, M. & Fadini, R. (2013). The Current Challenges of Efficient Immature Oocyte Cryopreservation. *J Assit. Reprod. Genet.*; 30(12): 1531-1539.

Braslavsky, S.E. (2003). Chapter 19 – Phytochrome & Photochromism (Revised Edition); 738-755.

Brar. G.A. & Amon, A. (2009). Emerging Roles for Centromeres in Meiosis I Chromosome Segregation. *Nat. Rev. Genet.*; 9(12): 899-910.

Breehl L, Caban O. Physiology, Puberty. StatPearls, Treasure Island (FL): StatPearls Publishing; 2023.

Born M, Wolf E. Principles of Optics. Cambridge, United Kingdom: Cambridge University Press; 1982: 1–30.

Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F. & Dressel, M.A. (1982). Normal Development Following In Vitro Fertilization in the Cow. *Biol. Reprod.*; 27: 147-158.

Brar, G.A., Hochwagen, A. & Amon, A. (2009). The Multiple Roles of Cohesion in Meiotic Chromosome Morphogenesis and Pairing. *Mol. Biol. Of the Cell*; 20(3): 1030-1047.

Bremer, J. (1983). Carnitine – Metabolism and Functions. *Physiol. Reviews*; 63: 1420–1480.

Buckman, J. F., Hernandez, H., Kress, G.J., Votyakova, T.V., Pal, S. & Reynolds, I.J. (2001). MitoTracker Labeling in Primary Neuronal and Astrocytic Cultures: Influence of Mitochondrial Membrane Potential and Oxidants. *Neuorsci Methods*; 104(2): 165-176.

Buravlev, E.A., Zhidkova, T.V., Osipov, A.N., Vladimirov, Y.A. (2015). Are the Mitochondrial Respiratory Complexes Blocked by NO the Targets for the Laser and LED Therapy? *Lasers in Med. Sci.*; 30:173-180.

Cagin, U. & Enriquez, J.A. (2015). The Complex Crosstalk Between Mitochondria and The Nucleus: What Goes Between? *Int. J. Biochem. & Cell Biol.*; 63: 10-15.

Callesen, H., Greve, T. & Christensen, F. (1987). Ultrasonically Guided Aspiration of Bovine Follicular Oocytes. *Theriogen.*; 27(1): 217.

Cao, L., Shitara, H., Horii, T., Nagao, Y., Imai, H., Abe, K., Hara, T., Haysahi, J.I., Yonekawa, H. (2007). The Mitochondrial Bottleneck Occurs Without Reduction of mtDNA Content in Female Mouse Germ. *Nat. Genet.*; 39(3): 386-390.

Carbone, M.C., Tatone, C., Delle Monache, S., Marci, R., Caserta, D., Colonna, R. & Amicarelli, F. (2003). Antioxidant Enzymatic Defences in Human Follicular Fluid: Characterization and Age-Dependent Changes. *Mol. Hum. Reprod.*; 9(11): 639-643.

Cetica, P., Pintos, L., Dalvit, G. & Beconi, M. (2002). Activity of Key Enzymes Involved in Glucose and Triglyceride Catabolism During Bovine Oocyte Maturation In Vitro. *Reprod.*; 124(5): 675-681.

Checura, C.M. & Siedel, G.E. (2007). Effect of Macromolecules in Solutions for Vitrification of Mature Bovine Oocytes. *Theriogen.*; 67(5): 919-930.

Chen, G., Chen, X., Niu, X., Sun, F., Shang, C. & Yu, B. (2023). Cell Cryopreservation Solution Used for Applying in Cryopreservation of Mammalian Cells, Comprises Cryopreservation Solution A Containing Disodium Hydrogen Phosphate, Potassium Chloride, Protective Agent and Dimethyl Sulfoxide, and Cryopreservation Solution B Containing Apoptosis Inhibitors. *Excel Bitech.*; 295380.

Chodos, E. (2003). This Month in Physics History. APS News; 12: 11.

Chow, R.T., Johnson, M.I., Lopes-Martins, R.A.B. & Bjordal, J.M. (2009). Efficacy of Low-Level Laser Therapy in the Management of Neck Pain: A Systematic Review and Meta-Analysis of Radomized Placebo or Active-Treatment Controlled Trials. *Lancet.*; 374(9705): 1897-1908.

Cohen, P. (2018). Meiosis Overview. *Encyclopedia of Reproduction*; 3, 5–12.

Conti, M., Andersen, C.B., Richard, F.J., Mehats, C., Chun, S.Y., Horner, L., Jin, C. & Tsafriri, A. (2002). Role of Cyclic Nucleotide Signaling in Oocyte Maturation. *Mol. & Cell. Endocrin*.; 187: 153-159.

Conti, M., Andersen, C.B., Richard, F.J., Shitsukawa, K. & Tsafriri, A. (1998). Role of Cyclic Nucleotide Phosphodiesterase in Resumption of Meiosis. *Mol. & Cell. Endocrin*,; 145: 9-14.

Coticchio, G., Dal Canto, M., Fadini, R., Renzini, M., Guglielmo, M.C., Miglietta, S., Palmerini, M.G., Macchiarelli, G. & Nottola, S.A. (2015). Ultrastructure of Human Oocytes After *In Vitro* Maturation. *Mol. Hum. Reprod.*; 22(2): 110-118.

Cox E., & Takov V. (2020). Embryology, Ovarian Follicle Development. *StatPearls*.

Cree, L.M., Samuels, D.C., Chuva de Sousa Lopes, S., Rajasimha, H.K., Wonnapinij, P., Mann, J.R., Dahl, H-H.M. & Chinnery, P.F. (2008). A Reduction of Mitochondrial DNA Molecules During Embryogenesis Explains the Rapid Segregation of Genotypes. *Nature Genet*.; 40: 249–254.

Crosier, A.E., Farin, P.W., Dykstra, M.J., Alexander, J.E. & Farin, C.E. (2000). Ultrastructural Morphometry of Bovine Compart Morulae Produced In Vivo or In Vitro. *Biol. Reprod.*; 62(5): 1459-1465.

Cummins, J.M. (2004). The Role of Mitochondria in the Establishment of Oocyte Functional Competence. *European J Obstert. & Gyneco. & Reprod. Biol.*; 115(1): S23-S29.

Currin, L., Baldassarre, H. & Bordignon, V. (2021). In Vitro Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential. *Animals*; 11(8): 2275.

Dalton, C.M. & Carroll, J. (2013). Biased Inheritance of Mitochondria During Asymmetric Cell Division in the Mouse Oocyte. *Cell Sci.*; 126(13): 2955-2964.

Dalton, C.M., Szabadkai, G. & Carroll, J. (2014). Measurement of ATP in Single Oocytes: Impact of Maturation and Cumulus Cells on Levels and Consumption. *Cell. Physiol.;* 229: 353-361.

Deluao, J.C., Winstanley, Y., Robker, R.L., Pacella-Ince, L., Gonzalez, M.B. & McPherson, N.O. (2022). Oxidative Stress and Reproduction: Reactive Oxygen Species in the Mammalian Pre-Implantation Embryo. *Reprod.*; 164: F95-F108.

Demetrio, D., Demetrio, C., Oliveira, M., Rais, R. & Santos, R.. (2022). From Oocyte to Calf: Practical Aspects of In Vitro Embryo Production. *Clin. Theriogen.*; 14: 193-201.

Dielman, S.J., Hendriksen, P.J.M., Viuff, D., Thomsen, P.D., Hyttel, P., Knijn, H.M., Wrenzycki, C., Kruit, T.A.M., Neimann, H., Gadella, B.M., Bevers, M.M. & Vos, P.L.A.M. (2002). Effects of In Vivo Prematuration and In Vivo Final Maturation on Developmental Capacity and Quality of Pre-Implantation Embryos. *Theriogen.*; 57: 5-20.

Dompe, C., Moncrieff, L., Matys, J., Grzech-Lesniak, K., Kocherova, I., Bryja, A., Bruska, M., Dominiak, M., Mozdziak, P., Skiba, T.H.I., Shibli, J.A., Volponi, A.A., Kempisty, B., & Dyskiewixz-Konwinska, M. (2020). Photobiomodulation – Underlying Mechanisms and Clinical Applications. *J. Clin. Med.*; 9(6): 1724.

Donahue, R.P. & Stern, S. (1968). Follicular Cell Support of Oocyte Maturation – Production of Pyruvate *In Vitro*. *Reprod. Fert.*; 17(2): 395-398.

Dumollard, R., Duchen, M. & Carroll, J. (2007). The Role of Mitochondrial Function in Oocyte and Embryo. *Curr. Top. in Dev. Biol.*; 77: 29-41.

Dunning, K.R., Cashman, K. Russell, D.L., Thompson, J.G., Norman, R.J. & Robker, R.L. (2010). Beta-Oxidation Is Essential for Mouse Oocyte Developmental Competence and Early Embryo Development. *Biol. Reprod.*; 83: 909-918.

Dunning, K.R., Russell, D.L. & Robker, R..L. (2014). Lipids and Oocyte Developmental

Competence: The Role of Fatty Acids and β-Oxidation. *Reprod.*; 148(1): R15-R27.

Enright, B.P., Lonergan, P., Dinnyes, A., Fair, T., Ward, F.A., Yang, X. & Boland, M.P. (2000).

Culture of *In Vitro* Produced Bovine Zygotes *In Vitro* vs *In Vivo*: Implications for Early

Embryo Development and Quality. *Theriogen.*; 54(5): 659-673.

Fair, T., Lonergan, P., Dinnyes, A., Cottell, .D.C., Hyttel, P., Ward, F.A. & Boland, M.P. (2001).

Ultrastructure of Bovine Blastocysts Following Cryopreservation: Effect of Method of

Blastocyst Production. *Mol. Reprod. Dev.*; 58(2): 186-195.

Fan H.Y. & Sun, Q.Y. Oocyte Meiotic Maturation. Elsevier Inc.; 2019: 181–203.

Ferre, L.B., Kjelland, M.E., Strobech, L.B., Hyttel, P., Mermilod, P. & Ross, P.J. (2020). Review:

Recent Advances in Bovine In Vitro Embryo Production: Reproductive Biotechnology

History and Methods. *Animals*; 14(5): 991-1004.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A. & Navarro, P.A.A.S.

(2009). Cytoplasmic Maturation of Bovine Oocytes: Structural and Biochemical

Modifications and Acquisition of Developmental Competence. *Theriogen.*; 71(5): 836-848.

Foley, J., Vasily, D.B., Bradle, J., Rudio, C. & Calderhead, R.G. (2016). 830 nm Light-Emitting

Diode (LED) Phototherapy Significantly Reduced Return-To-Play in Injured University

Atheletes: A Pilot Study. *Laser Ther.*; 25(1): 35–42.

Gad, A., Hoelker, M., Besenfelder, U., Havlicek, V., Cinar, U., Rings, F. Held, E., Dufort, I., Sirard,

M.A., Schellander, K. & Tesfaye, D. (2012). Molecular Mechanisms and Pathways Involved

in Bovine Embryonic Genome Activation and Their Regulation by Alternative In Vivo and In

Vitro Culture Conditions. *Biol. Reprod.*; 87(4): 100, 1-13.

Gaspar, L. (2009). Professor Endre Mester, the Father of Photobiomodulation. *J. Laser Dent*; 17(3): 146-148.

Gershon, E. & Dekel, N. (2020). Newly Identified Regulators of Ovarian Folliculogenesis and Ovulation. *Int. J. Mol. Sci.*; 21(12): 4565.

Ghanem, N., Ha, A.N., Fakurzzaman, Md. Bang, J.I., Lee, S.C. & Kong, I.K. (2014). Differential Expression of Selected Candidate Genes in Bovine Embryos Produced In Vitro and Cultured With Chemicals Modulating Lipid Metabolism. *Theriogen.*; 82: 238-250.

Gilmore, K. & Wilson, M. (1999). The Use of Chloromethyl-X-Rosamine (MitoTracker Red) to Measure Loss of Mitochondrial Membrane Potential in Apoptotic Cells Is Incompatible with Cell Fixation. *Cytomet*; 36: 355-358.

Ginsburg M., Snow M.H., McLaren A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Dev.;* 110:521–528.

Gledhill, J.R., Montgomery, M.G., Leslie, A.G.W. & Walker, J.E. (2007). How the Regulatory Protein IF_1 Inhibits F_1-ATPase From Bovine Mitochondria. *Proc. Natl. Acad. Sci. USA*; 104(40): 15671-15676.

Gonzalez, M.B., Robker, R.L., Rose, R.D. (2022). Obesity and Oocyte Quality: Significant Implications for ART and Emerging Mechanistic Insights. *Biol. Reprod.*; 106(2): 338-350.

Gordon, S.A. & Surrey, K. (1960). Red and Far-Red Action on Oxidative Phosphorylation. *Radiat. Res.*; 12(4): 325–339.

Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y. & Ogawa, K. (1988). Pregnancies After Co-Culture of Cumulus Cells with Bovine Embryos Derived from In-Vitro Fertilization of In-Vitro Matured Follicular Oocytes. *Reprod.*; 83(2): 753-758.

Greve, T., Xu, K.P., Callesen, H. & Hyttel, P. (1987). In Vivo Development of In Vitro Fertilized
Bovine Oocytes Matured In Vivo vs. In Vitro. *J. In Vitro Fert. & Embryo Trans*; 4: 281-285.

Grindler, N.M. & Moley, K.H. (2013). Maternal Obesity, Infertility and Mitochondrial Dysfunction:
Potential Mechanisms Emerging from Mouse Model Systems. *Mol. Hum. Reprod.*; 19(8):
486-494.

Guerin, P., Mouatassium, S.El., Menezo, Y. (2001). Oxidative Stress and Protection Against Reactive
Oxygen Species in the Pre-Implantation Embryo and Its Surroundings. *Hum. Reprod. Update*;
7(2): 175-189.

Hamblin, M.R. (2016). Photobiomodulation or Low-Level Laser Therapy. *J. Biophoton.*; 9(11-12):
1122–1124.

Hamblin, M.R. (2018). Mechanisms and Mitochondrial Redox Signaling in Photobiomodulation.
Photochem. Photobiol.; 94(2): 199–212.

Hamblin, M. & Demidova, T. (2006). Mechanisms of Low-Level Light Therapy. Proceedings of
SPIE BiOS: 614001.

Hardy, M.L.M, Day, M.L. & Morris, M.B. (2021). Redox Regulation and Oxidative Stress in
Mammalian Oocytes and Embryos Developed *In Vivo* and *In Vitro. Int J Environ Res Public
Health*; 18(21): 11374.

Hasler, J.F. (2000). *In-Vitro* Production of Cattle Embryos: Problems with Pregnancies and
Parturition. *Hum. Reprod.*; 15(5): 47-58.

Havlicek, V., Lopataova, M, Cech, S., Dolezel, R., Huber, T., Pavlok, A., Brem, G. & Besenfelder,
U. (2005). In Vivo Culture of Bovine Embryos and Quality Assessment of In Vivo vs. In
Vitro Produced Embryos. *Vet. Med. – Czech*; 50(4): 149-157.

Hawk, H.W. & Wall, R.J. (1994). Improved Yields of Bovine Blastocysts from In Vitro-Produced Oocytes I. Selection of Oocytes and Zygotes. *Theriogen.*; 41: 1571-1583.

He, M., Zhang, T., Yang, Y. & Wang, C. (2021). Mechanisms of Oocyte Maturation and Related Epigenetic Regulation. *Cell Dev. Biol.*; 9: 654028.

Heiskanen, V. & Hamblin, M.R. (2018). Photobiomodulation: Lasers vs. Light Emitting Diodes? *Photochem. Photobiol. Sci.*; 17(8): 1003-1017.

Hochi, S. (2022). Cryodevices Developed for Minimum Volume Cooling Vitrification of Bovine Oocytes. *Ani. Sci.*; 93: e13683.

Holm, P. & Cassen, H. (1998). In Vivo Versus In Vitro Produced Bovine Ova: Similarities and Differences Relevant for Practical Application. *Reprod. Nutr. Dev.*; 38: 579-594.

Holm, P., Shukri, N.N., Vajta, G., Booth, P., Bendixen, C., Callesen, H. (1998). Developmental Kinetics of the First Cell Cycles of Bovine In Vitro Produced Embryos in Relation to Their In Vitro Viability and Sex. *Theriogen.*; 50: 1285-1299.

Houten, S.M. & Wanders, R.J.A. (2010). A General Introduction to the Biochemistry of Mitochondrial Fatty Acid β-Oxidation. *J. Inherit. Metab. Dis.*; 33(5): 469-477.

Houten, S.M., Violante, S., Ventura, F.V. & Wanders, R.J.A. (2016). The Biochemistry and Physiology of Mitochondrial Fatty Acid β-Oxidation and Its Genetic Disorders. *Ann. Review Physiol.*; 78: 23-44.

Huang, Y.Y., Chen, A. C.H., Carroll, J.D. & Hamblin, M.R. (2009). Biphasic Dose Response in Low-Level Light Therapy. *Dose Respons.*; 7(4): 358–383.

Huang, Y.Y., Nagata, K., Tedford, C.E., McCarthy, T. & Hamblin, M.R. (2013). Low-Level Laser Therapy (LLLT) Reduces Oxidative Stress in Primary Cortical Neurons In Vitro. *J Biophoton.*; 6(10): 829–838.

Hwang, I-S. & Hochi, S. (2014). Recent Progress in Cryopreservation of Bovine Oocytes. *Biomed. Res. Int.*; 570647

Hyttel, P. Xu, K.P., Smith, S. & Greve, T. (1986). Ultrastructure of In-Vitro Oocyte Maturation in Cattle. *J. Reprod. Fert.*; 78: 615-625.

Hyttel, P., Fair, T. Callesen, H & Greve, T. (1997). Oocyte Growth, Capacitation and Final Maturation in Cattle. *Theriogen.*; 47(1): 23-32.

Itami, N., Kawahara-Miki, R., Kawana, H., Endo, M., Kuwayama, T. & Iwata, H. (2014). Age-Associated Changes in Bovine Oocytes and Granulosa Cell Complexes Collected from Early Antral Follicles. *Assit. Reprod. Genet.*; 31(1): 1079-1088.

Iwata, H., Goto, H., Tanaka, H., Sakaguchi, Y., Kimura, K., Kuwayama, T. & Monji, Y. (2011). Effect of Maternal Age on Mitochondrial DNA Copy Number, ATP Content and IVF Outcome of Bovine Oocytes. *Reprod, Fert. & Dev.*; 23(3): 424-432.

Iyer, E.S.S. (2020). The Role of Excited States in Deciphering Molecules and Materials: Time-Resolved Electronic Spectroscopic Studies. *Mol. and Laser Spectro. Advan. Applic.*; 2: 521–562.

Jang, T.H., Park, S.C., Yang, J.H., Kim, J.Y., Seok, J.H., Park, U.S., Choi, C.W., Lee, S.R. & Han, J. (2017). Cryopreservation and Its Clinical Applications. *Integ. Med. Res.*; 6(1): 12-18.

Jeseta, M., Knitlova, D.C., Hanzalova, K., Hulinska, P., Hanulakova, S., Nemcova, L., Kanka, J. & Machatkova, M. (2014). Mitochondrial Patterns in Bovine Oocytes with Different Meiotic Competence Related to Their In Vitro Maturation. *Reprod. In Dom. Ani.*; 49(3): 469-475.

Johnson, M.T., Freeman, E.A., Gardner, D.K. & Hunt, P.A. (2007). Oxidative Metabolism of Pyruvate is Required for Meiotic Maturation of Murine Oocytes *In Vivo*. *Biol. Reprod.*; 77(1): 2–8.

Jungheim, E.S., Travieso, J.L., Carson, K.R. & Moley, K.H. (2012). Obesity and Reproductive Function. *Obstet. Gynecol. Clin. North Am.*; 39(4): 479-493.

Karu, T. I. (1988) Molecular mechanism of the therapeutic effect of low-intensity laser radiation. *Lasers Life Sci.;* 2: 53–74.

Karu, T.I. (2008). Mitochondrial Signaling in Mammalian Cells Activated by Red and Near-IR Radiation. *Photochem. Photobiol.*; 84(5): 1091–1099.

Karu, T.I. & Kolyakov, S.F. (2005). Exact Action Spectra for Cellular Responses Relevant to Phototherapy. *Photomed. Laser Surg.*; 23: 355-361.

Karu, T.I., Pyatibrat, L. & Kalendo, G. (1995). Irradiation with He-Ne Laser Increases ATP Level in Cells Cultivated In Vitro. *J Photochem. & Photobiol.*; 27: 219-223.

Karu, T.I., Pyatibrat, L.V., Kolyakov, S.F. & Afanasyeva, N.I. (2005). Absorption Measurements of a Cell Monolayer Relevant to Phototherapy: Reduction of Cytochrome C. Oxidase Under Near IR Radiation. *J Photochem. & Photobiol.*; 81(2): 98-106.

Kim, E., Ra, K., Lee, M.S. & Kim, G.A. (2023). Porcine Follicular Fluid-Derived Exosome: The Pivotal Material for Porcine Oocyte Maturation in Lipid Antioxidant Activity. *Int. J. Mol. Sci.*; 24(12): 9807.

King, W.A., Bousquet, D., Greve, T. & Goff, A.K. (1986). Meiosis in Bovine Oocytes Matured In Vitro and In Vivo. *Acta Vet. Scand.*; 27: 267-279.

Kirillova, A., Smitz, J.E.J., Sukhikh, G.T. & Mazunin, I. (2021). The Role of Mitochondria in Oocyte Maturation. *Cells*; 10(9): 2484.

Knitlova, D., Hulimska, P., Jeseta, M. Hanzalova, K., Kempisty, B. & Machatkova, M. (2017). Supplementation of L-Carnitine During In Vitro Maturation Improves Embryo Development From Less Competent Bovine Oocytes. *Theriogen.*; 102: 16-22.

Koyama, K. Kang, S-S., Huang, W., Yanagawa, Y., Takahashi, Y & Nagano, M. (2014). Estimation
of the Optimal Timing of Fertilization for Embryo Development of *In Vitro*-Matured Bovine
Oocytes Based on the Times of Nuclear Maturation and Sperm Penetration. *J Vet. Med. Sci*.;
76(5): 653-659.

Krishner, R.L. & Bavister, B.D. (1998). Responses of Oocytes and Embryos to the Culture
Environment. *Theriogen.*; 49(1): 103-114.

Kruip, T., Cran, D.G., Van Beneden, T.H. & Dieleman, S.J. (1983). Structural-Changes in Bovine
Oocytes During Final Maturation *In Vivo*. *Gamete Res.*; 8(1): 29-47.

Labno, C. (2020). Two Ways to Count Cells with Imagej. Integrated Light Microscopy Core.
University of Chicago.

Lamkin-Kennard, K.A. & Popovic, M.B. (2019). Sensors: Natural and Synthetic Sensors. *Biomechat*;
81-107.

Leese, H.J., Sturmey, R.G., Baumann, C.G. & McEvoy, E.G.. (2007). Embryo Viability and
Metabolism: Obeying the Quiet Rules. *Hum. Reprod.*; 22(12): 3047-3050.

Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., Northey, D.L & First, N.L. (1987).
Development Potential of Bovine Oocytes Matured In Vitro or In Vivo. *Biol Reprod.*; (36):
376-383.

Leibfried-Rutledge, M.L., Critser, E.S., Parrish, J.J. & First, N.L. (1989). In Vitro Maturation and
Fertilization of Bovine Oocytes. *Theriogen.*; 31(1): 61-74.

Leyane, T.S., Jere, S.W. & Houreld, N.N. (2021). Cellular Signaling and Photobiomodulation in
Chronic Woung Repair. *In. J Mol. Sci.*; 22: 11223.

Li, R., Redersen, K.S., Liu, Y., Pedersen, H.S., Laegdsmand, M., Rickelt, L.R., Kulh, M. &Callensen, H. (2014). Effect of Red Light on the Development and Quality of Mammalian Embryos. *J Asst. Reprod. Genet*.; 31: 795-801.

Lin, F.H., Zhang, W.L., Li, H., Tian, X.D., Zhang, J., Li, X., Li, C.Y. & Tan, J.H. (2018). Role of Autophagy in Modulating Post-Maturation Aging of Mouse Oocytes. *Cell Death and Dise*.; 9: 308.

Liu C., Peng J., Matzuk M.M. & Yao H.H. (2015). Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat. Commun.* 2015; 6: 6934.

Liu, Y.J., Mclntyre, R.L., Janssens, G.E., Houtkooper, R.H. (2020). Mitochondrial Fission and Fusion: A Dynamic Role in Aging and Potential Target for Age-Related Disease. *Mech. Aging & Dev.*; 186: 111212

Livadas, S. & Chrousos, G.P. (2019). Molecular and Environmental Mechanisms Regulating Puberty Initiation: An Integrated Approach. *Fron. Endocrin.*; 10: 828.

Lopes, A.S., Lane, M. & Thompson, J.G. (2010). Oxygen Consumption and ROS Production are Increased at the Time of Fertilization and Cell Cleavage in Bovine Zygotes. *Hum. Reprod*.; 25(11): 2762-2773.

Luciano, A.M. & Sirard, M-A. (2018). Successful In Vitro Maturation of Oocytes: A Matter of Follicular Differentiation. *Biol. Reprod*.; 98(2): 162-169.

Machatkova, M., Jeseta, M., Hulinska, P., Knitlova, D., Nemcova, L., & Kanka, J. (2012). Characteristics of Bovine Oocytes with Different Meiotic Competence in Terms of Their Mitochondrial Status and Expression of Nuclear-Encoded Factors. *Reprod. Dom. Ani.*; 47: 806-814.

Majidi, M., Salehi, M., Salimi, M., Paktinat, S., Sefati, N., Montazeri, S., Jalili, A. & Norouzian, M. (2021). *Brazilian J Pharm. Sci*.; 57: e18882.

Malhi, P.S., Adams, G.P., Mapletoft, R.J. & Singh, J. (2007). Oocyte Developmental Competence in A Bovine Model of Reproductive Aging. *Reprod.*; 134(2): 233-239.

Marcovina, S.M., Sirtori, C., Peracino, A., Gheorghiade, Borum, P., Remuzzi, G. & Adehali, H. (2013). Translating the Basic Knowledge of Mitochondrial Functions to Metabolic Therapy: Role of L-Carnitine. *Transl. Research*; 161(2): 73-84.

Marei, W.F.A., Smits, A., Mohey-Elsaeed, O., Pintelon, I., Gineberge, D., Bols, P.E.J., Moerloose, K. & Leroy, J.L.M.R. (2020). Differential Effects of High Fat Diet-Induced Obesity on Oocyte Mitochondrial Functions in Inbred and Outbred Mice. *Sci. Rep.*; 10: 9806.

Marengo-Rowe, A.J. (2006). Structure-Function Relations of Human Hemoglobins. Proc. (Bayl Univ Med Cent).; 19(3): 239-245.

Martinez-Diez, M., Santamaria, G., Ortega, A.D. & Cuezva, J.M.. (2006). Biogenesis and Dynamics of Mitochondria During the Cell Cycle: Significance of 2'UTRs. *PLoS One*; 1(1): e107.

Mateusen, B., Van Soom, A., Maes, D.G.D., Donnay, I., Duchateau, L. & Lequarre, A-S. (2005). Porcine Embryo Development and Fragmentation and Their Relation to Apoptotic Markers: A Cinematographic and Confocal Laser Scanning Microscopic Study. *Reprod.*; 129(4): 443-452.

May-Panloup, P., Boucret, L., Chao de la Barca, J-M., Desquiret-Dumas, V., Ferre-L'Hotellier, V., Moriniere, C., Descamps, P., Procaccio, V. & Reynier, P.. (2016). Ovarian Aging: The Role of Mitochondria in Oocytes and Follicles. *Hum. Reprod. Update*; 22(5): 725-743.

Mel-Sterza, F.A. & Poehland, R. (2021). Lipid Metabolism in Bovine Oocytes and Early Embryos Under In Vivo, In Vitro and Stress Conditions. *Int. J Mol. Sci.*; 22(7): 3421.

Mermillod, P. (1999). Aspects of Follicular and Oocyte Maturation that Affect the Developmental
    Potential of Embryos. *Reprod. & Fert.*; 54: 449-460.

Mermillod, P., Dalibies-Tran, R., Uzbekova, S., Thelie, A., Traverso, J.M., Perreau, C., Papillier, P.
    & Monget, P. (2008). Factors Affecting Oocyte Quality: Who is Driving the Follicle?.
    Reprod. Dom. Ani.; 43(2): 393-400.

Merton, J.S., de Roos, A.P.W., Koenen, E.P.C., Roelen, B.A.J., Vos, P., Mullaart, E. & Knijn, H.M.
    (2012). Bovine Opu-derived oocytes Can be Matured *In Vitro* for 16-28h with Similar
    Developmental Capacity. *Reprod. Dom. Ani.*; 47(6): 1037-1042.

Merton, J.S., de Roos, A.P.W., Mullaart, E., de Ruigh, L., Kaal, L., Vos, P.L.A.M. & Dieleman, S.J.
    (2003). Factors Affecting Oocyte Quality and Quantity in Commercial Application of Embryo
    Technologies in the Cattle Breeding Industry. *Theriogen.*; 59(2): 651-674.

Mester, E., Lundany, G., Selyei, M., Szende, B., Total, G.J.. (1968). The Stimulating Effect of Low
    Power Laser Rays on Biological Systems. *Laser Rev.; 1(3)*.

Mester, E., Szende, B. & Gartner, P. (1968). The Effect of Laser Beams on the Growth of Hair in
    Mice. *Radiobio. Rediother.*; 9:

Michaels, G.S., Hauswirth, W.W. & Laipis, P.J.. (1982). DNA Copy Number in Bovine Oocytes and
    Somatic Cells. *Dev. Biol*, 94, 246-251.

Mietkiewska, K., Kordoqitzki, P. & Pareek, C.S. (2022). Effects of Heat Stress on Bovine Oocytes
    and Early Embryonic Development – An Update. *Cells*; 11(24)

Mikhaylova, A.G., Mikhailova, A.A., Ushakova, K., Tretiakov, E.O., Shamansky, V., Yurchenko, A.,
    Zazhytska, M., Zdobnov, E., Makeev, V., Yurov, V., Tanaka,M., Gostimskaya, I., Fleischmann,
    Z., Annis, S., Franco, M., Wasko, K., Kunz, W.S., Knorre, D.A., Mazunin, I., Nikolaev, S.,
    Fellay, J., Reymonds, A., Khrapko, K., Gunbin, K., & Popadin K. (2019). Mammalian

Mitochondrial Mutational Spectrum as a Hallmark of Cellular and Organismal Aging. *bioRxiv*; doi:10.1101/589168

Minke, B. (2010). The History of the *Drosophila* TRP Channel: The Birth of a New Channel Superfamily. *J Neurogenet*.; 24(4): 216-233.

Mitchell, P. (2011). Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation. *Biol. Reviews*; 41: 445-501.

Reid, R.A., Moyle, J., Mithcell, P. (1966). Synthesis of Adenosine Triphosphate by A Protonmotive Force in Rat Liver Mitochondria. *Nature*; 212(5059): 257.

Mitra, J. & Schultz, R.M. (1996). Regulation of the Acquisition of Meiotic Competence in the Mouse: Changes in the Subcellular Localization of CDC2, Cyclin B1, CDC25C, WEE1, and in the Concentration of These Proteins and Their Transcripts. *Cell Sci*.; 109: 2407-2415.

Montell, C. (2011). The History of TRP Channels, A Commentary and Reflection. *European J Physiol*.; 461: 499–506.

Moreira, F., Paula-Lopes, F.F., Hernandez-Ceron, J., Moore, K. & Hansen, J.P. (2001). Protocol to Count Cell Number of Preimplantation Embryos Using Nuclear Staining with Hoechst 33342 or DAPI. PJ Hansen Laboratory. University of Florida.

Motta, P.M. Familiari, G., Nottola, S.A. & Micara, G. (1988). Ultrastructure of the Zona Pellucida and of the Cumulus Cells Surrounding Human Unfertilized Oocytes and *In Vitro*-Fertilized Polypronuclear Ova. *Anatom. Record*.; 220(4); A65.

Motta, P.M., Nottola, S.A., Makabe, S. & Heyn, R. (2000). Mitochondrial Morphology in Human Fetal and Adult Female Germ Cells. *Hum. Reprod.*; 15(2): 129-147.

Mroginski M, Adam S, Amoyal GS, Barnoy A, Bondar A, Borin VA, Church JR, D.omratcheva T, Ensing B, Fanelli F, Ferré N, Filiba O, Pedraza-González L, González R, González-Espinoza

CE, Kar RK, Kemmler L, Kim SS, Kongsted J, Krylov AI, Lahav Y, Lazaratos M, NasserEddin Q, Navizet I, Nemukhin A, Olivucci M, Haugaard Olsen JM, de Alba Ortíz AP, Pieri E, Rao AG, Rhee YM, Ricardi N, Sen S, Solov'yov IA, De Vico L, Wesolowski TA, Wiebeler C, Yang X, Schapiro I. (2021). Frontiers in Multiscale Modeling of Photoreceptor Proteins. *Photochem. Photobiol*; 97:243-269.

Oerly, A., Johnson, M. & Soule, J. (2022). Economic, Social, and Environmental Impacts of Cattle on Grazing Land Ecosystems. *Rangelands*; 44(2): 148-156.

Oh, S.J., Gong S.P., Lee S.T., Lee E.J. & Lim, J.M. (2007). Light Intensity and Wavelength During Embryo Manipulation are Important Factors for Maintaining Viability of Preimplantation Embryos In Vitro. *Fert. & Steril.*; 88:1150-1157.

Oliveira, C.S., de Barros, B.A.F., Monteiro, C.A.S., Rosa, P.M.S., Leal, G.R., Serapiao, R.V. & Camargo, L.S.A. (2019). Individual Assessment of Bovine Embryo Development Using a Homemade Chamber Reveals Kinetic Patterns of Success and Failure to Reach Blastocyst Stage. *Syst Biol. Reprod. Med.*; 65(4) 301-311.

Olzmann, J.A. & Carvalho, P. (2019). Dynamics and Functions of Lipid Droplets. *Nat. Rev. Mol. Cell Biol.*; 20(3): 137-155.

Pallotti, F., Bergamini, C., Lamperti, C. & Fato, R. (2022). The Roles of Coenzyme Q in Diseases: Direct and Indirect Involvement in Cellular Functions. *Int. J Mol. Sci.*; 23(3): 128.

Palma, G.A., Tortonese, D.J. & Sinowatz, F. (2008). Developmental Capacity *In Vitro* of Prepubertal Oocytes. *J Physiol*; 30(5): 295-300.

Parrish, J.J. (2015). Bovine In Vitro Fertilization: In Vitro Oocyte Maturation and Sperm Capacitation with Heparin. *Theriogen.*; 81(1): 67-73.

Park, Y-S., Kim, S-S., Kim, J-M., Park, H-D. & Byun, M-D. (2005). The Effects of Duration of In Vitro Maturation of Bovine Oocytes on Subsequent Development, Quality and Transfer of Embryos. *Theriogeno.*; 64(1): 123-134.

Passarella, S., Casamassima, E., Molinari, S., Pastore, D., Quagliariello, E. & Catalano, I.M. & Cingolani, A. (1984). Increase of Proton Electro-Chemical Potential and ATP Synthesis in Rat Liver Mitochondria Irradiated In Vitro by He-Ne Laser. *FEBS Letters*; 175(1): 95–99.

Passarella, S., Karu, T. (2014). Absorption of Monochromatic and Narrow Band Radiation in the Visible and Near IR by Both Mitochondrial and Non-Mitochondrial Photoacceptors Results in Photobiomodulation. *J. Photochem. & Photobiol. B-Biol.*; 140: 344-358.

Pavlok, A., Lucashahn, A. & Niemann, H. (1992). Fertilization and Developmental Competence of Bovine Oocytes Derived from Different Categories of Antral Follicles. *Mol. Reprod. Dev.*; 31(1): 63-67.

Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B. & Gelbard, H.A. (2011). Mitochondrial Membrane Potential Probes and the Proton Gradient: A Practical Usage Guide. *Biotechni*; 50(2): 98-115.

Pfeiffer, K. Gohil, V., Stuart, R.A., Hunte, C., Brandt, U., Greenburg, M.L. & Schagger, H. (2003). Cardiolipin Stabilizes Respiratory Chain Supercomplexes. *J Biol. Chem.*; 278(52): 52873-52880.

Phillips, P.H. & Lardy, H.A. (1940). A Yolk-Buffer Pabulum for the Preservation of Bull Semen. *J Dairy Sci.*; 23(5): 399–404.

Phongnimitr, T. Liang, Y.Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetampinich, C. & Parnpai, R. (2013). Effect of L-Carnitine on Maturation, Cryotolerance and Embryo Developmental Competence of Bovine Oocytes. *Anim. Sci.*; 84(11): 719-725.

Plante, L. & King, W.A. (1994). Light and Electron Microscopic Analysis of Bovine Embryos

    Derived By In Vitro and In Vivo Fertilization. *Assit. Reprod. & Genet.*; 11: 515-529.

Polge, C., Smith, A.U. & Parkes, A.S. (1949). Revival of Spermatozoa After Vitrification and

    Dehydration at Low Temperatures. *Nature*; 164(4172): 666.

Purcell, S.H. & Moley, K.H. (2011). The Impact of Obesity on Egg Quality. *Assit. Reprod. Genet.*;

    28(6): 517-524.

Pursel, V.G., Wall, R.J., Rexroad, C.E., Hammer, R.E. & Brinster, R.L. (1985). Rapid Whole-Mount

    Staining Procedure for Nuclei of Mammalian Embryos. *Theriogen.*; 24(6): 687-691.

Quinn, P.J. (1985). A Lipid-Phase Separation Model of Low-Temperature Damage to Biological

    Membranes. *Cryobiol.*; 22: 128–146.

Read, C.C., Bhandari, S., Moorey, S.E. (2021). Concurrent Measurement of Mitochondrial DNA

    Copy Number and ATP Concentration in Bovine Oocytes. *Methods Protoc.*; 4(4): 88.

Reaume, A.G., De Sousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C. Juneja, S.C.,

    Kidder, G.M. & Rossant, J. (1995). Cardiac Malformation in Neonatal Mice Lacking

    Connexin43. *Sci.*; 267: 1831-1834.

Remington, L.A. (2012). Chapter 4 – Retina. *Clin. Anant. and Physiol. of the Visual System*; 3: 61–

    92.

Reusch, T.B.H. & Boyd, P.W. (2013). Experimental Evolution Meets Marine Phytoplankton. *Evol.*;

    67(7): 1849-1859.

Richani, D., Dunning, K.R., Thompson, J.G. & Gilchrist, R.B. (2021). Metabolic Co-Dependence of

    the Oocyte and Cumulus Cells: Essential Role in Determining Ooycte Developmental

    Competence. *Hum. Reprod, Update*; 27(1): 27–47.

Richani, D. & Gilchrist, R.B. (2022). Approaches to Oocyte Meiotic Arrest *In Vitro* and Impact on Oocyte Developmental Competence. *Biol. Reprod.*; 106(2): 243-252.

Richard, F.J., Fortier, M.A. & Sirard, M.A. (1997). Role of the Cyclic Adenosine Monophosphate-Dependent Protein Kinase in the Control of Meiotic Resumption in Bovine Oocytes Cultured with Thecal Cell Monolayers. *Biol. Reprod.*; 56(6): 1363-1369.

Rieger, D., Loskutof, N.M., Betteridge, K.J. (1992). Developmentally Related Changes in the Uptake and Metabolism of Glucose, Glutamine and Pyruvate by Cattle Embryos Produced In Vitro. *Reprod, Fert & Dev.*; 4(5): 547-557.

Rimon-Dahari N., Yerushalmi-Heinemann L., Alyagor L. & Dekel, N. (2016). Ovarian Folliculogenesis. *Results Prob. Cell. Differ.*; 58:167–190.

Rivera, R.M., Goldkamp, A.K., Patel, B.N., Hagen, D.E., Soto-Moreno, E.J., Li, Y., Kin, C.N., Miller, C., Williams III, F., Jannaman, E., Xiao, Y., Tribulo, P., Estrada-Cortes, E., Brau-Rdriguez, A.R., Hansen, P.J., Wu, Z., Spinka, C.M., Martin, N. & Elsik, C.G. (2022). Identification of Large Offspring Syndrome During Pregnancy Through Ultrasonography and Maternal Blood Transcriptome Analyses. *Sci Rep.*; 12: 10540.

Rizos, D., Ward, F., Duffy, P., Boland, M.P. & Lonergan, P. (2002). Consequences of Bovine Oocyte Maturation, Fertilization or Early Embryo Development in Vitro Versus In Vivo: Implications for Blastocyst Yield and Blastocyst Quality. *Mol. Reprod. & Dev.*; 61(2): 234-248.

Rizos, D., Clemente, M, Bermejo-Alvarez, P., de La Fuente, J., Lonergan, P. & Gutierrez-Adan, A. (2008). Consequences of *In Vivo* Culture Conditions on Embryo Development and Quality. *Reprod. Dom. Anil.*; 43(4): 44-50.

Rossi, A., Pizzo, P. & Filadi, R. (2019). Calcium, Mitochondria and Cell Metabolism: A Functional Triangle in Bioenergetics. *Biochem Biophysica (BBA) – Mol. Cell Research.*; 1866(7): 1068-1075.

Sanchez, F., Romero, S., De Vos, M., Verheyen, G. & Smitz, J. (2015). Human Cumulus-Enclosed Germinal Vesicle Oocytes From Early Antral Follicles Reveal Heterogeneous Cellular and Molecular Features Associated with *In Vitro* Maturation Capacity. *Hum. Reprod.*; 30(6): 1396-1409.

Sanz, A. & Stefanatos, R.K.A. (2008). The Mitochondrial Free Radical Theory of Aging: A Critical View. *Curr. Aging Sci.*; 1(1): 10-21.

Schenkel, L.C. & Bakovic, M. (2014). Formation and Regulation of Mitochondrial Membranes. *Int. J Cell. Biol.*; 709828

Schultz, R.M. (2007). Of Light and Mouse Embryos: Less is More. Proceedings of the National Academy of Science, USA; 104(37): 14547-14548.

Seidel, G.E. (2022). Translating Basic Research to Animal Agriculture. *Ann. Rev. of Anim. Biosci.*; 10: 1–15.

Senger PL. Pathways to Pregnancy and Parturition. Pullman, WA: Current Conceptions Inc.; 2006

Serrage, H., Heiskanen, V., Palin, W., Cooper, P.R., Milward, M.R., Hadis, M. & Hamblin, M.R. (2019). Under the Spotlight: Mechanisms of Photobiomodulation Concentrating on Blue and Green Light. *Photochem. Photobiol. Sci.*; 18(8): 1877-1909.

Sharma, V., Karlin, K. & Wilkstrom, M. (2013). Computational Study of the Activated O_H State in the Catalytic Mechanism of Cytochrome C. Oxidase. *PNAS*; 110(42): 16844-16849.

Shichida, Y. & Matsuyama, T.. (2009). Evolution of Opsins and Phototransduction. *Philos Trans. R Soc. Long. B. Biol. Sci.*; 364(1531): 2881-2895.

Shields, H.J., Traa, A. & Van Raamsdonk, J.M. (2021). Beneficial and Detrimental Effects of

    Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and

    Experimental Studies. *Front. Cell Dev. Biol.*; 9: 628157.

Shteinfer-Kuzmine, A., Verma, A., Arif, T., Aizenberg, O., Paul, A. & Shoshan-Barmaz, V. (2020).

    Mitochondria and Nucleus Cross-Talk: Signaling in Metabolism, Apoptosis, and

    Differentiation and Function in Cancer. *IUBMB LIFE*; 73(3): 492-510.

Sies, H. (2019). Chapter 13 – Oxidative Stress: Eustress and Distress in Redox Homeostasis. *Physiol.,*

    Biochem. & Pathol.; 3: 153–163.

Sirard, M.A., First, N.L. (1988). In Vitro Inhibition of Oocyte Nuclear Maturation in the Bovine.

    Biol. Reprod.; 39: 229-234.

Sirard, M.A., Florman, H.M., Leibfried-Rutledge, M.L., Barnes, F.L., Sims, M.L. & First, N.L.

    (1989). Timing of Nuclear Progression and Protein-Synthesis Necessary for Meiotic

    Maturation of Bovine Oocytes. *Biol. Reprod.*; 40(6): 1257-1263.

Sirard, M.A., Richard, F., Blondin, P. & Robert, C. (2006). Contribution of the Oocyte to Embryo

    Quality. *Theriogen.*; 65(1): 126-136.

Sirard, M.A. (2018). 40 Years of Bovine IVF in the New Genomic Selection Context. *Reprod.*

    156(1): R1-R7.

Sirard, M.A. (2019). Folliculogenesis and Acquisition of Oocyte Competence in Cows. *Ani. Reprod.*;

    16(3): 449-454.

Smith, K. (2014). What is Photobiology? American Society for Photobiology. Los Catos, CA.

Spinelli, J.B. & Haigis, M.C. (2018). The Multifaceted Contributions of Mitochondria to Cellular

    Metabolism. *Nat. Cell Biol.*; 20(7); 745–754.

Stocklein, K.S., Ortega, M.S., Spate, L.D., Murphy, C.N. & Prather, R.S. (2021). Improved Cryopreservation of In Vitro Produced Bovine Embryos Using FGF2, LIF, and IGF1. *PLoS ONE*; 16(2): e0243727.

Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonclaves, P.B. & Wolf, E. (2001). Mitochondrial Distribution and Adenosine Triphosphate Content of Bovine Oocytes Before and After In Vitro Maturation: Correlation with Morphological Criteria and Developmental Capacity After In Vitro Fertilization and Culture. *Biol. Reprod.*; 64(3): 904-909.

Suigura, K., Pendola, F.L. & Eppig, J.J. (2005). Oocyte Control of Metabolic Cooperativity Between Oocytes and Companion Granulosa Cells: Energy Metabolism. *Dev. Biol.*; 279(1): 20-30.

Sun, W-S., Jang, H., Park, M-R., Oh, K.B., Lee, H., Hwang, S., Xu, L-J., Hwang, I-S. & Lee, J-W. (2021). N-Acetyl-L-Cysteine Improves the Developmental Competence of Bovine Oocytes and Embryos Cultured In Vitro by Attenuating Oxidative Damage and Apoptosis. *Antioxid.*; 10(6): 860.

Sviridova-Chailakhyan, T.A., Fakhranova, L.I., Simnova, N.B., Khramov, R.N., Manokhin, A.A., Paskevich, S.I. & Chailakhyan, L.M. (2008). Photobiomodulation of Early Mouse Embryo Development. Proc. SPIE 6991, Biophotonics: Photonic Solutions for Better Health Care: 69912B.

Takahashi, N., Davy, P.M., Gardner, L.H., Matthews, J., Yamazaki, Y & Allsopp, R.C. (2016). Hypoxia Inducible Factor 1 Alpha Is Expressed in Germ Cells Throughout the Murine Life Cycle. *PLoS ONE*; 11(15): e0154309.

Tanner, J.M. (1985). Early Quantum Physics. Physics for Students of Science and Engineering; 691–716.

Tarazona, A.M., Rodriguez, J.I., Restrepo, L.F. & Olivera-Angel, M. (2006). Mitochondrial Activity, Distribution and Segregation in Bovine Oocytes and In Embryos Produced In Vitro. *Reprod. In Dom. Ani.*; 41(1): 5-11.

Thibault, C. (1977). Are Follicular Maturation and Oocyte Maturation Independent Processes? *J Reprod. Fert.*; 51: 1-15.

Tilly, J.L., Sinclair, D.A. (2013). Germiline Energetics, Aging and Female Infertility. *Cell Metab.*; 17(6): 838–850.

Toogood, H.S. & Scrutton, N.S. (2020). Flavin-Dependent Enzymes: Mechanisms, Structures and Applications. *The Enzymes*; 47: 491–515.

Trebichalska, Z., Kyjovska, D., Kloudova, S., Oteverel, P., Hampl, A. & Holubcova, Z. (2020). Cytoplasmic Maturation in Human Oocytes: An Ultrastructural Study. *Biol. Reprod.*; 104(1): 106-116.

Tsafriri, A., Lindner, H.R. & Lamprec, S.A. (1972). In-Vitro Induction of Meiotic Division in Follicle-Enclosed Rat Oocytes by LH, Cyclic AMP and Prostaglandin E_2. *Reprod.*; 31(1): 39-50.

Tsafriri, A., Cao, X., Ashkenazi, H., Motola, S., Popliker, M. & Pomerantz, S.H. (2005). Resumption of Oocyte Meiosis n Mammals: On Models, Meiosis Activating Sterols, Steroids and EGF-Like Factors. *Mol. & Cell. Endo.*; 234: 37–45.

Tsafriri, A., Chun, S.Y., Zhang, R., Hsueh, A.J.W. & Conti, M. (1996). Oocyte Maturation Involves Compartmentalization and Opposing Changes of cAMP Levels in Follicular Somatic and Germ Cells: Studies Using Selective Phosphodiesterase Inhibitors. *Dev. Biol.*; 178: 393-402.

Turner, J. & Hode, L. (1998). It's All in the Parameters: A Critical of Some Well-Known Negative Studies on Low-Level Laser Therapy. *J Clin. Laser Med. & Surg.*; 16(5):245-248.

Twig, G., Elorza, A., Molina, A.J.A., Mohamed, H., Wikstrom, J.D., Walzer, G., Stiles, L., Haigh, S.E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B.F., Yuan, J., Deeney, J.T., Corkey, B.E., Shirihai, O.S. (2008). Fission and Selective Fusion Govern Mitochondrial Segregation and Elimination by Autophagy. *Embryo J*; 27(2): 433–446.

Unnikrishnan, V., Kastelic, J. & Thundathil, J. (2021). Intracytoplasmic Sperm Injection in Cattle. *Genes (Basel)*; 12(2): 198.

Van Blerkom, J. (2004). Mitochondria in Human Oogenesis and Preimplantation Embryogenesis: Engines of Metabolism, Ionic Regulation and Developmental Competence. *Reprod.*; 128(3): 269-280.

Van Leeuwen, J., Rawson, P., Berg, D.K., Wells, D.N. & Pfeffer, P.L. (2020). On the Enigmatic Disappearance of Rauber's Layer. *Proc. Of the Nat. Acad. Of Sci. of the United States of America*; 117(28): 16409-16417.

VanSoom, A., Ysebaert, M.T. & DeKruif, K. (1997). Relationship Between Timing of Development, Morula Morphology, and Cell Allocation to Inner Cell Mass and Trophectoderm in *In Vitro* Produced Bovine Embryos. *Mol. Reprod. Dev.*; 47(1): 47-56.

Van Wagtendonk-de Leeuw, A.M. (2006). Ovum Pick Up and In Vitro Production in the Bovine After Use in Several Generations. *Theriogen.*; 65: 914-925.

Viana, J.H.M. (2022). 2021 Statistics of Embryo Production and Transfer in Domestic Farm Animals. *Embryo Tech. News.*; 40(4): 22-40.

Wagtendonk-de Leeuw, A.M. (2006). Ovum Pick Up and *In Vitro* Production in the Bovine After Use in Several Generations: A 2005 Status. *Theriogen.*; 65(5): 914-925.

Wai, T. Teoli, D. & Shoubridge, E.A. (2008). The Mitochondrial DNA Genetic Bottleneck Results from Replication of a Subpopulation of Genomes. *Nat. Genet.*; 40: 1484-1488.

Wai, T., Ao, A., Zhang, X., Cyr, D., Dufort, D. & Shoubridge, E.A. (2010). The Role of Mitochondrial DNA Copy Number in Mammalian Fertility. *Biol Reprod*, 83(1), 52-62.

Walker, J.E. (2013). The ATP Synthase: The Understood, The Uncertain and The Unknown. *Biochem. Soc. Trans.*; 41(1): 1–16.

Wang, F., Tian, X., Zhang, L., Gao, C., He, C., Fu, Y., Ji, P. & Liu, G. (2014). Beneficial Effects of Melatonin on In Vitro Bovine Embryonic Development are Mediated by Melatonin Receptor 1. *J Pineal Research*; 56(3): 333–342.

Wang, F. Tian, X., Zhou, Y., Tan, D., Zhu, S., Dai, Y. & Liu, G. (2014). Melatonin Improves the Quality of In Vitro Produced (IVP) Bovine Embryos: Implications for Blastocyst Cryotolerance, and Modifications of Relevant Gene Expression. *PLoS ONE*; 9(4): e93641.

Wang, J. Diao, Z., Fang, J., Zhu, L., Xu, Z., Lin , F., Zhang, N. & Chen, L. (2022). The Influence of Day 3 Embryo Cell Number on the Clinical Pregnancy and Live Birth Rates of Day 5 Single Blastocyst Transfer from Frozen Embryo Transfer Cycles. *BMC Pregn. Childb.*; 22: 980.

Ward, F., Enright, B., Rizos, D., Boland, M. & Lonergan, P. (2002). Optimization of In Vitro Bovine Embryo Production: Effect of Duration of Maturation, Length of Gamete Co-Incubation, Sperm Concentration and Sire. *Theriogen.*; 57(8): 2105-2117.

Warzych, E., Pawlak, P., Pszczola, M., Cieslak, A. & Lechniak, D. (2017). Prepubertal Heifers Versus Cows – The Differences in the Follicular Environment. *Theriogen.*; 1(87): 36-47.

Wiltbank, M.C., Mezera, M.A, Toledo, M.Z., Drum, J.N., Baez, G.M., Garcia-Guerra, A. & Sartori, R. (2018). Physiological Mechanisms Involved in Maintaining the Corpus Luteum During the First Months of Pregnancy. *Ani. Reprod.*; 15(1): 805-821.

Winterhager, E. & Kidder, G.M. (2015). Gap Junction Connexins in Female Reproductive Organs: Implications for Women's Reproductive Health. *Hum. Reprod. Update*; 21(3): 340-352.

Wu, B., Ignotz, G., Currie, B. & Yang, X. (1997).Dynamics of Maturation-Promoting Factor and Its Constituent Proteins During In Vitro Maturation of Bovine Oocytes. *Biol. Reprod.*; 56: 253-259.

Wu, C. & Sirard, M-A. (2020). Parental Effects on Epigenetic Programming in Gametes and Embryos of Dairy Cows. *Front. Genet.*; 11: 1–13.

Xu, K.P. Yadav, B.R., Rorie, R.W., Plante, L., Betteridge, K.J. & Kong, W.A. (1992). Development and Viability of Bovine Embryos Derived From Oocytes Matured and Fertilized In Vitro and Co-Culutred with Bovine Oviductal Epithelial Cells. *Reprod. Fert.*; 94: 33–43.

Yamada, T., Imani, H. & Yamada, M. (2005). Beneficial Effects of Acetyl-L-Carnitine Treatment During IVM on Post-Fertilization Development of Bovine Oocytes In Vitro. *Reprod. Fert. & Dev.*; 18(2): 280-281.

Yanez-Ortiz, I., Catalan, J., Rodiguez-Gil, J.E., Miro, J., & Yeste, M. (2022). Advances in Sperm Cryopreservation in Farm Animals: Cattle, Horse, Pig and Sheep. *Anim. Reprod. Sci.*; 246: 106904.

Yang, M-Y., Chang, C-J. & Chen, L-Y. Blue Light-Induced Reactive Oxygen Species from Flavin Mononucleotide and Flavin Adenine Dinucleotide on Lethality of *HeLa* Cells. *J Photochem. & Photobiol. B: Biol.*; 173: 325-332

Yin, H. & Zhu, M. (2012). Free Radical Oxidation of Cardiolipin: Chemical Mechanisms, Detection, and Implications in Apoptosis, Mitochondrial Dysfunction and Human Disease. *Free Rad. Research*; 46(8): 959–974.

Youle, R.J. & van der Bliek, A.M. (2012). Mitochondrial Fission, Fusion and Stress. *Sci.*; 33 (6098): 1062-1065.

Young, L.E., Sinclair, K.D. & Wilmut, I. (1998). Large Offspring Syndrome in Cattle and Sheep. *Reviews of Reprod.*; 3: 155–163.

Zeng, H.T., Yeung, W.S.B., Cheung, M.P.L., Ho, P.C., Lee, C.K.F, Zhang, G.L., Liang, X.Y., & O, W-S. (2009). In Vitro-Matured Rat Oocytes Have Low Mitochondrial Deoxyribonucleic Acid and Adenosine Triphosphate Contents and Have Abnormal Mitochondrial Redistribution. *Fert. & Steril.*; 91: 900-907.

Zhang. Y., Song, S., Fong, C-C., Tsang, C-H., Yang, Z. & Yang, M. (2003). cDNA Microarray Analysis of Gene Expression Proteins in Human Fibroblast Cells Irradiation with Red Light. *J Invest. Dermatol.*; 120: 849-857.

Zhang, X., Wu, X.Q., Lu, S., Guo, Y.L. & Ma, X.. (2006). Deficit of Mitochondrial-Derived ATP During Oxidative Stress Impairs Mouse MII Oocyte Spindles. *Cell Research,* 16, 841–850.

Zhang, W., Yi, K., Yan, H. & Zhou, X. (2012). Advances on *In Vitro* Production and Cryopreservation of Porcine Embryos. *Ani. Reprod. Sci.*; 132: 115–122.

Zhang, H.X., Esposito, M., Pezet, M.G., Aryaman, J., Wei, W., limm, F., Calabrese, C., Burr, S.P., Macabelli, C.H., Viscomi, C., Saitou, M., Chiaratti, M.R., Stewart, J.B., Jones, N. & Chinnery, P.F. (2021). Mitochondrial DNA Heteroplasmy is Modulated During Oocyte Development Propagating Mutation Transmission. *Sci. Advan.*; 7(50): 5657.

Zhang. CH., Liu, X.Y. & Wang, J. (2023). Essential Role of Granulosa Cell Glucose and Lipid Metabolism on Ooyctes and the Potential Imbalance in Polycystic Ovarian Syndrome. *Int. J Mol. Sci.*; 24(22): 16247.

Zhao, J. & Li, Y. (2012). Adenosine Triphosphate Content in Human Unfertilized Oocytes, Undivided Zygotes and Embryos Unsuitable for Transfer or Cryopreservation. *J. Int. Med. Research*.; 40: 734–739.

Zohuri, B. (2018). First Law of Thermodynamics. *Physics of Cryogen*.; 119–163.

Zolini, A.M., Carrascal-Triana, E., Ruiz de King, A., Hansen, P.J., Torres, C.A.A. & Block, J. (2019). Effect of Addition of L-Carnitine to Media for Oocyte Maturation and Embryo Culture on Development and Cryotolerance of Bovine Embryos Produced In Vitro. *Theriogen*.; 133(15): 135-143.

Zorova, L.D., Popkov, V.A., Plotnikov., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., Scollott, S.J. & Zorov, D.B. (2018). Mitochondrial Membrane Potential. *Anal. Biochem.*; 552: 50-59