Inland Norway University of Applied Sciences

Department of Biotechnology

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# Master thesis

## Oocyte parameters important for embryo survival and development during *in vitro* embryo production

**Experimental Biotechnology**

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# Abbreviation

**AI:** Artificial Insemination

**ARTs:** Assisted reproductive technologies

**ATP:** Adenosine triphosphate

**CASA:** Computer-assisted sperm analysis

**CCs:** Cumulus cells

**CL:** corpus luteum

**COCs:** Cumulus oocyte complex

**DMSO:** Dimethyl sulfoxide

**EGA**: Embryo genome activation

**EGF:** epidermal growth factor

**ER:** Endoplasmic reticulum

**FSH:** Follicle-stimulating hormone

**GCs:** Granulosa cells

**GEBV:** Genomic Estimated Breeding Values

**GnRH:** Gonadotropin-releasing hormone

**GSH:** Glutathione

**GV**: Germinal vesicle

**GVBD:** Germinal vesicle breakdown

**$H_2O_2$:** Hydrogen peroxide

**ICSI**: Intracytoplasmic sperm injection

**INN**: Inland Norway University of Applied Sciences

**IVC:** *In vitro* culture

**IVEP**: *In vitro* embryo production

**IVF:** *In vitro* fertilization

**IVM:** *In vitro* maturation

**IVP:** *In vitro* production

**LH:** Luteinizing Hormone

**MGCs:** Mural granulosa cells

**MI:** Meiosis I

**MII:** Metaphase II

**MOET:** Multiple Ovulations Embryo Transfer

**mRNAs:** Messenger RNA

**mtDNA**: Mitochondrial DNA

**PB:** Polar Body

**PBS:** Phosphate buffer saline

**PFA:** Paraformaldehyde

**POM**: Porcine Oocyte Medium

**PVS**: Perivitelline space

**PXM:** Porcine X media

**RLU:** relative luminescence units

**ROS:** Reactive oxygen species

**SEM**: Standard error of mean

**TCs:** theca cells

**ZP:** Zona pellucida

# Acknowledgment

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Mahsa Abdi

# Abstract

*In vitro* fertilization (IVF) in cattle has been impeded by technological constraints. These limitations are related to the quality of *in vitro* matured (IVM) oocytes. Numerous studies have investigated factors affecting oocyte quality, and our research aimed to assess critical parameters impacting oocytes and, consequently, IVF outcomes. MitoTracker and Hoechst staining were employed to visualize the distribution pattern of mitochondria and DNA stages before and after maturation. Confocal microscopy images revealed a reorganization in mitochondria distribution from the peripheral region to the inner part of the cytoplasm during the transition of the germinal vesicle (GV) stage to the metaphase II (MII) stage. Additionally, ATP content in bovine and porcine oocytes was measured at different maturation time points. Bovine oocytes exhibited an almost stable increasing trend in ATP levels, while pig oocytes showed fluctuations, suggesting a need for further investigation. Based on ATP content results in the bovine oocytes, three time points were selected for oocyte maturation (20, 25, and 30 hours), and subsequently, IVF was performed for these three time points. Cleavage and blastocyst rates were measured two and seven days after IVF, respectively. Although no significant differences were observed in cleavage rates, the 20-hour maturation group showed the highest blastocyst yield, significantly differing from the 30-hour group ($p < 0.0001$). Oocytes matured for 30 hours also showed the lowest four-cell plus stage rate, with significant differences compared to the 20-hour group ($p < 0.0001$) as well as with the 25-hour group ($p < 0.0001$). This study also compared the slashing and aspiration methods for retrieving oocytes from bovine ovaries. No significant differences were observed between these two methods regarding ATP content, suggesting that the slashing method might be a possible approach for retrieving more and younger oocytes from the ovaries. In conclusion, our study highlights dynamic changes in mitochondria distribution and ATP levels during oocyte maturation. Moreover, there might be an optimal time for performing the IVF, and prolonging *in vitro* maturation adversely affected IVF outcomes since the 20-hour time point demonstrated the highest blastocyst yield. These findings provide valuable insights for optimizing IVF protocols in cattle.

# 1. Introduction

## 1.1 Project background

The conducted study comprises 60 credits and is a project for the master's thesis for the Experimental Biotechnology program at Inland Norway University of Applied Sciences (INN). This project is part of a larger project named Zygote, where different academic and industry partners, including SINTEF, Oslo University Hospital, Norsvin, and Geno, are collaborating. The primary objective of the Zygote project is to build a strong and globally competitive research environment for the livestock and fish breeding industry and applied biotechnology in the Inland region. The objectives include understanding physiological mechanisms for gamete production, developing methods for characterizing ejaculates, advancing knowledge in oocyte and embryo identification, improving cryopreservation methods, and effective collaboration and communication between scientific and industrial partners. This master project is connected to bovine and porcine oocytes and embryo research.

## 1.2 Livestock breeding industry

In the livestock industry, one of the goals is to optimize genetic advancement throughout breeding programs (Armstrong et al., 1997). The dairy industry is a pioneer in the food animal sector in applying advanced breeding techniques successfully (Wiggans et al., 2017). There is still a growing demand for methods to improve dairy cattle production (Sanches et al., 2019). Genetic progress previously was restricted because genetically superior cows could only produce one calf per breeding season (Vasques et al., 1995). Livestock breeding has developed a lot due to the use of assisted reproductive technologies (ARTs), including *in vitro* fertilization (IVF) and *in vitro* embryo production (IVEP) (Allan, 2018), which made it possible to increase the number of calves generated from both superior bulls and cows (Baruselli et al., 2018).

Furthermore, livestock breeding has experienced a dramatic shift due to genomic selection. Genomic selection relies on using genotypic information to choose an ideal phenotype (Seidel, 2010). Due to genomic selection, females, particularly heifers, play a crucial role in reducing the generation interval thereby further increasing genetic progress across cattle herds. Animals with desirable traits are valuable for performing genomic selection for commercial applications (Moore & Hasler, 2017).

## 1.3 Reproductive technologies in cattle breeding

The use of ART techniques has played a significant role in livestock breeding. There are three primary generations of ARTs, comprising artificial insemination (AI) alongside gamete and embryo cryopreservation, multiple ovulations, and embryo transfer (MOET), and the third generation introduces techniques such as semen sexing and IVEP. Additionally, intracytoplasmic sperm injection (ICSI) represents another technique within the third category, but its practicality is limited. Therefore, due to these advancements, biotechnologists are conducting extensive scientific research to enhance the effectiveness of this technology in breeding programs (Gonźalez-Bulnes et al., 2004).

*In vitro* embryo production is considered a crucial research tool for animal embryology, with the possibility of accelerating genetic advancement in cattle production (Camargo et al., 2006). Commercial embryo companies are progressing in using the IVP method, and a large part of all bovine embryos produced globally are IVP embryos (Thibier, 2005). Although considerable enhancements have been made to improve the hormone levels, molecular nutrition, and media quality for the *in vitro* maturation of oocytes, the quality of oocytes matured *in vitro* still differs from that of their *in vivo* counterparts (Yang & Chian, 2017). This difference may stem from several factors, such as an incomplete understanding of the mechanisms involved in oocyte maturation, distinctions between *in vitro* culture conditions and the natural *in vivo* condition, unresolved issues related to the transcriptome, and epigenetic changes during oocyte progression (Jiang et al., 2023). Therefore, as the environment has a direct impact on phenotypes and IVP outcomes (Marsico et al., 2019) for successfully producing embryos, it is crucial to mimic the maturation, fertilization, and culture medium with the conditions found *in vivo* (Sudano et al., 2012).

## 1.4 Anatomy of the reproductive tract in cattle

The reproductive system of cattle consists of the ovaries, oviducts, uterus, cervix, and vagina (Figure 1). The uterus and ovaries play an essential role, during embryo implantation and growth (Carson et al., 1998). The ovary serves as the primary organ for reproduction in females. The two ovaries of a cow are egg-shaped to bean-shaped structures that are between 2.5 and 4 cm in length and are found in the abdominal cavity (Deutscher, 1980). The ovary serves two main purposes. Initially, the ovaries produce hormones essential for the

functioning of the female reproductive system. Additionally, it manages the maturation, selection, and ovulation of a mature oocyte for potential fertilization (Cox & Takov, 2023).

The fallopian tube, or oviduct, is the place where fertilization occurs and is linked to the ovaries (Rosales & Ametaj, 2021). After fertilization, ciliary action makes developing zygotes conveyed into the uterus, where implantation occurs (Senger, 2003). The reproductive tract anatomy of females in different livestock species share similarities with that of cows, differing primarily in the uterus and cervix structure (Thomas & Ellis, 2021).

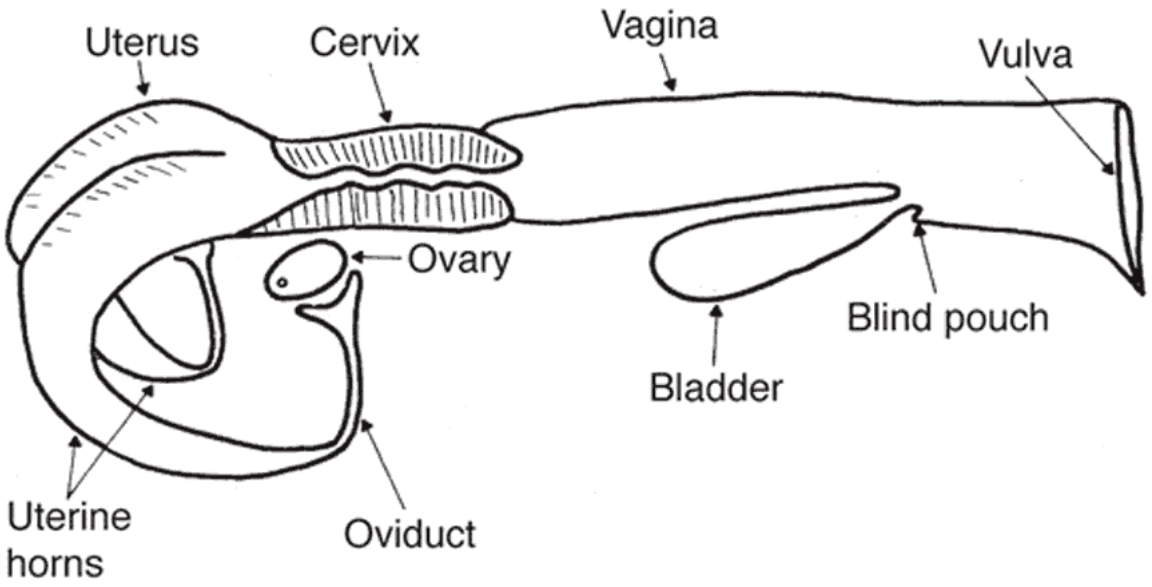


*Figure 1. Schematic illustration of a cow's reproductive system. The system consists of the ovaries as the main reproductive organ and different networks of tubes such as the cervix, uterus, uterine horns, and oviducts (Thomas & Ellis, 2021).*

## 1.5 Folliculogenesis

In cattle, the estrous cycle takes between 18 and 24 days. It is divided into two phases, which include the follicular phase, lasting 4-6 days, and the luteal phase, lasting 14–18 days (Forde et al., 2011). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropin hormones that are essential during ovarian development and the start of puberty (Orisaka et al., 2021). The follicle-stimulating hormone helps with the growth of ovarian follicles, while LH controls the ovulation process, preovulatory oocyte maturation, and the development of the corpus luteum (CL) (Lee et al., 2021).

Ovarian folliculogenesis (Figure 2) is a complex process that involves the progression of the immature oocyte to the ovulation stage with the support of surrounding somatic cells (Morton et al., 2023). Granulosa cells (GCs) and theca cells (TCs) are two types of these somatic cells (Lee et al., 2021). Folliculogenesis is initiated during fetal development (Cox & Takov, 2023) and involves a significant alteration in the volume and number of cells in the ovary (Pangas & Rajkovic, 2015). The ovary contains a set number of primordial follicles at birth. The initial recruitment of primordial follicles leads to the formation of primary follicles and their subsequent development into secondary follicles. This process is regulated by intraovarian factors with the independent function of gonadotropins (Lee et al., 2021). As secondary follicles progress to the preantral stage, their development becomes responsive to FSH. The further growth of preantral follicles into early antral and then antral stages relies on FSH stimulation. After follicle selection, they become LH-dependent, and the LH surge leads to the formation of Graafian follicles (Lee et al., 2021). The selection process is related to overcoming inhibitory mechanisms in one follicle and preparing it for ovulation (Johnson, 2015).

LH signaling is essential for the last phase of oocyte maturation, ovulation, and luteinization of granulosa cells (Lee et al., 2021). After ovulation, the cells inside the follicle go through the luteinization process and form CL, which plays a significant role in progesterone secretion (Thomas & Ellis, 2021). Progesterone is essential for priming the uterus to accept the presumptive zygote and sustaining the optimal uterine conditions for ongoing pregnancy (Thomas & Ellis, 2021).

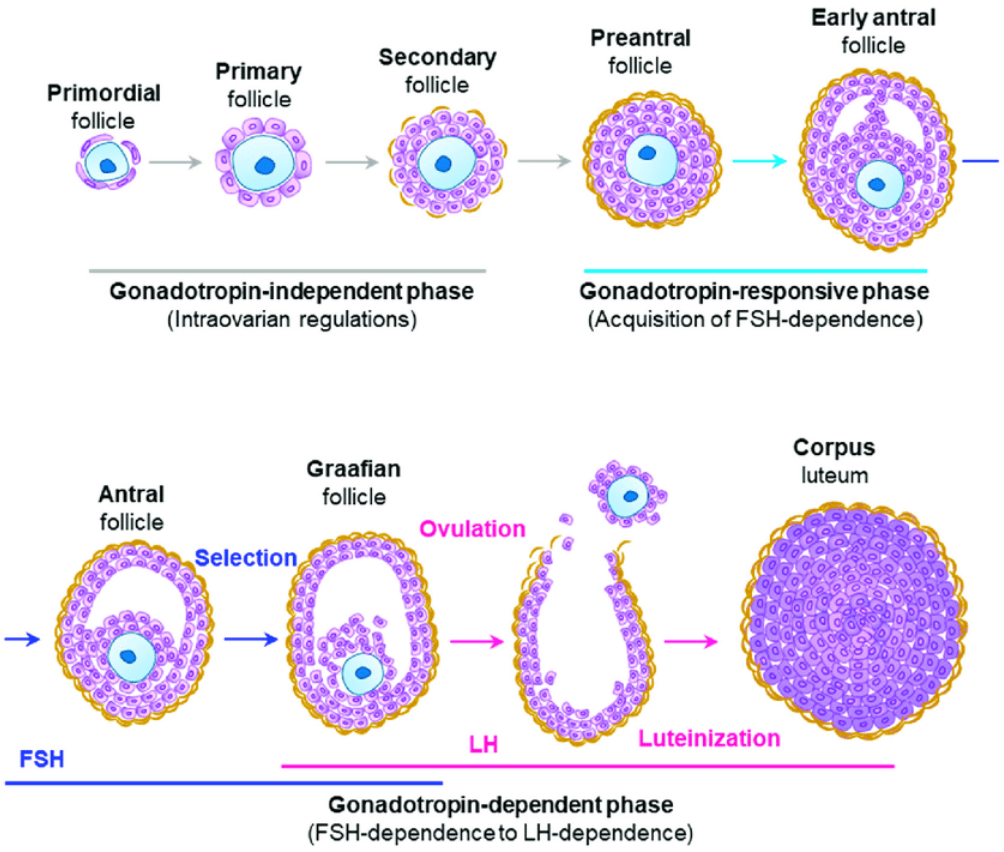


*Figure 2. The growth of ovarian follicles and the process of ovulation. the follicle development process started with Primordial follicles, and developed to Primary follicles, Secondary follicles, Antral follicles, and Graafian follicles, and finally, ovulation (Lee et al., 2021).*

## 1.6 Oocyte morphology

The critical requirement for successful IVP is high-quality oocytes (Boni, 2012). During ovulation, matured oocytes paused in MII stage of meiosis are released from the Graafian follicle, enclosed by two to three layers of cumulus cells and the Corona radiata (interior cumulus cells) (Wassarman & Litscher, 2022). These ovulated oocytes with normal morphology (Figure 3) possess a transparent cytoplasm, a single polar body (PB), an appropriate thickness of the zona pellucida (ZP), a suitable perivitelline space (PVS) (Rienzi et al., 2008). In comparison, oocytes that exhibit abnormalities in shape are typically distinguished by their unusual cytoplasm (such as dark or cytoplasmic granularity), abnormal ZP, abnormal perivitelline space (such as a large PVS or debris in the PVS), and abnormal PB (Rienzi et al., 2011).

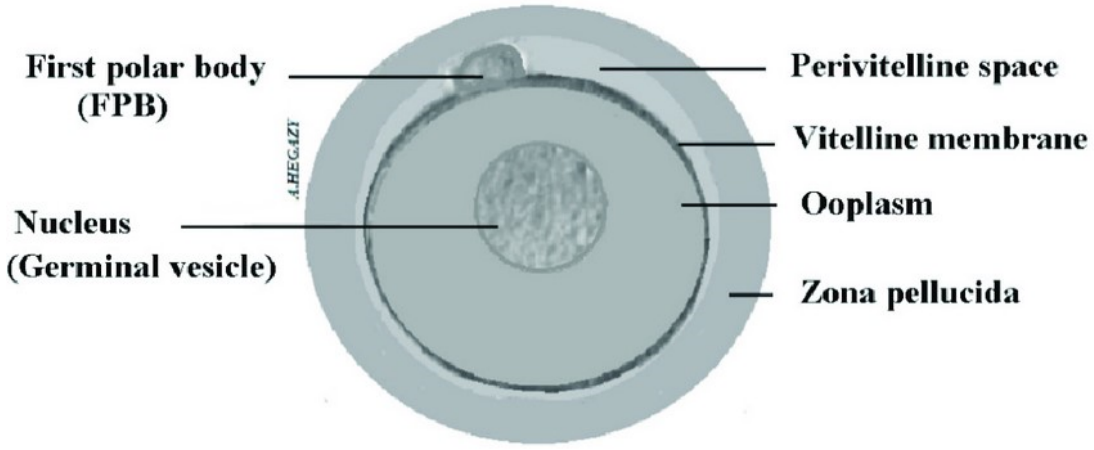


*Figure 3. Illustration of the different parts of the denuded matured oocyte with extruded polar body (Hegazy et al., 2023).*

## 1.7 *In vitro* embryo production

*In vitro* embryo production has three essential stages: maturation of oocytes, fertilization of the oocyte with motile and capacitated sperm, and subsequent cultivation of the embryo to reach the blastocyst stage (Abd El-Aziz et al., 2016). These *in vitro-produced* embryos have some typical features like darker pigmentation and a less compact cellular mass, in addition to gene expression and metabolism modifications compared to *in vivo* embryos (Lonergan et al., 2006).

### 1.7.1 *In vitro* oocyte maturation

Many efforts have been made to improve the *in vitro* production of cattle embryos, like refining the conditions of the culturing systems, particularly during IVM. The term "*in vitro* maturation" refers to the development of an oocyte from the diplotene stage of prophase I (GV), to MII stage, and following cytoplasmic maturation (Trounson et al., 2001). As mentioned before this is an important step because *in vitro*-matured oocytes acquire less developmental capacity compared to oocytes matured *in vivo* (Rocha-Frigoni et al., 2016). Thus, the maturation of the oocyte is the process by which the oocytes gradually obtain the essential capacity to support future phases of growth and finally activate the embryo genome (Ferreira et al., 2009). The complicated procedure during oocyte maturation relies on different factors, for instance, accurate separation of the chromosomes during nuclear maturation, reorganization of the organelles during cytoplasmic maturation, and also the storage of

mRNA, proteins, and transcription factors, which are essential for the accomplishment of the maturation process (Ferreira et al., 2009).

## *Nuclear maturation*

Nuclear maturation is the process (Figure 4) of changing chromatin during the maturation of the oocytes from germinal vesicle breakdown (GVBD) throughout meiosis I (MI) and finally reaching the MII stage (Marteil et al., 2009). Oocyte maturation initiates with the resuming of meiosis division. Oocytes progress through the primary meiotic division and pause at the metaphase stage of the second meiotic division until fertilization occurs (Marteil et al., 2009). An arrested oocyte in meiotic prophase I possesses a large nucleus enclosed by a nuclear membrane, commonly known as the GV stage. In response to an increase in LH secretion, events linked to the nuclear maturation of the oocyte take place within fully developed follicles, including chromatin condensation and germinal vesicle breakdown (He et al., 2021). Following GVBD, oocytes progress to the MI stage (Moor et al., 1998). The oocyte later completes Meiosis I, following the extrusion of the first polar body (PB1), which contains a small amount of cytoplasm. After this stage, Meiosis II initiates immediately until mature oocytes stop again at the MII stage until fertilization. The oocyte completes the second meiosis only if fertilization occurs (He et al., 2021).

Oocytes in the GV stage are not fully developed and are considered immature. Those classified in the MII stage are considered fully developed and have a metaphase plate and first polar body. Oocytes that are in the process of maturing but have not reached the MII stage yet, including metaphase I, anaphase I, and telophase I, are classified as intermediate stages of meiosis (Rocha-Frigoni et al., 2016).

## *Cytoplasmic maturation*

In addition to chromosome rearrangement and reduction, the maturation process also includes changes in cytoplasmic composition. The processes of nuclear and cytoplasmic maturation happen simultaneously but separately (Yamada & Isaji, 2011). The three main events that constitute cytoplasmic maturation are the redistribution of cytoplasmic organelles (Figure 4), the dynamics of the cytoskeletal filaments, and molecular growth and development (Ferreira et al., 2009). Chromosomal segregation primarily occurs during nuclear maturation, whereas organelle reorganization and storage of transcription factors, proteins, and messenger RNA

(mRNAs) that are associated with overall maturation, fertilization, and early embryogenesis take place during cytoplasmic maturation (Ferreira et al., 2009). Throughout the maturation process, the oocyte accumulates enough mRNA, ribosomes, proteins, and cytoplasmic organelles, such as mitochondria and microtubules, which will be utilized after fertilization (Mermillod, 2014).

The oocyte's volume increases throughout the maturation process. In mammals, there is a clear connection between oocyte size and their ability for development (Bhardwaj et al., 2016). Cattle and pigs have been reported to exhibit enhanced oocyte developmental competence associated with larger follicular size (Bagg et al., 2004; Marchal et al., 2002). This could be why only a limited number of oocytes with comparable morphology can reach viable blastocysts (Chandra & Sharma, 2020). Moreover, this may also be a rationale for the higher capability of oocytes developed *in vivo* compared to those derived from *in vitro* cultured preantral follicles (Sharma et al., 2013).

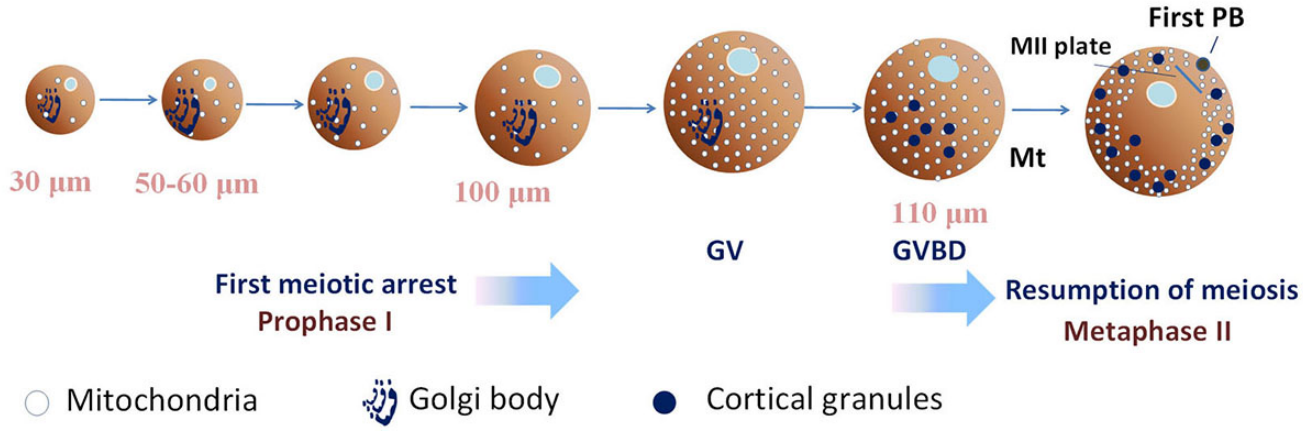


*Figure 4. Illustration of oocyte growth and acquisition of developmental competence: The size of the oocyte increases along with the follicle's growth, reaching approximately 110 µm at GVBD. The development process involves the redistribution and multiplication of mitochondria. The Golgi body is distributed across the cytoplasm during the GV stage. Upon GVBD, it undergoes fragmentation, creating cortical granules scattered in the outer region of the ooplasm. Chromosomes are compacted during the GV stage and decondensed and separated during the GVBD stage. The polar body extrudes during the metaphase plate stage, followed by chromosome alignment. PB stands for polar body, GV for germinal vesicle, and GVBD for germinal vesicle breakdown (Chandra & Sharma, 2020).*

### 1.7.2 Cumulus cells and grading oocyte quality

The somatic cells encompassing the oocyte are called cumulus cells (CCs). In mammals, they have a crucial function in the development of the oocyte, the process of meiotic maturation, ovulation, and fertilization (Demiray et al., 2019). Cumulus cells are derived from undifferentiated GCs in the follicles at the primordial to preantral stages. These GCs are the primary cell type in the ovary that provides the microenvironment and physical support needed for the developing oocyte (Huang & Wells, 2010). A larger considerable cumulus expansion during *in vitro* maturation positively correlates with a higher progression ability of oocytes to the blastocyst stage in various species (Han et al., 2006; Qian et al., 2003). Surges in FSH and LH result in cumulus cells producing an extracellular matrix, and the expansion of CCs aids in resuming the meiotic growth of the oocyte (Turathum et al., 2021).

The capacity of an oocyte to mature, fertilize, and generate typical offspring is referred to as oocyte quality (Duranthon & Renard, 2001). The cumulus-oocyte complex (COCs) comprises cumulus cells near the oocyte (Kidder & Mhawi, 2002). Cumulus cell morphology can be applied to assess the quality and maturity state of the oocytes (Torner et al., 2004). Compressed, complete cumulus layers and transparent uniform ooplasm define high-quality immature oocytes. In contrast, a cumulus with fewer than three layers or gloomy heterogeneous ooplasm indicates poor-quality immature oocytes (Blondin & Sirard, 1995). The cumulus-oocyte complex has four different classifications (Figure 5). Class 1 comprises COCs characterized by uniform oocyte cytoplasm and a full, compressed, multilayered cumulus. In Class 2, the cumulus cells are smaller than Class 1 COCs and possess more than five layers of tightly compacted cumulus cells. In addition, the cytoplasm is uniform, with very few areas displaying inconsistent coloring. In Class 3, the cytoplasm is divided or heterogeneous. Three to five cumulus cell layers cover the ZP over small, denuded regions. Finally, Class 4 is distinguished with heterogeneous cytoplasm pigmentation, and the cumulus is either absent, largely absent, or expanded (Stojkovic et al., 2001).

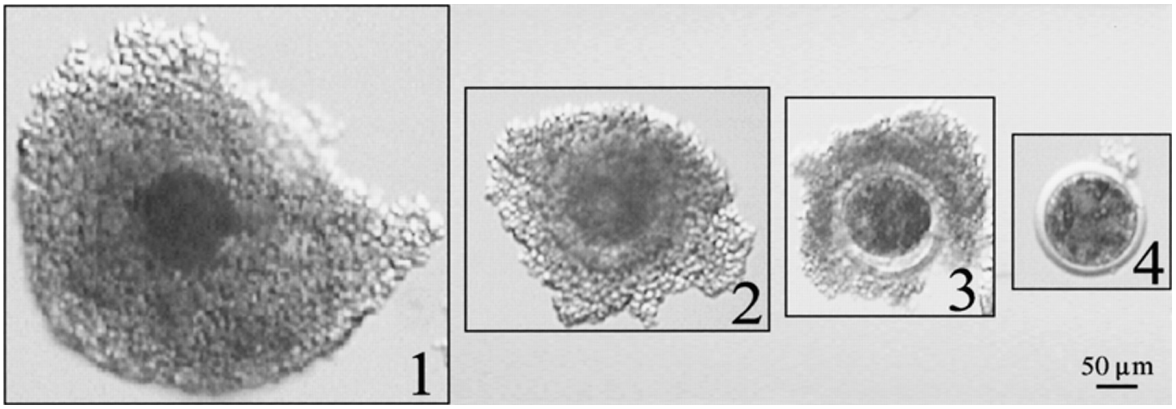


*Figure 5. Bovine cumulus-oocyte complexes (COCs) are divided into four different categories. Class 1 possesses perfect, thick, and multi-layered cumulus. Class 2 features smaller cumulus cells with over five layers of compacted cells. In Class 3, the cumulus covers the zona pellucida with small, denuded regions. An absent or expanded cumulus marks class 4 (Stojkovic et al., 2001).*

### 1.7.3 *In vitro* fertilization and *in vitro* culture

Since 1978, the IVF technique has been introduced and has received considerable public attention (Wang & Sauer, 2006). During the IVF procedure, bovine oocytes and spermatozoa are incubated together for up to 18 to 24 hours after the IVM is finished (Ferré et al., 2020). For successful IVF, it is essential to expose mature oocytes to sperm that has already undergone or is currently undergoing capacitation (Yanagimachi & Usui, 1974). When the male gamete passes through the female's reproductive tract in a process called "capacitation," it gives the sperm cells the ability to fertilize an egg *in vivo* (Yang & Chian, 2017). The penetration rate of oocytes will significantly increase by adding caffeine to the fertilization medium (Niwa & Ohgoda, 1988). This suggests that the combination of heparin and caffeine together acts cooperatively by inducing capacitation and acrosome reactions in spermatozoa and prompting the *in vitro* fertilization of cattle oocytes (Niwa & Ohgoda, 1988). The movement of each sperm cell can be automatically and precisely measured thanks to computer-assisted semen analysis (CASA) technology (Amann & Waberski, 2014).

The fertilized bovine oocytes from IVF are presented to *in vitro* development for seven days until they reach the blastocyst stage (Ferré et al., 2020). Two days after IVF, the fertilization or cleavage rate can be recorded, typically ranging from 70 to 85 percent (Ferré et al., 2020). Seven days after fertilization, the blastocyst rate should be recorded, and typically, 20%–40% of cultured presumptive zygotes will develop until blastocysts (Rizos et al., 2008). When an

embryo gets to the blastocyst stage, it can either be cryopreserved or transferred using similar methods to those used for *in vivo* blastocysts (Ferré et al., 2020).

Although development in the media and culture conditions of the bovine embryos has facilitated their progression to the blastocyst stage, most of these embryos cease development after a few cell stages after starting the cleavage process. This block usually occurs in bovine embryos at the eight-cell stage (Meirelles et al., 2004). This problem is possibly associated with an inadequate cytoplasmic maturation of the oocyte, and might furthermore be related to the maternal zygotic transition, also known as embryonic genome activation (EGA) (Meirelles et al., 2004). Oocytes contain all the necessary mRNAs and proteins for progressing to the fourth or fifth cell cycle (Barnes & First, 1991; Memili & First, 1998). Nonetheless, embryos that are unsuccessful in initiating their own genome transcription do not progress further in development (Meirelles et al., 2004).

### 1.7.4 Oxidative stress affecting IVP

Numerous studies have suggested that the culture environment in which oocytes and embryos are presented could influence their quality (Trounson et al., 2001). One critical factor affecting the cultural environment is the oxidative stress caused by increased oxygen levels, which has received specific consideration in recent years (Corrêa et al., 2008). Typically, the level of oxygen applied in IVM culture systems is higher than that which is naturally present in the female reproductive system (20% compared to 3-9% $O_2$), leading to varying levels of reactive oxygen species (ROS) including hydrogen peroxide ($H_2O_2$), hydroxyl radicals, and peroxyl radicals (Rocha-Frigoni et al., 2016). ROS generation is a natural process inside oocytes, embryos, and cumulus cells (Guérin et al., 2001). In typical biological circumstances, the primary source of ROS arises during oxidative phosphorylation in mitochondria (Feugang et al., 2004).

Oxidative stress can seriously damage the oocyte's functionality by causing oxidation of RNA, DNA, and proteins, destroying the integrity of the cell membrane by reducing the telomere length (Rodríguez-Varela & Labarta, 2020). The process of mitochondria in an oocyte generating superoxide is still unclear (Cobley, 2020). It is thought that a malfunction in mitochondrial function may contribute to the unexpected production of superoxide. Consequently, oxidative stress may reduce the survivability of *in vitro* embryos (Kitagawa et al., 2004) (Figure 6).

The primary strategy to avoid oxidative stress is adding antioxidants to IVM culture media (S. Soto-Heras & M.-T. Paramio, 2020). Antioxidants like glutathione (GSH) play an important role in controlling the production of ROS and defending against damage caused by oxidative stress (Calabrese et al., 2017). Furthermore, the production of meiotic spindles, the development of embryonic pronuclei following fertilization, and the cytoplasmic maturation of oocytes are all influenced by the intracellular production of GSH *in vivo*. (García-Martínez et al., 2020). Many studies have shown that the gametogenic development of bovine and porcine oocytes during IVM was restricted by their limited ability to synthesize GSH. Supplementing the IVM medium with exogenous GSH can mitigate this limitation (Ren et al., 2021) (Figure 6).

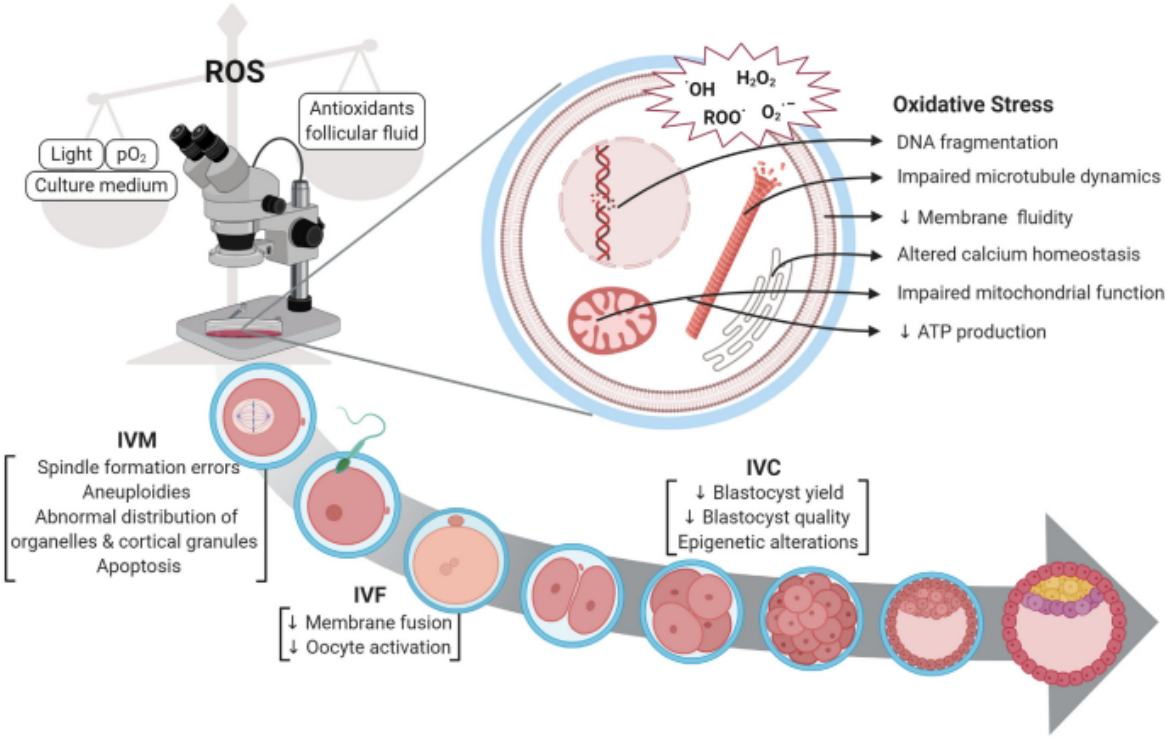


*Figure 6. Effect of oxidative stress on in vitro embryo production. Unbalanced amounts of ROS can cause oxidative stress, ultimately influencing all processes of in vitro embryo production, including IVM, IVF, and, finally, blastocyst development in IVC. Oxidative stress can have a negative impact on embryo development (S. Soto-Heras & M. T. Paramio, 2020).*

## 1.8 Insights into pig *in vitro* embryo production

*In vitro*, embryo production in pigs is an appealing choice for research domains like reproductive biotechnology and biomedicine (Fowler et al., 2018) since pigs contribute to approximately 40% of meat consumption worldwide (Tilman et al., 2002). Pigs are suitable for use as a case study bio model due to their similarities with human physiology. They are also helpful in generating transgenic animals, which could provide tissues and organs for transplantation. (Gil et al., 2010).

Pig reproduction is unique in that it ovulates 15–30 oocytes during one estrous period, and this is influenced by different factors such as age, breed, nutritional status, and other variables (Soede et al., 2011). The production of pig embryos has had significant advancements with IVM and IVF methodologies in the past few years. Furthermore, improvements in IVM protocols have resulted in better oocyte cytoplasmic maturation (Abeydeera, 2002b). *In vitro*, matured oocytes reach the MII stage with the polar body extrusion after 36–42 hours (Yuan & Krisher, 2012). Thus, in pigs, COCs are cultured for approximately 40–44 hours after maturation in IVM, and this is much longer compared to cattle, which are cultured for 20–24 hours (Figure 7).

During porcine in *vitro* embryo production, there are some challenges; polyspermic penetration, e.g., when an oocyte is fertilized by more than one sperm cell, is still a significant problem that requires a solution (Abeydeera, 2002b). It has been shown that polyspermic is more prevalent in the IVF process compared to *in vivo* conditions (Coy & Avilés, 2010; Tanihara et al., 2013). Decreasing the spermatozoa concentration during IVF could be a straightforward solution to reduce polyspermy, but studies indicate that lowering the spermatozoa concentration considerably minimizes IVF success rates (Abeydeera, 2002a). Another challenge for pigs is the relatively low and inconsistent blastocyst percentage during different IVP rounds. Approximately 15–30% of the pig oocytes progress to the blastocyst stage during the culture (Martinez et al., 2017). In pigs, EGA happens around the four-cell stage (Cao et al., 2014).

Another significant challenge in pig embryo production is related to the elevated inner lipid content. Under a microscope, this excess of lipid makes the oocytes and embryos appear much darker and less transparent than mouse or human cells (Genicot et al., 2005). This feature makes it more difficult to observe early signs of successful fertilizations, like pronuclear

formation and morphological evaluation, which are typically the first steps in the human embryology process. Research shows that, compared to bovine oocytes containing 58–59 ng lipids (Ferguson & Leese, 1999), porcine oocytes have more than twice the lipid content (135–156 ng) (McEvoy et al., 2000; Sturmey & Leese, 2003). It is important to carefully evaluate the intracellular lipid content of oocytes during oocyte maturation. Lipids can function in energy production as building blocks of steroid hormones and signaling molecules. Moreover, there is a strong correlation between the activation of oocytes and the mobilization of lipid reserves. Cumulus-oocyte complexes have a function in regulating lipid metabolism during the cytoplasmic maturation of the oocyte (Khan et al., 2021).

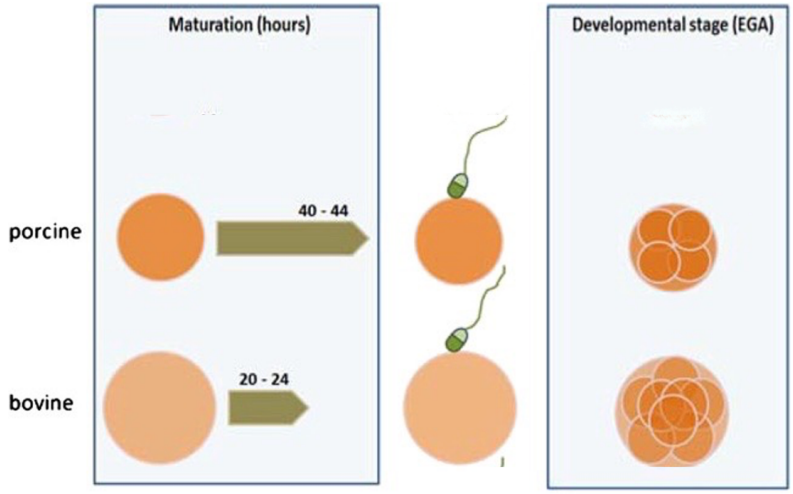


*Figure 7. Comparison between porcine and bovine oocytes regarding size, maturation duration, and embryonic genome activation (EGA) after fertilization. The differences in color between the oocytes indicate a higher lipid content in porcine oocytes. EGA in pigs usually occurs at the fourth cell stage, while in bovines, it occurs at the eight-cell stage. Picture adapted from Santos et al. (2014).*

## 1.9 Mitochondria and ATP content in oocytes

Mitochondria are essential organelles for oocyte function and serve as an important marker for indicating oocyte quality. As a result, they are a significant factor for successful fertilization and subsequent development (Schatten et al., 2014). Mitochondria have their own genetic material called mitochondrial DNA (mtDNA), which entirely comes from the maternal mtDNA (Cummins et al., 1997). The mtDNA copies in a mammalian oocyte range from 100,000 to 200,000, and they are present in all daughter cells as the growth of embryos proceeds (Reynier et al., 2001). Furthermore, cellular activity is frequently indicated by the quantity of mitochondria within cells. For example, compared to other somatic cells, neurons,

muscle cells, and mature oocytes possess numerous copies of mitochondrial DNA (Chappel, 2013).

Oocytes are more vulnerable to any type of mitochondrial dysfunction because mtDNA replication does not start until post-implantation (Spikings et al., 2007). Previous research has indicated that mitochondrial malfunction, like structural, spatial, and genetic anomalies in the oocyte, could affect normal embryonic development. As a result, mitochondrion-related traits and other changes can be used as indicators of oocyte quality (Wang et al., 2009).

The oocyte's mitochondria can store intracellular calcium, proapoptotic factors, and adenosine triphosphate (ATP) for fertilization and embryonic development (Torner et al., 2004). The ability to generate ATP is essential for successfully maturing the cytoplasm and nucleus, preparing for fertilization, and completing meiosis II (May-Panloup et al., 2005; St John et al., 2010). During the maturation process of the oocyte, from the primordial follicle to the appearance of the metaphase plate, the mtDNA copy number significantly increases (Figure 8). There are two high points during the transition from early to later follicle stages and from the GV stage to metaphase II, showing that the number of mtDNA copies goes up. Alongside the increase in mtDNA, the ATP content also rises in parallel for continuous transcription and translation in the oocyte (Kirillova et al., 2021). Previous research demonstrated that high-quality oocytes with efficient mitochondrial numbers and adequate ATP content (at least 2 pM) (Van Blerkom et al., 1995) can generate blastocysts with better quality after fertilization.

### 1.9.1 Mitochondrial distribution patterns during oocyte maturation

The two main mitochondrial distribution patterns in oocytes are homogeneity and heterogeneity. According to Nishi et al. (2003), homogeneous distribution of mitochondria is more frequently seen in the cytoplasm of the GV stage, whereas heterogeneous distribution is regularly found in metaphase I and II oocytes (Torner et al., 2004). The indicator of oocyte maturation is the rising level of mitochondrial accumulation around the nucleus, at the same time as the distribution of the mitochondria changes from a homogenous pattern to a heterogenous one. More specifically, the mitochondrial distribution switched from granular aggregation to clustered aggregation in the heterogeneous pattern (Wang et al., 2009). The embryos with low morphological quality can only be identified by their homogenous distribution of mitochondria (Wilding et al., 2001).

Since ATP synthesis and calcium supply are the two primary tasks of mitochondria, mitochondrial redistribution in the oocyte may be caused by the significant need for these nutrients during cytoplasmic maturation (Krisher, 2004). The breakdown of the nuclear envelope in mammalian oocytes requires a lot of ATP, but it also needs intracellular calcium, which comes from the endoplasmic reticulum (ER) and mitochondria (Krisher, 2004). In the last stage of oocyte maturation, the cumulus cells provide nutrients to the developing oocytes (Gilchrist et al., 2008).

The orientation of mitochondria impacts the production of ATP and the regulation of calcium in the oocytes (Van Blerkom et al., 2008). As a result, any malfunction of mitochondria can trigger different physiological issues like neural dysfunction, cell death, and even cancer (Shao et al., 2020; Signorile et al., 2020). Numerous techniques have been created for structural and functional assessment of mitochondria (Kumagai et al., 2019; Momozane et al., 2018). For instance, the shape of mitochondria can be visualized with some dyes like rhodamine 123 and Mito Tracker (Calarco, 1995).

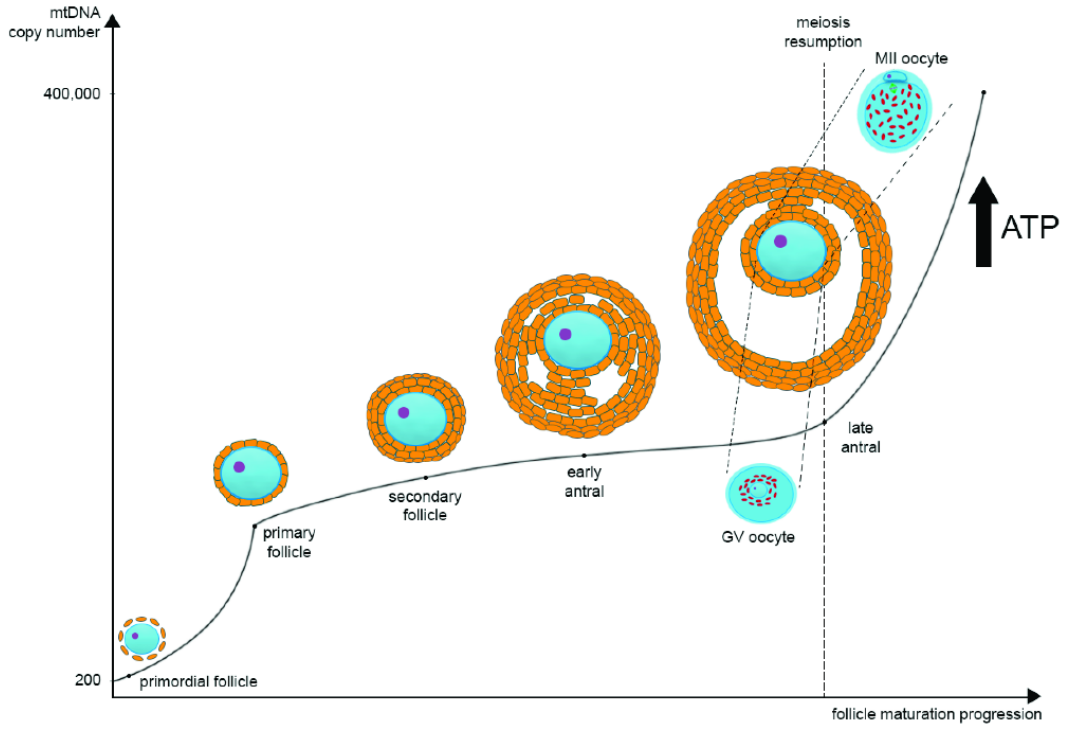


*Figure 8. Mitochondrial distribution and increasing mtDNA copy number during oocyte oogenesis. The amount of mtDNA and ATP content has increased during the maturation process. The maturation process begins from the primordial follicle to the MII stage. The copy number of the mtDNA has a dramatic surge at two different points. The first rise is in the transition from the primordial follicle to the primary follicle, and the second is in the transition from the GV stage to the MII stage (Kirillova et al., 2021).*

### 1.9.2 ATP content measurement by Luminescence assay

The bioluminescent luciferin-luciferase reaction (Figure 9) is one of the most effective and valuable methods for ATP measurement in living cells. Luciferase is an enzyme that generates light through the oxidation of the small-molecule substrate, d-luciferin. The reaction, catalyzed by luciferase in the presence of magnesium ions, involves the conversion of d-luciferin into oxyluciferin, resulting in a yellow-green light flash. This flash is directly proportional to the amount of ATP present, with a peak emission at 560 nm (Morciano et al., 2017).

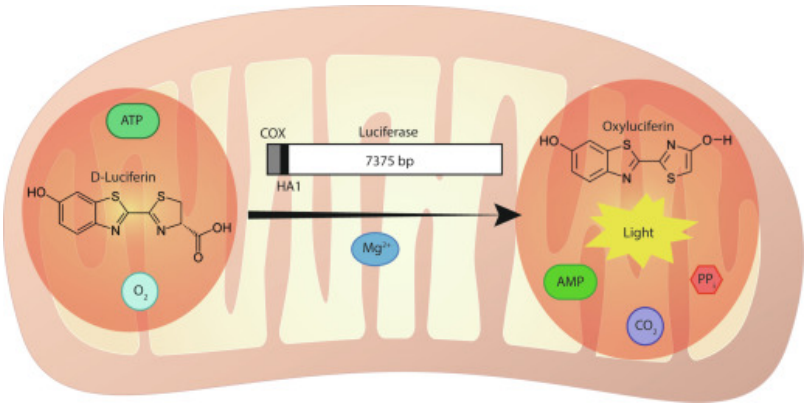


*Figure 9. Schematic illustration of the Luciferin-luciferase. Luciferase generates energy by oxidizing luciferin, releasing light photons. The emitted light, proportional to intracellular ATP concentration, is detectable by a sensitive detector (Morciano et al., 2020).*

## 1.10 Confocal microscopy to analyse mitochondria distribution

Confocal microscopy is employed to visualize DNA at different cell stages and observe the mitochondrial pattern both before and after oocyte maturation. A confocal microscope aims to produce a focused light source and remove unwanted blurry light. This helps create detailed 3D reconstructions and capture high-resolution images deep inside tissues (Elliott, 2020). Confocal microscopy makes it possible to capture sharp, focused images from thin sections within thick samples, reducing unwanted blurring and background noise. Both the biomedical sciences and the field of materials science make extensive use of this technique. A sample is positioned on the microscope stage during optical sectioning, and images are sequentially captured at various focal planes by adjusting the stage or objective position. The result of this procedure is a volumetric image called a "z-stack," which provides 3D spatial information about the sample. When the data is collected, quantitative analysis of the data, including measurements of volume, localization, and surface area, is achievable (Elliott, 2020).

## 1.11 Aim of the study

Oocyte maturation is critical for the success of IVF and subsequent embryonic development. The number of mitochondria and their distribution pattern inside the oocyte, as well as the level of ATP are two significant factors important for oocyte maturation and thereby the outcome of IVF. The main objective of this master project was to investigate bovine and porcine oocytes during maturation and to explore possible parameters important for IVF and embryo development.

To achieve the main objective the following tasks were conducted:

- Comparison of two methods for collection of oocytes from ovaries
- Measurements of oocyte ATP content at different stages during maturation
- Staining and visualisation of distribution of mitochondria and maturity state during oocyte maturation
- Performing IVF at different time points in the maturation process
- Evaluation of blastocyst yield from the different IVF time points

# 2. Study methods

## 2.1 Experimental design

After collecting bovine ovaries from the slaughterhouse, oocytes were extracted from the ovaries using two methods: aspiration and slashing. High-quality oocytes (those with three to five layers of cumulus cells) were selected and washed, and then COCs were transferred to IVM media for further maturation. A primary objective of our study was to assess the ATP content, mitochondrial distribution patterns, and DNA stages before and after oocyte maturation. To investigate the impact of extended maturation time on IVF outcomes, COCs were matured in IVM media for three different periods (20, 25, and 30 hours), which were selected based on ATP content results. Following each maturation time, COCs were fertilized with sperm cells in IVF media. Seventeen hours post-fertilization, the oocytes were transferred to IVC media. The percentage of cleaved embryos was then observed 48 hours after fertilization. Additionally, the percentage of embryos reaching the blastocyst stage was recorded seven days after fertilization (Figure 10). Similarly, porcine oocytes were collected using aspiration, and high-quality oocytes were selected. Then, ATP measurements for porcine oocytes were conducted before and after maturation at different times. A corresponding study on IVF will also be conducted on porcine oocytes.

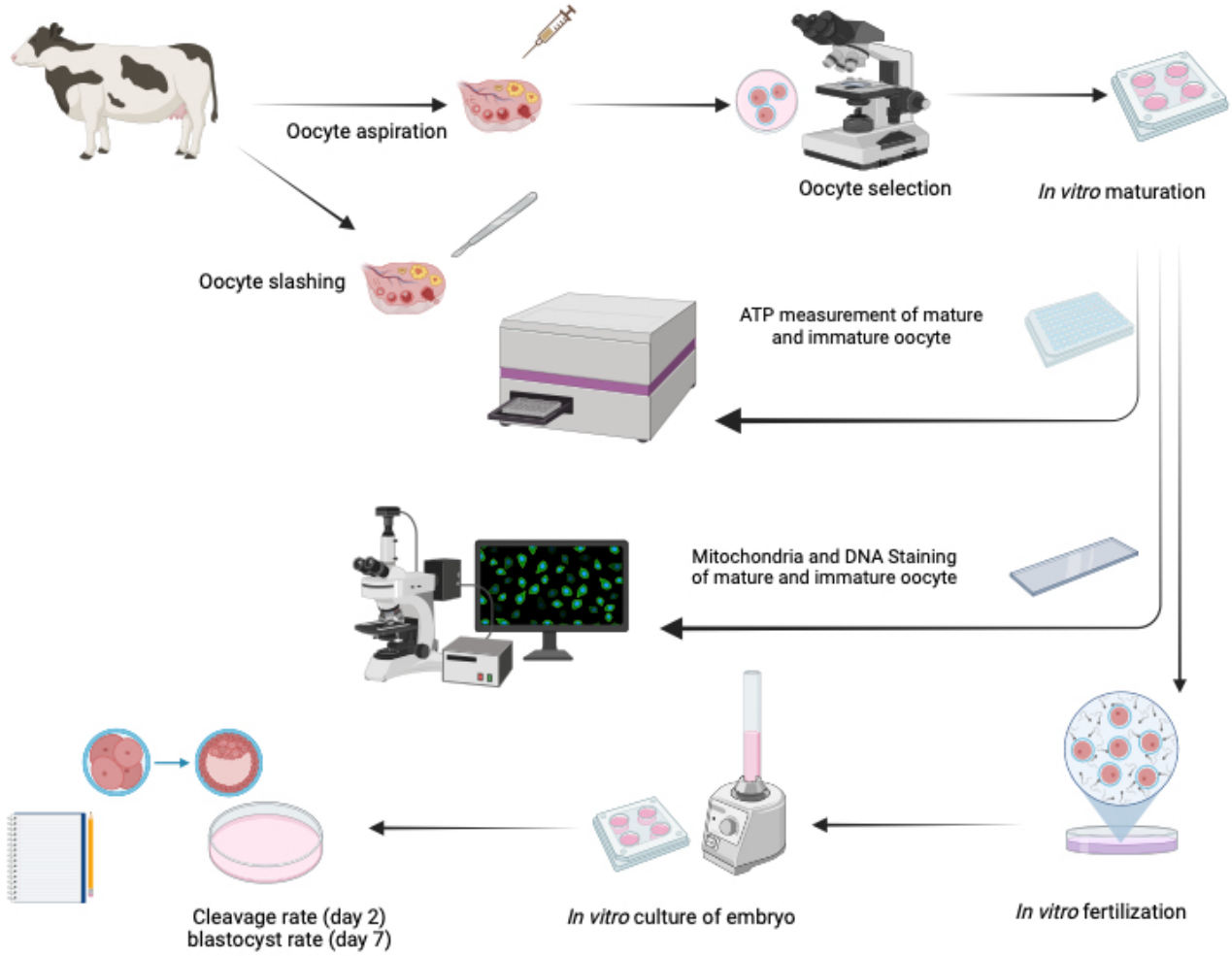


*Figure 10. Illustration of the present study's workflow, which begins with the collection of bovine ovaries, followed by retrieving the oocytes with aspiration or slashing and transferring them to in vitro maturation media. ATP measurement and staining of the DNA and mitochondria were performed both before and after oocyte maturation. Then, matured oocytes were transferred to in vitro fertilization (IVF) media for insemination with sperm cells. Eighteen hours after fertilization, the presumptive zygotes were cultured in in vitro culture (IVC) media. Two days and seven days after fertilization, the cleavage rate and blastocyst rate of the embryos were recorded, respectively. The picture was created with BioRender.com.*

## 2.2 Chemicals and solutions

All chemicals and reagents used were purchased from Sigma-Aldrich (Oslo, Norway) unless otherwise stated. All media used for bovine IVP were obtained from IVF Biosciences (Falmouth, UK). For transportation and washing of ovaries, 0.9% saline solution (0.9% NaCl with of 1% kanamycin per litter) was utilized. Heparin (5000IU/ml) was used for the collection of COCs. COCs were washed using PXM solution (porcine X media (PXM), 0.004 g/ml BSA bovine serum albumin (BSA), and 0.2 mM Na-pyruvate). PXM medium was composed of base stock, 5.00 mM bicarbonate, 25.0 mM hepes stock, 2,0 mM Ca-lactate, 0.01 mg/ml Gentamicin (10 mg/ml) and water. BO-IVM medium (oocyte maturation medium, IVF Biosciences, Falmouth, UK) was used as the maturation medium for bovine oocytes, and BO-IVF$^{TM}$ medium (Fertilisation Medium, IVF Biosciences, Falmouth, UK) was used for *in vitro* fertilization. Before the IVC step, wash media (IVF Biosciences, Falmouth, UK) was used to vortex and wash the zygotes. The BO-IVC medium (Embryo Culture Medium, IVF Biosciences, Falmouth, UK) supplemented with a BO-Oil (Oil for Medium Overlay, IVF Biosciences, Falmouth, UK) served as the embryo culture medium. Bovine semen was washed using BO-SemenPrep$^{TM}$ (Semen Medium Preparation, IVF Bioscience, Falmouth, UK). MitoTracker orange$^{TM}$ CMTMRos, (M7510, Thermos Fisher Scientific, Waltham, MA, USA) and Hoechst (H-33342, B2261, Sigma) were used for mitochondria and DNA staining, respectively. For washing the oocytes in the staining process, 0.004 g/ml BSA 0.01% triton X-100 in phosphate-buffered saline (PBS) were used, and oocytes were fixed in 4% paraformaldehyde (PFA) followed by mounting on slides using a mounting medium (Dako, Glostrup, Denmark). ATP measurement was conducted using Cell Titer-Glo® Reagent (Promega G7570).

For porcine experiments, COCs were washed using PXM solution, and *in vitro* maturation was performed using Porcine Oocyte Medium (POM), both supplemented with 4.0 mg/ml BSA (Yoshioka et al., 2008). POM medium was composed of 108 mM NaCl, 10 mM KCl, 0.35 mM KH2PO4, 0.4 mM MgSO4.7H2O, 25 mM NaHCO3, 5.0 mM glucose, 0.91 mM Na-pyruvate, 2.0 mM Ca-(lactate)2 · 5H2O, 2.0 mM L- glutamine, 5.0 mM hypotaurine, 20 ml/l BME amino acids, 10.0 ml/l MEM non-essential amino acid, 0.6 mM L-cysteine, 0.01 mg/ml gentamicin, 4.0 mg/ml BSA, serum substitute, 10 ng/ml epidermal growth factor (EGF) and 50 μM Mercaptoethanol (Gibco) (Jochems et al., 2021).

## 2.3 Collection and *in vitro* maturation of bovine oocyte

Ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermos containing warm saline solution (31-33 °C). In the laboratory, ovaries were further washed three to four times by gentle massaging in a fresh, warm saline solution, and transferred to a beaker containing the solution, and kept in a water bath at 33 °C during the aspiration or slashing process. The oocytes were aspirated from 2–8 mm follicles using an 18 G pink needle fixed to a 10 mL syringe (Figure 11 A). The aspirated fluid was incubated at 36 °C for 10 minutes in a 50-ml falcon tube containing 140 µl of 5000 IU/ml heparin to allow the oocytes to settle. The settled oocytes were aspirated from the bottom of the tube with a 1 ml pipette and transferred to 7 ml of prewarmed (36 °C) PXM wash media in a 90-mm petri dish. Oocytes with 3-5 cumulus cell layers were selected and washed three times in a 35-mm petri dish containing 2 ml of PXM wash media. Selected bovine oocytes were then washed once in a 200µl drop of prewarmed BO-IVM media. After washing, COCs were transferred to the 200 µL IVM droplet and rinsed. Oocytes were then transferred in groups of 30 into each well of a Nunc four-well dish (Thermo Scientific$^{TM}$) containing 500 µl of pre-heated BO-IVM media using a 15-µL pipette. The plate was subsequently incubated at 38.8 °C and 6% CO2 in a humidified atmosphere. In this study, oocytes were incubated in IVM media in 3 different maturation groups for 20,25 and 30 hours, and subsequent IVF was performed for each group (as explained in section 2.6). For each time point, a total of 230 oocytes were matured during six replicates. The standard incubation time of bovine oocytes in IVM media is 20-24 hours, both in INN and other IVF laboratories.

## 2.4 Collection and *in vitro* maturation of porcine oocytes

The procedure for collecting and washing the ovary was the same as for the bovine ovaries (Figure 11 B) from 3 to 8 mm follicles using an 18-gauge needle and a 10 ml syringe. Later, those oocytes with compact cumulus and homogeneous cytoplasm were selected and washed three times in PXM, followed by two times in POM medium, both supplemented with 4.0 mg/ml BSA (Yoshioka et al., 2008). Selected COCs were transferred, in groups of 40 to 50, to each well containing 500 µl of pre-equilibrated POM medium in a NUNC 4-well plate. During the initial 20 hours, COCs were matured in POM added with 0.05 IU/ml porcine FSH and LH (Insight Biotechnology Ltd., Wembley, UK) and 0.1 mM dbcAMP. Later, COCs were matured for an additional 28 hours in POM without hormones and dbcAMP. The oocytes

were cultured for 48 hours at 38.8 °C in in humid atmospheric air with 6% CO2 (Jochems et al., 2021).

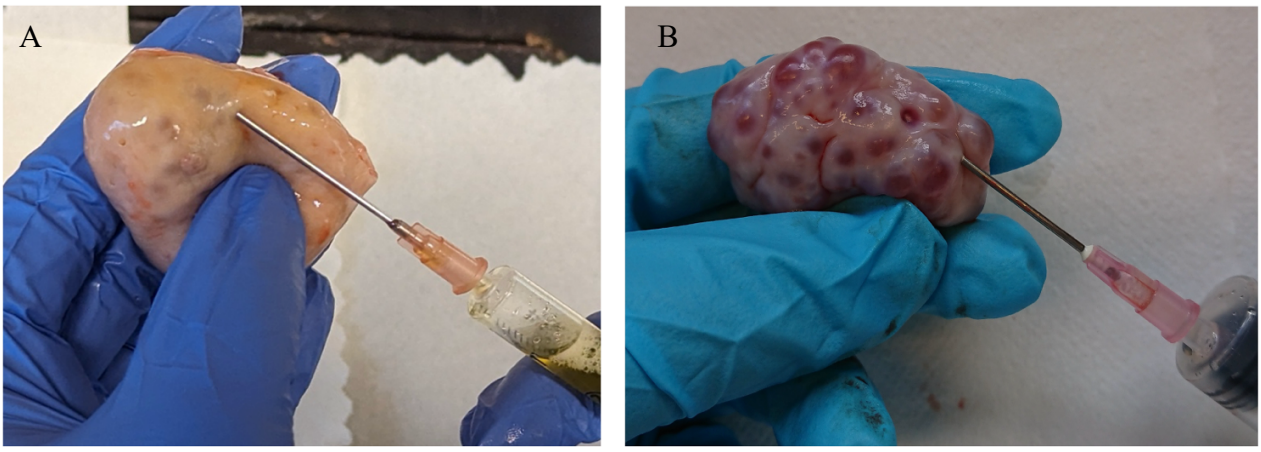


*Figure 11. Aspiration method of bovine and pig oocyte. A: aspiration of the bovine ovary (Picture was taken in INN laboratory). B: aspiration porcine ovary (R. Jochems).*

## 2.5 Collection of bovine oocytes with slashing method

In this study, our aim was to collect a larger number of young oocytes for research purposes from the inner parts of the ovary by performing the slashing method after aspirating the follicles on the same ovary. Therefore, the ATP content of the oocytes collected using the slashing method was compared with those collected by the aspiration method. In this procedure, the ovaries were washed (as mentioned above), and after performing the aspiration, the ovarian surface was scraped with a surgical blade. After making several deep scratches in different parts of the ovaries, they were later squeezed into a beaker containing PXM wash media to release the oocytes, even from the inner parts of the ovary. The subsequent steps for collecting and selecting the oocytes followed the same procedure as the aspiration method (Figure 12).

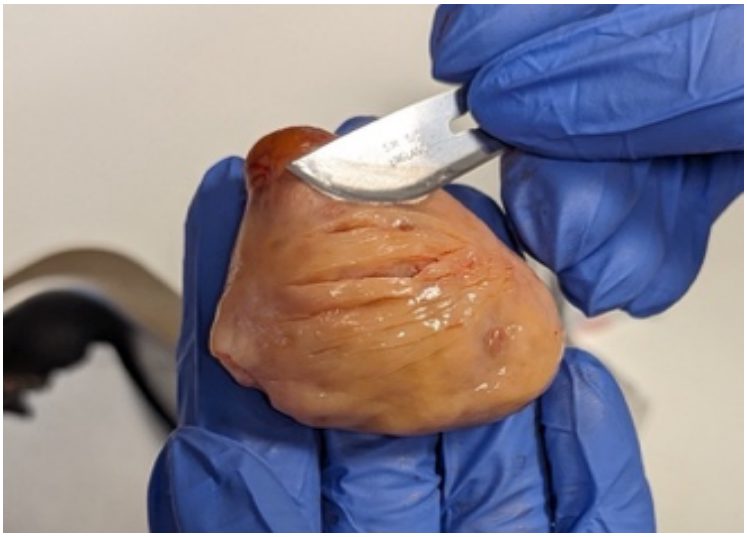

*Figure 12. Slashing method for oocyte collection with surgical blade for bovine ovaries (Picture was taken in INN laboratory).*

## 2.6 Sperm preparation and *in vitro* fertilization of bovine oocytes

Following completion of the maturation period for each time points (20, 25 and 30 hours), matured COCs were transferred to a 35-mm petri dish containing a 200 µL BO-IVF™ droplet using a 15 µl pipette. Then COCs were transferred in volume of 60 µl from the droplet to each well containing 400 µl of BO-IVF™ media and placed in the incubator (38.8 °C, 6% $CO_2$, 21% $O_2$) while the semen was prepared.

Regarding sperm preparation procedures (Figure 13), one frozen semen straw was thawed in a water bath (37 °C for 1 minute). Thawed semen was washed twice in a 15-ml falcon tube containing 2 ml of preheated BO-SemenPrep™ medium by centrifugation for 5 minutes at 330 x g. After the second wash, the supernatant was removed, leaving 250 µL of the sperm suspension for quality testing using CASA (Microptics, Barcelona, Spain). After evaluation of the sperm motility and concentration, the suspension was diluted in BO-SemenPrep™, and 2 x $10^6$ sperm cells/ml was transferred in a volume of 40µl to the IVF wells with matured oocytes. The wells were then incubated for 18 hours at 38.8 °C with 6% $CO_2$ in humidified atmospheric air.

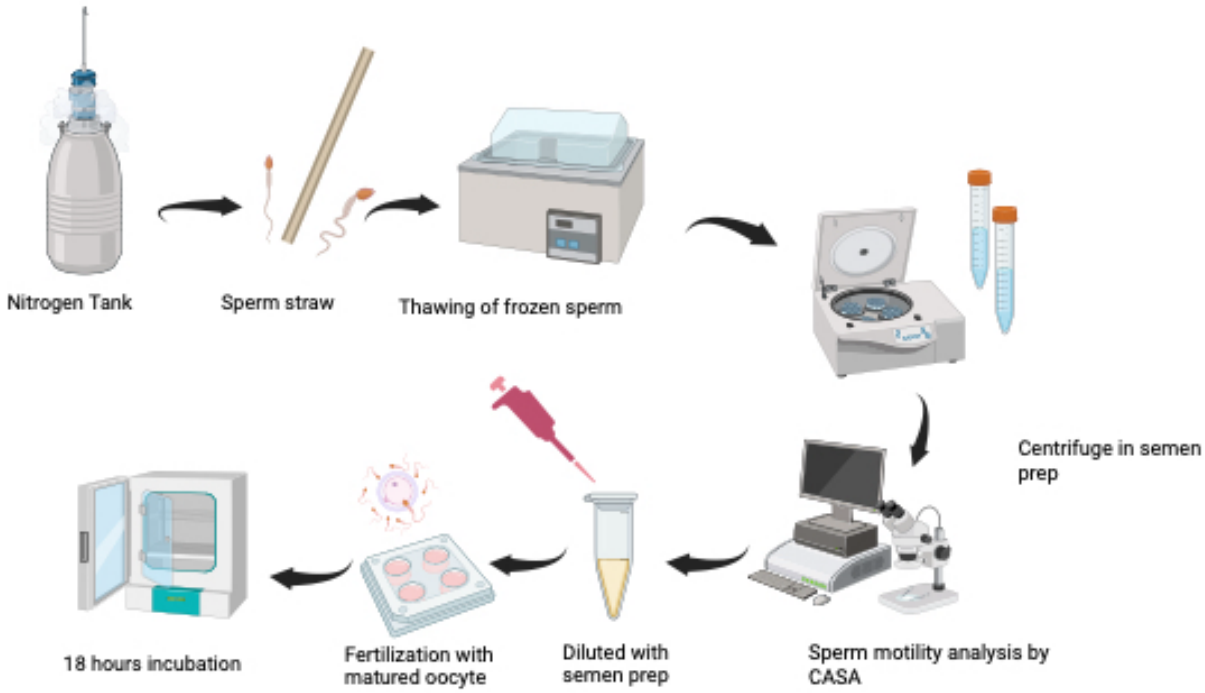


*Figure 13. Schematic illustration of the sperm preparation and IVF process (created by Biorender.com).*

## 2.7 *In vitro* culture of embryos

18 hours after fertilization, presumptive zygotes were retrieved from the IVF media and transferred to 2 ml of preheated wash medium (36 °C) for vortexing for 2 minutes to remove cumulus cells. Later, denuded zygotes were washed twice in a 35-mm petri dish containing preheated wash medium. Washed presumptive zygotes were rinsed in 200 µl of BO-IVC™ media droplet and then transferred to 500 µl of pre-equilibrated BO-IVC™ media, covered with 400 µl of Oil, and incubated for seven days at 38.8 °C in a humidified atmosphere of 7% $O_2$, 6% $CO_2$, and 87% $N_2$. The cleavage rates and blastocyst rates were evaluated 2 and 7 days after fertilization, respectively, using a Leica DM-IL inverted microscope. The rates were determined by dividing the number of cleaved embryos, or blastocysts, by the total number of cultured embryos (Figure 14).

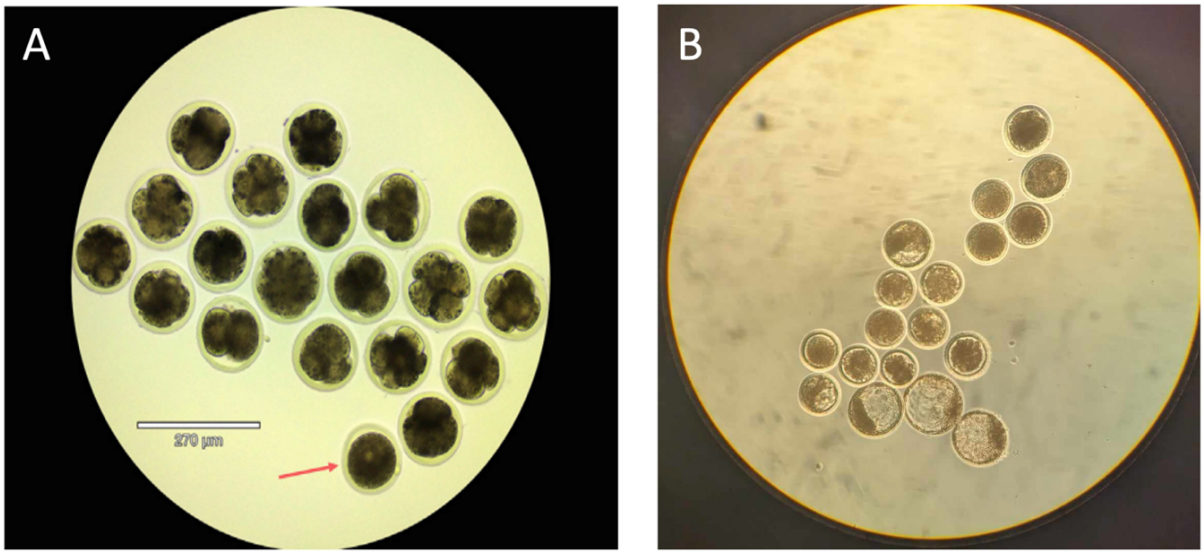


*Figure 14. A: Cleavage assessment of bovine embryos at different developmental stages on day 2 after fertilization. Unfertilized oocyte is marked with a red arrow. The scale bar represents 270 mm (Speckhart et al., 2023). B: Represent embryos in the blastocyst stage, 7 days after fertilization (picture taken at the INN laboratory).*

## 2.8 Analysis of ATP content of bovine and pig oocytes

The ATP content of bovine and porcine oocytes was measured at different maturation times using CellTiter-Glo® Luminescent Cell Viability Assay. Oocytes were denuded of the cumulus cells by vortexing (bovine oocytes for 2 minutes and porcine oocytes for 1 minute) in 2 ml of PXM wash media in a 15-ml Falcon tube. Denuded oocytes were then rinsed in a 35-mm petri dish containing 2 ml of PXM wash media and then allocated in different numbers and replicates (Table 1 B and C) in a 1.5 ml Eppendorf tube containing 50 µL of PBS. Subsequently, 50 µL of CellTiter-Glo® Reagent (Promega G7570) was added to each tube containing the oocytes. Then, the suspension in each tube was transferred to a Pierce™ 96-Well (White Opaque) Polystyrene Plates in 3 replicates and mixed using an orbital shaker (300 rpm) for 2 minutes to lyse the oocytes. Later, the luminescence signal was collected as relative luminescence units (RLU) using a Fluostar Optima plate reader (BMG Labtech, Germany) after 15 minutes of signal stabilization at room temperature. The ATP level in ng/µl was calculated from corresponding RLU values using a standard curve generated from a serial dilution of ATP disodium salt (Cat#P1132, Sigma) mixed with PBS (Table 1 A) and 50 µL of CellTiter-Glo®. All components were incubated at room temperature before utilization.

*Table 1.: A) Standard curve components. B) Replicates and total oocyte count for each time point of bovine oocytes. C) Replicates and total oocyte count for each time point of porcine oocytes.*

A

| Mix (dilution) | 1 | 3 | 5 | 7 | 9 | 10 |
|---|---|---|---|---|---|---|
| PBS (µl) | 100 | 96 | 92 | 80 | 20 | 0 |
| Stock solution (µl) | 0 | 4 | 8 | 20 | 80 | 100 |
| Final Conc.(nM.) | 0 | 40 | 80 | 200 | 800 | 1000 |

B

| Bovine oocyte maturation time points | 0 | 5 | 15 | 20 | 22 | 24 | 26 | 28 | 25 | 30 | 38 | 43 | 48 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oocytes per replicate | 2,3 | 1,2,3 | 2,3 | 1,2,3 | 1,2 | 1,2 | 1,2 | 2 | 2,3 | 1,2,3 | 2,3 | 1,2,3 | 1,2,3 |
| Replicates | 11 | 12 | 10 | 19 | 6 | 5 | 5 | 4 | 11 | 22 | 17 | 11 | 16 |
| Total oocyte number | 25 | 26 | 23 | 41 | 10 | 8 | 9 | 8 | 25 | 46 | 37 | 25 | 35 |

C

| Porcine oocyte maturation time points | 0 | 20 | 24 | 28 | 30 | 34 | 36 | 40 | 42 | 48 |
|---|---|---|---|---|---|---|---|---|---|---|
| Oocytes per replicate | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Replicates | 6 | 8 | 8 | 3 | 7 | 4 | 11 | 3 | 13 | 3 |
| Total oocyte number | 12 | 16 | 16 | 6 | 14 | 8 | 22 | 6 | 26 | 6 |

## 2.9 DNA and mitochondria staining of the oocytes

Cytoplasmic maturation of the oocytes was evaluated using mitochondria staining with MitoTracker orange™, and nuclear maturation was evaluated using DNA staining with Hoechst-33342 (an example shown in Figure 15), both before and after maturation at different time points (20, 24 and 30 hours). For each time point, 15-20 oocytes were denuded of the cumulus cells by vortexing for 2 minutes. For mitochondria staining, denuded oocytes were transferred to wells of a 4-well plate containing 500 µL of wash media with 200 nM MitoTracker orange (10 µl per 500 µl well) and incubated for 30 minutes at 38.5°C with 6% CO2. The working solution of 0.01 mM was prepared by diluting 1µM MitoTracker orange

in 99µl of dimethyl sulfoxide (DMSO) beforehand. After 30 minutes, oocytes were washed three times in 100 µl PXM solution and fixed in 50 µL drops of 4% paraformaldehyde covered with 3 ml of mineral oil for 30 minutes at room temperature in the dark. Fixed oocytes were washed again three times in PBS and then stained with 18 µg/ml Hoechst-33342 in a 50 µl PBS droplet for 10 minutes in the dark. Stained oocytes were mounted on a glass slide with 6 µL of mounting medium (Dako, Glostrup, Denmark), and a coverslip (18 mm square) was gently placed on the droplet and kept in the dark for drying. All the slides were analyzed using Leica SP8 laser confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Hoechst (H-33342, B2261, Sigma) staining was evaluated with a 420–480 nm excitation laser and a 410–480 nm emission filter. MitoTracker Orange was excited using a 532 nm laser and an emission peak at 575 nm.

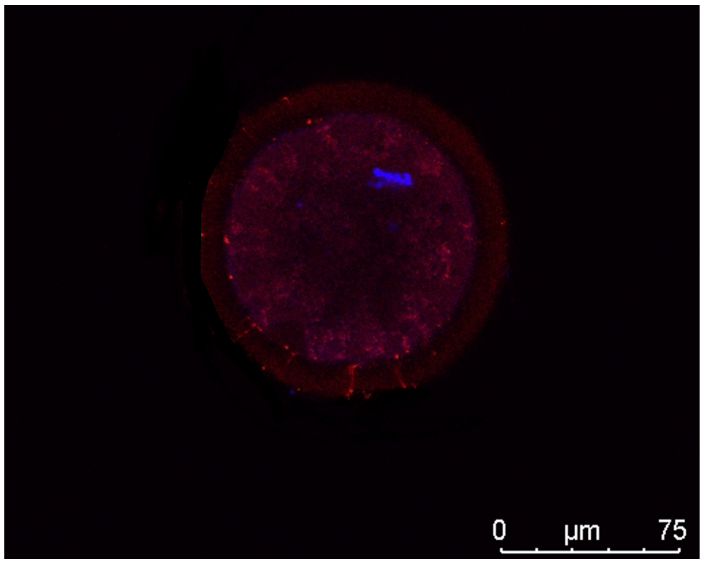


*Figure 15. Indication of mitochondria staining with MitoTracker Orange (red area) and DNA staining with Hoechst (blue) on a matured oocyte. The scale bar represents 75 µm.*

## 2.10 Statistical analysis

All the data in the current master thesis were analysed using one-way ANOVA, except for the data related to bovine embryo developmental rate (Figure 22), which was analysed with two-way ANOVA. Results are presented as the mean ± SEM. All the figures were plotted using GraphPad Prism v.10.0 (GraphPad Software, San Diego, USA).

# 3. Result

## 3.1 ATP content of bovine oocytes during maturation

The ATP level was measured in bovine oocytes at different maturation time points during incubation in IVM media (Figure 16) to determine the optimal time point for performing IVF. An upward trend in ATP content was observed from 0 hours to 22 hours of maturation time, increasing from 29.6 nM to 46 nM. However, after 22 hours, no significant changes were observed until the last measurement at 48 hours, and the ATP content remained relatively consistent at around 46–50 nM. Statistical analysis revealed significant differences among some of the groups ($p < 0.0001$). Details of these differences, along with corresponding p-values, are provided in Table A 1 in the Appendix.

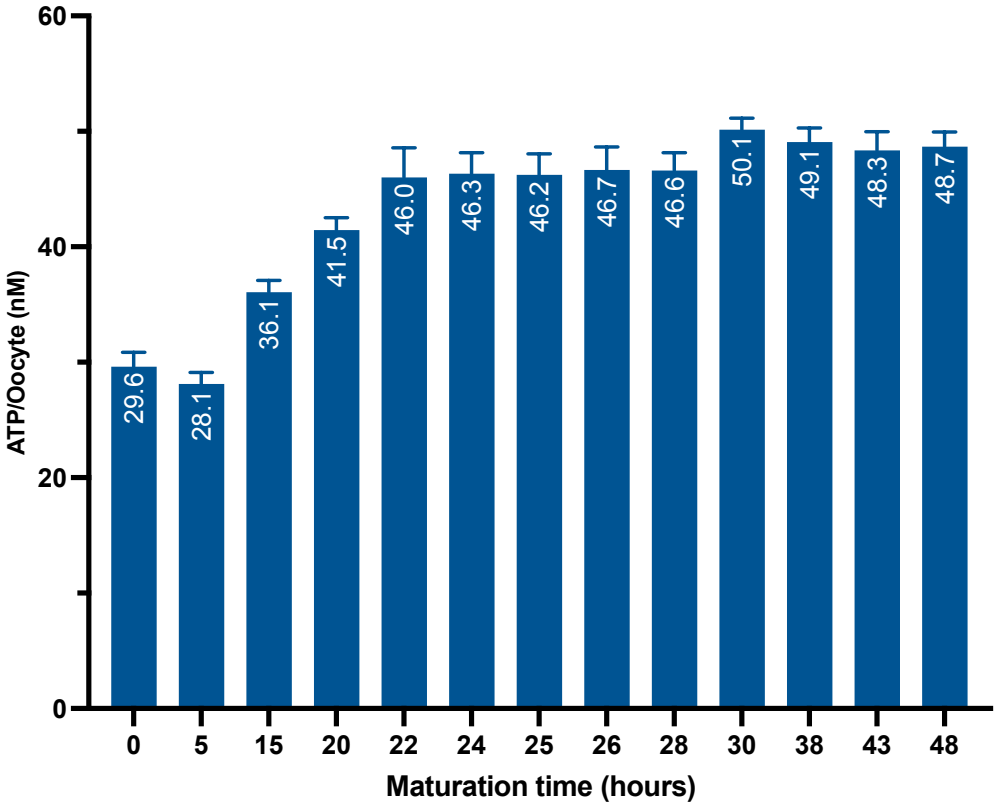


*Figure 16. Changes in ATP content (nM) per bovine oocytes (n = 318) during different maturation times (from 0 to 48 hours) in IVM medium. The data are presented as the mean ± SEM.*

## 3.2 ATP content of porcine oocytes in different maturation time points

The ATP levels of porcine oocytes at different maturation times in IVM media were evaluated (Figure 17) with the same objective as the study conducted on bovine. The results showed fluctuations in ATP content during the maturation of porcine oocytes. From zero to 20 hours, ATP levels remained relatively stable around 35–37 nM. However, after 20 hours, an upward trend was observed, reaching its peak at 24 hours with a concentration of 43.6 nM. Following this peak, there was a decrease in ATP levels, remaining consistently at a low level until 36 hours, which indicated another peak at almost 42 nM. After 36 hours, the same trend of drop and rise appeared again until the last measurement at 48 hours. Despite these fluctuations in ATP content at different maturation time points, statistical analysis did not reveal significant differences (P = 0.3300) among these maturation time points.

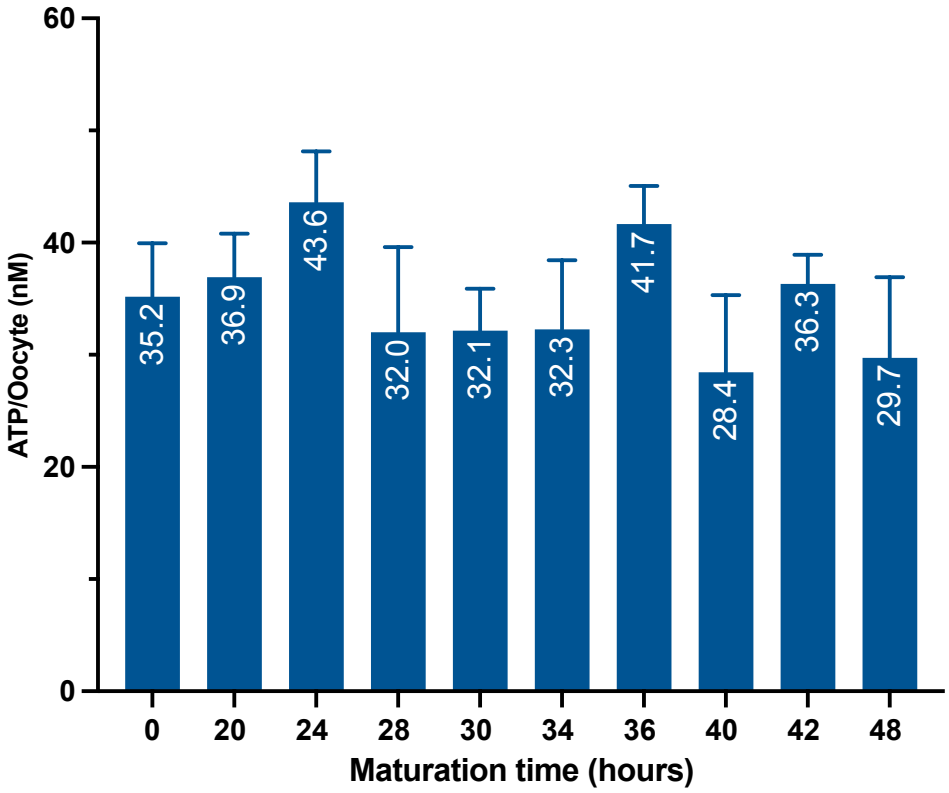


*Figure 17. Fluctuations in the ATP content (nM) per porcine oocytes (n = 132) during different maturation time points (from 0 to 48 hours). The data are presented as the mean ± SEM. There was no significant difference observed between all the time points.*

## 3.3 ATP content of collected bovine oocyte with aspiration and slashing methods

The ATP levels of bovine oocytes were assessed before and after maturation in IVM media using both aspiration and slashing methods to compare the quality of oocytes retrieved by these methods (Figure 18). Results indicated that the highest ATP level for both methods was observed after maturation. For the aspiration method, it was 4.1 nM, and for the slashing method, it was 39 nM. Interestingly, no significant differences were observed regarding the ATP level between the two methods ($p = 0.0024$).

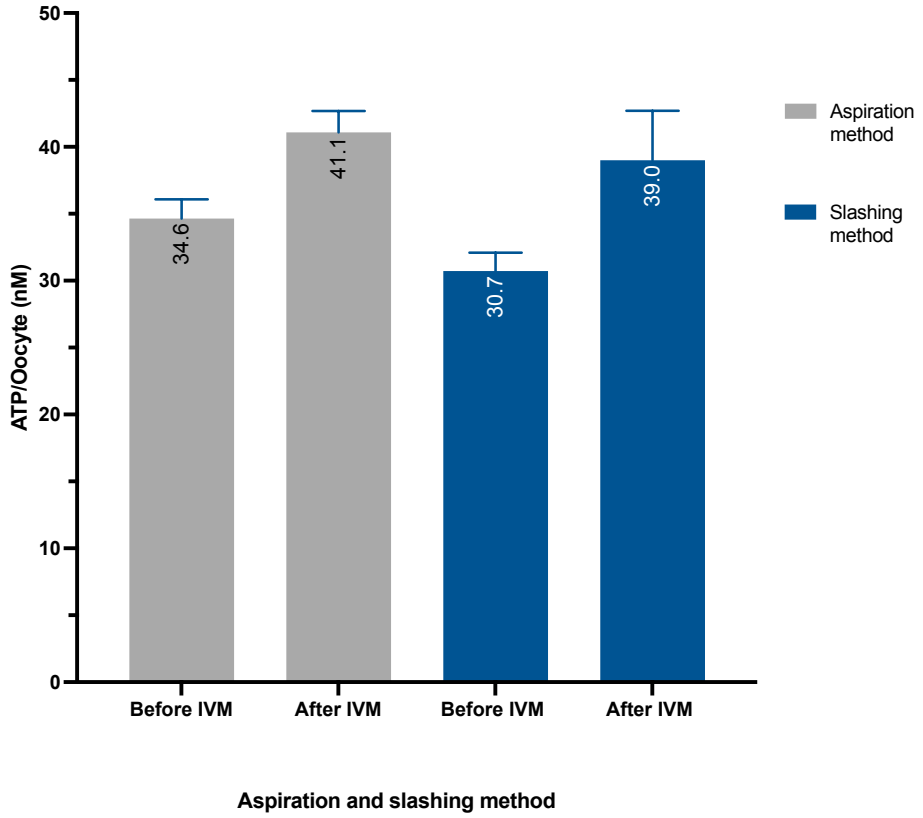


*Figure 18. Comparison of ATP levels per bovine oocyte (nM) using aspiration and slashing methods before and after maturation in IVM media. Different collection methods are represented by colors. The data are presented as the mean ± SEM.*

## 3.4 Bovine oocyte DNA and mitochondria staining

The nuclear maturation stage of bovine oocytes, including GVBD and MII stages was visualized using DNA Hoechst staining and confocal microscopy (Figure 19). In the immature oocyte at the GVBD stage, chromatin was visible in a diffused form (Figure 19 A). The DNA became condensed in the MII stage, and the metaphase plate and polar body were also visible, indicating maturation (Figure 19 B). In addition, mitochondria distribution patterns were observed using MitoTracker oranges at different time points during maturation (Figure 20). In immature oocytes in the GV stage, a higher mitochondrial volume in the peripheral region with a relatively uniform distribution was observed (Figure 20 A). As oocytes progressed to the GVBD stage (Figure 20 B), the peripheral distribution of mitochondria remained consistent. In matured oocytes at the MII stage, mitochondria were distributed in a cluster pattern toward the inner part of the cytoplasm (Figure 20 C). In addition, the effects of prolongation of the maturation times in IVM media on mitochondrial distribution were evaluated. The mitochondria distribution pattern was visualized for oocytes, which incubated for 20 hours (Figure 20 D), 24 hours (Figure 20 E), and 30 hours (Figure 20 F) in the IVM media. At the 20-hour maturation time (Figure 20 D), the mitochondria distribution pattern was similar to that of matured oocytes in Figure 20 C with cluster pattern, while in 24 and 30 hours, their distribution pattern had become more scattered.

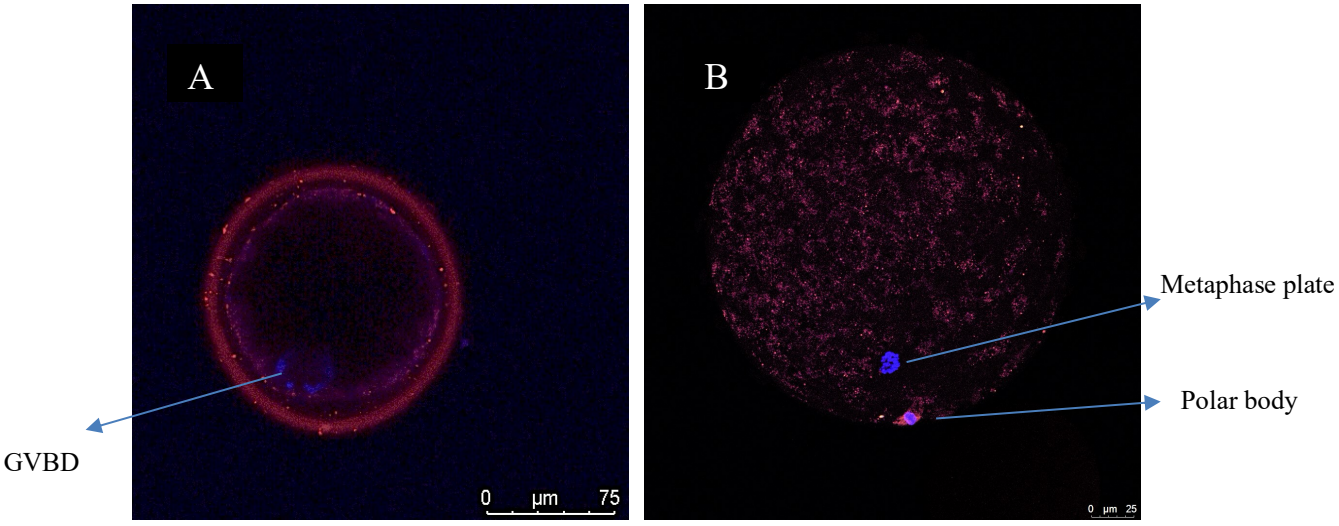


*Figure 19. Illustration of DNA Hoechst staining. A. Immature oocyte at the GVBD stage. The scale bar represents 75 µm, and the picture was captured from the middle section of the oocyte. B. Matured oocyte with extruded polar body and metaphase plate. The scale bar represents 25 µm. The image was captured from the top view of the oocyte.*

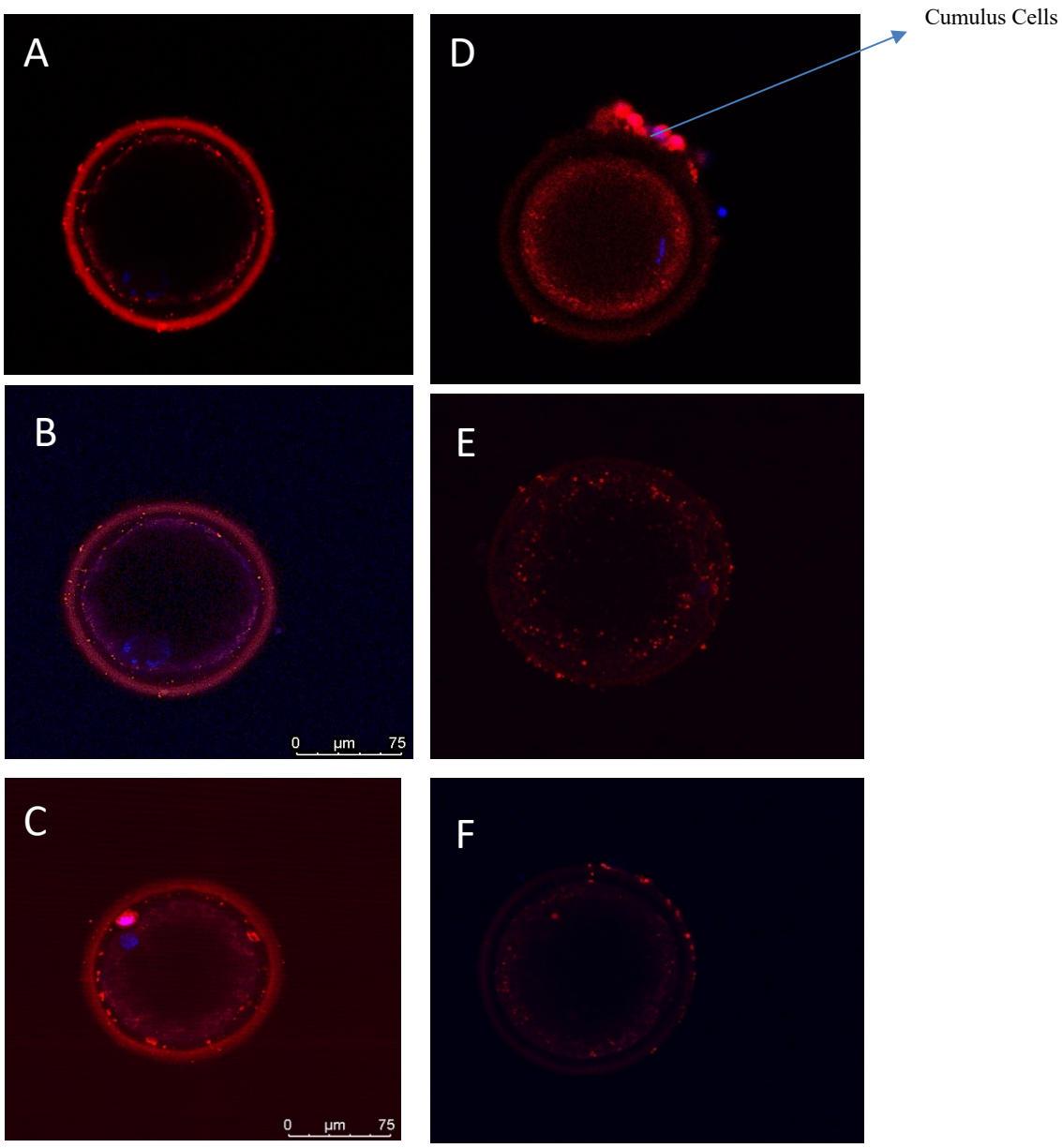


*Figure 20. Mitochondrial distribution patterns in bovine oocytes at different maturation stages. A: Immature oocyte at GV stage; B: Immature oocyte at GVBD stage; C: Mature oocyte at MII stage; D: Oocyte matured for 20 hours; E: Oocyte matured for 24 hours; F: Oocyte matured for 30 hours. All images were captured from the middle section of the oocyte. The scale bar represents 75 µm for all images.*

## 3.5 Cleavage rate of bovine embryo in different maturation time points

The percentage of cleaved embryos was assessed (Figure 21) two days after fertilization to investigate the effect of different maturation time points (20, 25, and 30 hours) in IVM media on cleavage rate. The data revealed that the percentage of cleaved embryos at 20 and 25 hours was nearly the same, around 86 percent. This percentage slightly decreased for the 30-hour time point, representing the lowest rate among all the time points. However, statistical analysis indicated no significant differences in the cleavage rate among the three time points (P = 0.0625).

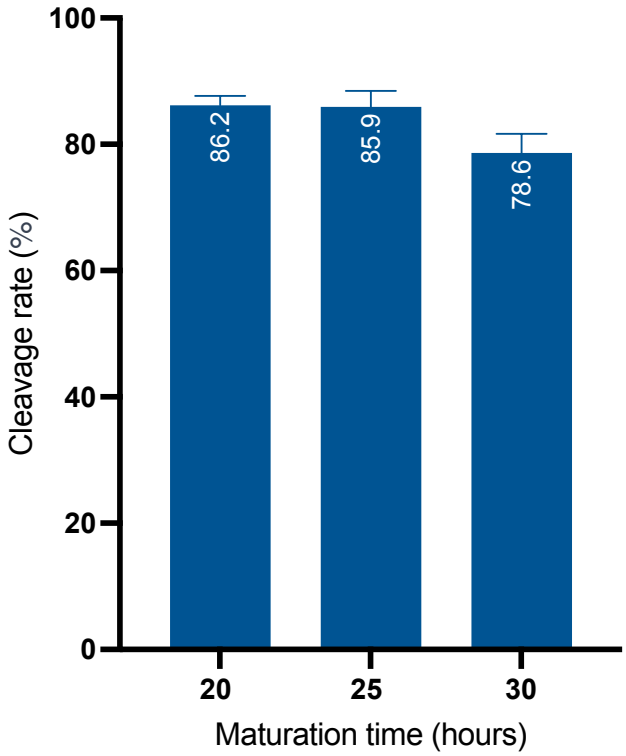


*Figure 21. The cleavage rate of embryos matured at different time points (20, 25, and 30 hours) after in vitro fertilization. The cleavage rate was calculated by dividing the number of cleaved embryos by the total number of cultured embryos, with the lowest rate observed at 30 hours. The data are presented as the mean ± SEM. Statistical analysis revealed no significant difference in cleavage rate between all maturation time points (p = 0.0625)*

## 3.6 Bovine embryo developmental rate

The embryo progression rate at different developmental stages was recorded two days after fertilization (Figure 22) for embryos that matured at different time points (20, 25, and 30 hours). The highest percentage of embryos reaching the four-cell plus stage was observed at the 20-hour maturation time point, at approximately 50 percent, while this stage showed its lowest rate at the 30-hour time point. Statistical analysis did not reveal significant differences in the percentages of embryos at the one-cell and two-cell stages between all the time points. However, significant differences were observed in the percentage of embryos at the four-cell stage between the 20- and 30-hour maturation groups ($p = 0.0001$). Additionally, significant differences were observed in the percentage of embryos at the four-cell plus stage between 20 and 30 hours ($p < 0.0001$), as well as between 25 and 30 hours ($p < 0.0001$).

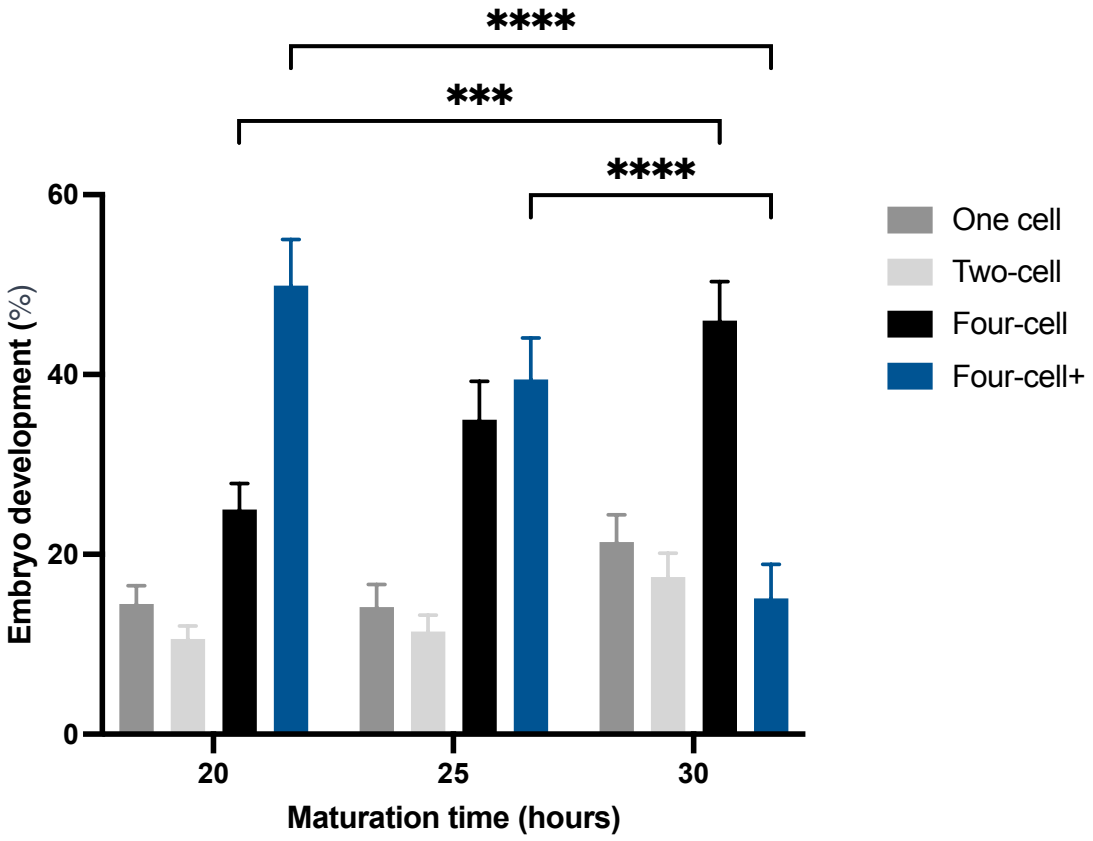


*Figure 22. Comparison of embryo progression to different cell stages at 20, 25, and 30 hours of maturation time. At each maturation time point, each column represents the one-cell, two-cell, four-cell, and four-cell plus stages, respectively. **** = $p < 0.0001$, *** $p = 0.0001$. The data are presented as the mean ± SEM.*

## 3.7 Blastocyst rate of bovine embryos in different maturation time

A comparison of blastocyst rates between different maturation time points was conducted to investigate the effect of prolonging the IVM maturation time on the IVF outcome (Figure 23). As shown in the figure, the highest blastocyst rate, approximately 42 percent, was observed at 20 hours, while the 30-hour maturation time showed the lowest rate among all time points. Statistical analysis revealed significant differences in blastocyst rates between 20 and 30 hours ($p < 0.0001$) and between 25 and 30 hours ($p = 0.0034$) of maturation time. No significant differences were observed between blastocyst rates at 20 and 25 hours of maturation time.

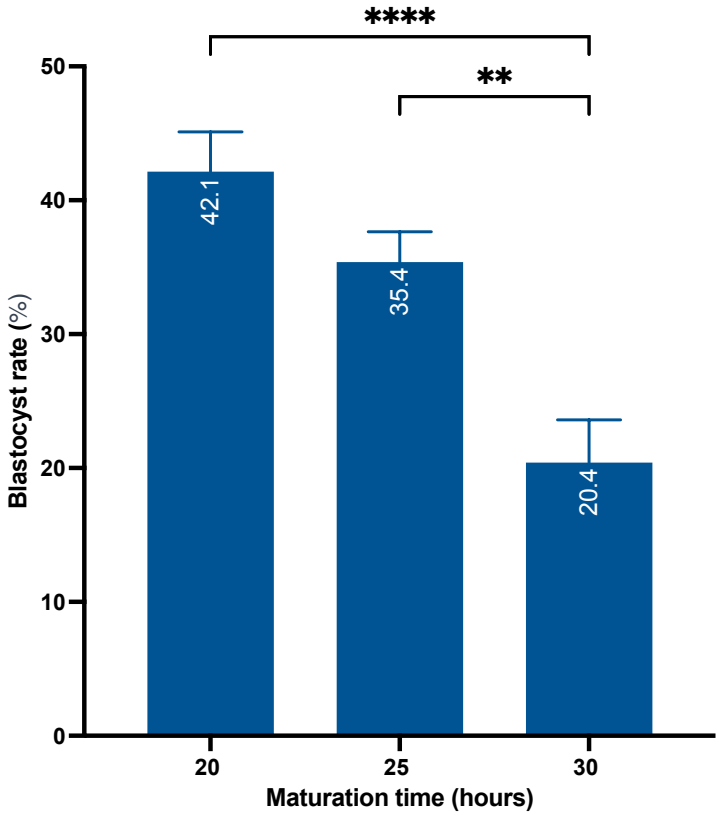


*Figure 23. The percentage of the bovine embryos reaching the blastocyst stage was assessed seven days after fertilization at different maturation time points (20, 25, and 30 hours). The highest rate was at 20 hours, and the lowest percentage of blastocyst was at the 30-hour maturation time point. The data are presented as the mean ± SEM. *** P<0.0001; ** P=0.0034.*

# 4. Discussion

Although numerous elements influence the success of IVF, oocyte quality has been suggested to be the most crucial and challenging aspect of this process (Sirard, 2019). This study aimed to identify critical parameters indicative of oocyte quality and factors influencing the success of IVF outcomes and subsequent embryonic progression. Previous studies revealed that the oocyte quality and maturation could significantly impact both the fertilization process and the success of embryonic production. Apart from this, oocyte characteristics can have a long-lasting effect on the subsequent growth and development of the fetus (Keefe et al., 2015). In addition to the factors affecting oocyte quality, the impact of different maturation times in IVM media on blastocyst rate was evaluated in the current study. As reported by Koyama et al. (2014) determining the ideal timing for fertilizing *in vitro*-matured bovine oocytes could enhance IVP outcome.

## 4.1 Mitochondria as a energy supplier during maturation

The research conducted by Nagai et al. (2006) revealed the essential role of an adequate quantity of mitochondria not only in cytoplasmic maturation but also in fertilization and embryonic development. In our study, the qualitative results of mitochondria staining of bovine oocytes at different cell stages revealed a significant difference in mitochondria distribution pattern between immature oocytes in the GV and GVBD stages compared to mature oocytes at the MII stage. Before maturation, in the GV and GVBD stages, mitochondria were distributed in the peripheral region of the cytoplasm. However, after maturation, particularly at the MII stage, they migrated to the inner side of the cytoplasm and formed clusters, especially around the nucleus. Our observation aligns with the findings of Wang et al. (2009), who reported that the accumulation of mitochondria around the nucleus is the oocyte maturation signature. In addition, Sun et al. (2001) reported that the same reallocation of the mitochondria pattern was observed in pig oocytes during maturation. Krisher (2004) hypothesized that the probable reason for this redistribution in mitochondrial patterns could be the oocyte's increased requirement for ATP and calcium as energy sources during cytoplasmic maturation, given that mitochondria serve as the primary source for ATP production.

In another section of this study, our observations at different maturation time points did not indicate significant differences in terms of mitochondria distribution between 20, 24, and 30-hour maturation group. However, as depicted in Figure 20, at the 30-hour maturation time point, the aggregation of mitochondria was lower compared to the patterns observed at 20 and 24 hours. This observation could possibly be correlated with our findings on blastocyst rates (as will be discussed later), suggesting a potential correlation between mitochondrial accumulation and the success of IVF. This aligns with the research by Nagai et al. (2006), which confirmed that a spread or fragmented distribution of mitochondria can disrupt the mitochondrial network, thereby hindering their role as suppliers of ATP required for cell development and thereby potentially reducing embryo developmental competence.

## 4.2 Dynamics of ATP production during maturation

As previously discussed, mitochondria play a crucial role in ATP production, which is vital for various cellular functions, including motility, maintaining cellular homeostasis, and regulating cell survival (St John, 2002). This study assessed ATP levels in bovine and pig oocytes at different maturation times. The data regarding the bovine oocytes demonstrated a significant increase in ATP levels during the maturation process, particularly when the oocyte reached the MII stage. This finding aligns with the study conducted by Kirillova et al. (2021), which observed a rise in ATP content during bovine oocyte maturation in oogenesis, likely attributable to the increased cellular demand for energy required in processes such as transcription and translation. In contrast, a study by Nazmara et al. (2014) on mouse oocytes did not reveal significant alteration regarding the ATP level between the GV stage before and the MII stage after maturation. These differences in ATP level patterns might be due to differences between species.

The data on ATP levels during pig oocyte maturation indicated fluctuations at different time points. As observed, there was a decrease after 24 hours, followed by a peak around 36 hours of maturation in terms of ATP levels. This fluctuation could be attributed to the fact that the pig oocyte was in the GVBD stage around 24 hours and in the metaphase plate stage, undergoing polar body extrusion, around 36 to 42 hours. This aligns with findings by Dalton et al. (2014), suggesting alterations in ATP levels during different maturation states, possibly due to varying ATP consumption in different stages. ATP content was notably higher in MI and MII stages compared to the GV stage, indicating increased energy demand during MII

stage (Dalton et al., 2014). Consequently, this fluctuation in ATP levels during different maturation stages might be influenced by the energy requirements. Despite the hypothesis that ATP levels could serve as a marker for oocyte health and maturation capability, the data did not support this hypothesis (Dalton et al., 2014). Our results might have been influenced by the porcine ovaries received, both from gilts and sows, due to their availability at the slaughterhouse. The variation in the ATP content of porcine oocytes might suggest that more repetitions are needed to confirm our findings.

## 4.3 Oocyte againg and embryonic geneme activation

The data on cleaved bovine embryos two days after fertilization indicated no significant differences in cleavage rates among the 20-, 25-, and 30-hour maturation groups. Therefore, this result alone might not reliably predict IVF success. However, data from other studies were in contrast with our findings, suggesting that oocytes with the ability to cleave early after fertilization could exhibit a higher blastocyst rate (Fenwick et al., 2002; Lundin et al., 2001). Early zygotic cleavage is claimed not only to be considered a marker for embryonic progression but also an indicator for selecting high-quality embryos (Lechniak et al., 2008).

From a genetic perspective, a study by Zhang et al. (2010) showed that embryos classified based on maturation time had a higher percentage of chromosomal anomalies when derived from maturation times up to 48 hours after collection compared to those maturing *in vivo* and within 24 hours after collection (Zhang et al., 2010). This finding may provide a possible explanation for our observation, elucidating why embryos at the 30-hour maturation time point exhibited lower cleavage and blastocyst rates compared to other time points.

As our data revealed, the percentage of the four-cell plus stage at 30-hour maturation groups was almost 15 percent, which was significantly lower than the other time point. This could indicate that the embryos showed lower competence for progression in the higher cell stage at these time points. Several factors can disrupt embryonic development in cattle and lead to an arrest in the embryo cleavage process. Many embryos are arrested during the transition from the fourth to the fifth cell stage (Memili & First, 2000). For instance, in the bovine embryo, developmental arrest occurs at the eight-cell stage and is probably linked to the cytoplasmic quality of the oocyte. Although oocytes contain all the necessary mRNAs and proteins for reaching the fourth or fifth cell cycle, those embryos unable to transcribe their own genome might be hindered from further development (Meirelles et al., 2004).

In addition, the study by Grisart et al. (1994) suggested two different developmental arrests in bovine embryos, referred to as the early lag phase, occurring between the 4 to 7-cell stage, and the late lag phase, appearing at the 8-cell stage. This research indicated that embryos arrested in the late lag phase showed a higher likelihood of reaching the blastocyst stage compared to those arrested in the early phase. The suggested reason for this correlation might be the abundance and quality of RNA and proteins accumulated in the oocyte or other factors associated with EGA. This finding aligns with our study, which revealed that although the 30-hour maturation group showed a higher four-cell stage compared to other groups, this group indicated the lowest blastocyst rate among the three maturation groups. In contrast, the 20-hour maturation group, which indicated the highest four-cell plus stage (potentially arrested in the late lag phase), also showed the highest blastocyst rate.

According to Rispoli et al. (2011) and Somfai et al. (2011), bovine oocytes are considered aged or slightly aged approximately 30 hours after the initiation of IVM. Consequently, it is hypothesized that the decreased percentages of cleavage and blastocyst development stem from the lack of essential EGA processes in aged oocytes (Koyama et al., 2014). Thus, the lower percentage of embryos at the four-cell plus stage after 30 hours of maturation in our study might be the reason for this developmental failure. Furthermore, Luo et al. (2023) mentioned that aneuploidy, an abnormal chromosomal division, is another possible age-related issue that decreases the success rate of fertilization. This finding may provide a possible explanation for our observation, elucidating why embryos at the 30-hour maturation time point exhibited lower cleavage and blastocyst rates compared to other time points.

## 4.4 Other possible factors that might reduce the blastocyst yield

The data regarding the blastocyst rate showed that, although the ATP content remained high in the 30-hour maturation group, the blastocyst rate was significantly lower than at other time points. Previous studies have emphasized the importance of ATP levels in IVF, suggesting that low ATP content may be correlated with fertilization failure (Zhao & Li, 2012). However, our study suggests that, in addition to high ATP levels, other factors should be considered as well. This section will discuss some of these factors that might affect the blastocyst rate, such as the optimal time for the IVF process, the presence of elevated levels of ROS in IVM media,

the overexpansion of cumulus cells due to the prolonged *in vitro* maturation and the oocyte aging process which was previously discussed.

As mentioned previously, suboptimal timing for oocyte fertilization could contribute to the diminished developmental competence of *in vitro*-matured bovine oocytes (Koyama et al., 2014). In addition, a study by Miao et al. (2009) suggests that oocytes not fertilized in the optimal fertilization time might undergo gradual degradation and programmed cell death. Our data suggest that the 20-hour maturation time showed the highest blastocyst rate. Therefore, it could be considered an optimal time for maturation and subsequent fertilization. This finding aligns with the data reported by Koyama et al. (2014), which reported that the ideal time for the *in vitro* maturation process is around 21 hours.

Maturation media are enriched with antioxidants like GSH (Deleuze & Goudet, 2010), melatonin (Remião et al., 2016), and β-mercaptoethanol (Choe et al., 2010) to inhibit oxidative stress in oocytes. However, there is a potential concern that after 25 hours of maturation, the oocyte culture media might be deficient in these antioxidants. Consequently, the unnaturalized production of reactive oxygen species could lead to an elevated level of ROS, resulting in oxidative stress, which is one of the interfering factors in the developmental competence of the oocyte (S. Soto-Heras & M. T. Paramio, 2020). Previous research by S. Soto-Heras and M. T. Paramio (2020) has demonstrated that oocyte oxidative stress acts as a disruptive factor in both IVM and IVF processes, ultimately compromising blastocyst development during *in vitro* embryo culture.

The study by Tatemoto et al. (2000) proposed that CCs play a crucial role in protecting oocytes from apoptosis induced by oxidative stress. This protection is achieved by increasing the glutathione content in oocytes throughout the IVM process (Tatemoto et al., 2000). In addition, CCs might facilitate sperm penetration during IVF, indicating a potential direct impact on sperm capacitation and acrosome reaction (Chian et al., 1995). Therefore, excessive expansion of the cumulus cells after a prolonged incubation time in the IVM media is another rationale for the decrease in blastocyst rate in the 30-hour group, as their cumulus might have been overexpanded after this maturation time.

Our data on bovine blastocyst rates indicate the adverse effect of prolonged maturation time on IVF outcomes. To justify this, further research is needed in another area, such as

metabolomics analysis of the media to investigate changes in the media environment, as well as measuring the level of ROS in the media across different maturation groups.

## 4.5 Oocyte collection with aspiration and slashing method

Our results regarding the ATP content of bovine oocytes, collected using the slashing method, showed no significant difference in ATP content compared to the aspiration method. The rationale for this experiment was to explore the possibility of using the slashing method for collecting more and younger oocytes for research purposes. Typically, the slashing and cutting method is suitable for mouse ovaries due to their small size, making it challenging to extract all the oocytes. Although the data regarding the aspiration and slashing methods revealed no remarkable differences in ATP, a slight difference exists. This difference may be due to the oocytes collected from the slashing method being extracted from the inner side of the ovaries. Therefore, the possible reason for the slight difference in ATP levels with the slashing method is that these oocytes are immature, leading to lower ATP levels. As demonstrated by Saleh (2017), in the aspiration method, more than 80 percent of the collected oocytes had good quality, while in the slashing method, more than 55 percent of the oocytes showed good quality. In this article, it is mentioned that an oocyte with good quality possesses layers of cumulus with uniform cytoplasm (Rahman et al., 2008). Further studies should be performed on oocytes retrieved using the slashing method to determine if the blastocyst rate of these small, young oocytes is lower than that of oocytes aspirated, given the slight difference in ATP levels observed in immature oocytes collected using the slashing method.

# 5. Conclusion

In conclusion, it was found that the ATP content of bovine oocytes increased as they matured and stabilized afterward. However, for porcine oocytes, no clear trend was observed during maturation, and the experiment might need to be repeated due to high variations. A reorganization of mitochondria after bovine oocyte maturation was noted, indicating an increased demand for ATP produced by mitochondria during various maturation steps. Although high ATP content was observed after 30 hours of maturation, it did not correspond to successful IVF outcomes. The study suggests that there might be an optimal time for performing IVF, with data revealing that after 30 hours of maturation in IVM media, oocytes might undergo the aging process, leading to a significant decrease in blastocyst rate compared to those matured for 20 hours, which showed the highest blastocyst outcome. This indicates that prolonged *in vitro* maturation negatively affects IVF success. Additionally, our data on ATP content of oocytes retrieved using both slashing and aspiration methods showed the potential of the slashing method for retrieving more and younger oocytes, suggesting an alternative collection method.

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# 7. Appendix

## 7.1 Appendix A: Bovine oocyte ATP content differences

*Table A 1: Significant differences between bovine oocyte ATP content in different maturation time points.*

| Tukey's multiple comparisons test | mean diff. | 95.00% ci of diff. | below threshold? | summary | adjusted p value |
|---|---|---|---|---|---|
| **0 VS. 20** | -11.85 | -17.91 to -5.789 | Yes | **** | <0.0001 |
| **0 VS. 22** | -16.41 | -24.53 to -8.292 | Yes | **** | <0.0001 |
| **0 VS. 24** | -16.72 | -25.34 to -8.089 | Yes | **** | <0.0001 |
| **0 VS. 25** | -16.61 | -23.43 to -9.794 | Yes | **** | <0.0001 |
| **0 VS. 26** | -17.05 | -25.68 to -8.427 | Yes | **** | <0.0001 |
| **0 VS. 28** | -17 | -26.34 to -7.660 | Yes | **** | <0.0001 |
| **0 VS. 30** | -20.54 | -26.44 to -14.63 | Yes | **** | <0.0001 |
| **0 VS. 38** | -19.48 | -25.67 to -13.29 | Yes | **** | <0.0001 |
| **0 VS. 43** | -18.74 | -25.56 to -11.92 | Yes | **** | <0.0001 |
| **0 VS. 48** | -19.06 | -25.32 to -12.79 | Yes | **** | <0.0001 |
| **5 VS. 15** | -7.955 | -14.80 to -1.106 | Yes | ** | 0.0088 |
| **5 VS. 20** | -13.33 | -19.23 to -7.436 | Yes | **** | <0.0001 |
| **5 VS. 22** | -17.89 | -25.89 to -9.896 | Yes | **** | <0.0001 |
| **5 VS. 24** | -18.2 | -26.71 to -9.687 | Yes | **** | <0.0001 |
| **5 VS. 25** | -18.1 | -24.77 to -11.42 | Yes | **** | <0.0001 |
| **5 VS. 26** | -18.54 | -27.05 to -10.02 | Yes | **** | <0.0001 |
| **5 VS. 28** | -18.48 | -27.72 to -9.249 | Yes | **** | <0.0001 |
| **5 VS. 30** | -22.02 | -27.76 to -16.28 | Yes | **** | <0.0001 |
| **5 VS. 38** | -20.96 | -26.99 to -14.93 | Yes | **** | <0.0001 |
| **5 VS. 43** | -20.23 | -26.90 to -13.55 | Yes | **** | <0.0001 |
| **5 VS. 48** | -20.54 | -26.65 to -14.43 | Yes | **** | <0.0001 |
| **15 VS. 22** | -9.939 | -18.20 to -1.679 | Yes | ** | 0.0053 |
| **15 VS. 24** | -10.25 | -19.01 to -1.485 | Yes | ** | 0.008 |
| **15 VS. 25** | -10.14 | -17.13 to -3.155 | Yes | *** | 0.0002 |
| **15 VS. 26** | -10.58 | -19.34 to -1.823 | Yes | ** | 0.005 |
| **15 VS. 28** | -10.53 | -19.99 to -1.066 | Yes | * | 0.0153 |
| **15 VS. 30** | -14.07 | -20.17 to -7.968 | Yes | **** | <0.0001 |
| **15 VS. 38** | -13.01 | -19.38 to -6.633 | Yes | **** | <0.0001 |
| **15 VS. 43** | -12.27 | -19.26 to -5.282 | Yes | **** | <0.0001 |
| **15 VS. 48** | -12.59 | -19.03 to -6.140 | Yes | **** | <0.0001 |
| **20 VS. 30** | -8.689 | -13.70 to -3.680 | Yes | **** | <0.0001 |
| **20 VS. 38** | -7.629 | -12.97 to -2.289 | Yes | *** | 0.0003 |
| **20 VS. 43** | -6.892 | -12.95 to -0.8323 | Yes | * | 0.0116 |
| **20 VS. 48** | -7.209 | -12.64 to -1.782 | Yes | ** | 0.0011 |