Review Article

In-vitro Maturation of Bovine Oocytes and Recent Innovations by Harnessing the Power of Diverse Antioxidants

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Abstract

In-vitro maturation (IVM) refers to the process of maturing oocytes outside the body and is a crucial step in *in-vitro* embryo production (IVEP). Unfortunately, the pregnancy rates achieved with IVM oocytes are lower than those with oocytes matured in vivo. This discrepancy has hindered the adoption of IVM technology in assisted reproductive technology (ART) laboratories. Several factors contribute to the generally poor quality of *in-vitro*-matured oocytes, with oxidative stress (OS) being a significant concern. Antioxidants can play a crucial role in treating and preventing oxidative stress. This review aims to explore the various factors that influence IVM of oocytes and to discuss both the elements that enhance oocyte viability and those that may have harmful effects on the oocytes. Additionally, it will present the findings from scientific research conducted over the past few years, focusing on the effects of different antioxidants on bovine oocyte maturation.

Keywords: Antioxidants, Bovine oocytes, *In-vitro* maturation, Maturation related genes, Oxidative stress

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1. Introduction

Laboratory production of embryos from farm animals is still the most widely used protocol for genetic improvement. *For* this purpose, immature oocytes collected from ovaries undergo a process of *in-vitro* maturation (IVM) to be ready for fertilization and embryo production. Oocytes must have attained a crucial size to develop meiotic competence for IVM to be effective. The IVM system must be able to sustain oocytes' nuclear maturation and cytoplasmic differentiation in addition to growth. It is believed that 120 mm is the necessary size for achieving complete developmental capability **(Telfer et al., 2020).** The accumulation of proteins, mRNA and other materials required for development and fertilization is an indicator of cytoplasmic maturation **(Mao et al., 2014)**.

Reaching the metaphase II stage (MII) and continuing meiosis are indicated by oocyte nuclear maturation. In this procedure, the oocytes of mammals divide twice **(Sun and Kim 2013)**. When the oocyte reaches the diplotene stage, which contains a large nucleus known as the germinal vesicle (GV), it is first halted. A process known as germinal vesicle breakdown (GVBD) results in nuclear membrane disintegration and chromatin condensation **(Gosden and Lee 2010)**. After GVBD, oocytes resume meiosis and enter metaphase I (MI) **(Almonacid et al., 2019)**. When the first polar body is extruded and a haploid egg is produced, the first meiosis is completed. The first one is kept at MII till the time of fertilization **(Sun et al., 2011)**. However, inaccuracies in meiotic events can hinder oocytes from achieving normal maturity **(Zeng et al., 2018)** (Figure 1).

2. Factors Affecting Oocyte Maturation

A few of the several factors that influence oocyte maturation include the oocyte's quality, the media components used for maturation, and culture conditions **(Gatimel et al., 2020)**.

2.1. Oocyte Quality

Numerous internal and external variables can impact the quality of cumulus oocyte complexes (COCs). Reproductive state, nutritional and

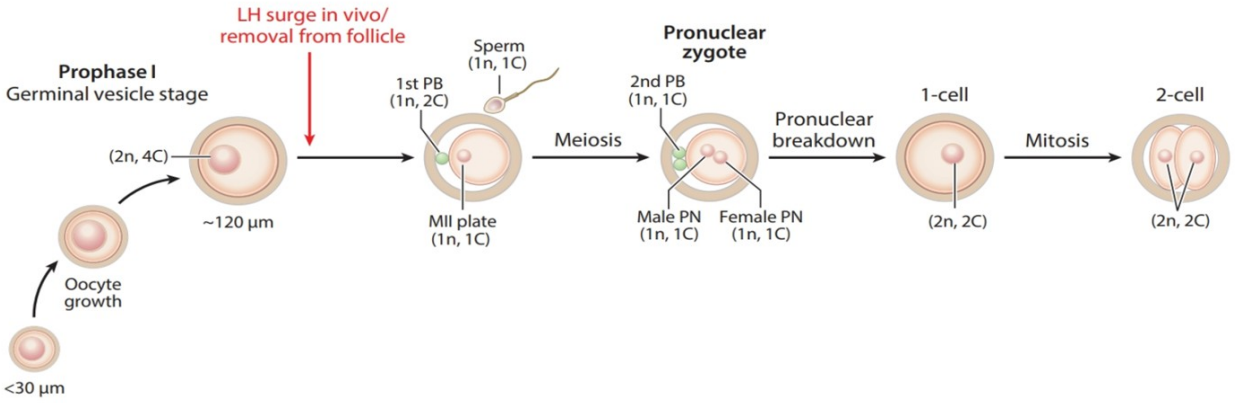

Figure 1: Prophase I arrest (GV stage), preovulatory LH surge and further arrest at the metaphase II **(Lonergan and fair, 2016).**

metabolic health, hormone levels, age, breed and oestrous cycle stage are examples of internal influences **(Moussa et al., 2015)**. However, important external influences include the interval between ovarian oocyte retrieval and slaughter, the morphology and methods of collecting COCs, the ovaries' storage temperature, the collection medium, and the operator's skill in micromanipulation **(Tello et al., 2020)**.

The success of IVM, fertilization, and embryo development is all impacted by the quality of the oocyte **(Liu et al., 2021)**. Cumulus oocyte complex morphology is a commonly used criterion for selecting and classifying bovine oocytes, as well as oocyte cytoplasmic characteristics like as texture or brightness. There are three different classifications for the quality of COCs: Class I is the healthiest, with compacted cell layers and a complete cumulus cover; Class II is the medium-quality, with a partial cumulus cover and/or slightly expanded cumulus that contains fewer than five cell layers; and Class III is the worst, with more intense cytoplasm, spots of darkness, and expanded cumulus, indicating follicular atresia (Figure 2) **(Aguila et al., 2020)**. Cumulus cells (CCs) are necessary for oocytes to mature *in-vitro*. Cumulus cells contribute to energy generation in the COC **(Lewis et al., 2020)** and protect oocytes from reactive oxygen species (ROS) damage **(von Mengden et al., 2020)**. For the categorization of medical images, a convolutional neural network (CNN) is a very useful tool. This methodology is used for categorizing pictures from different medical imaging modalities since it can complete classification jobs more quickly and accurately **(Cavusoglu et al., 2023)**.

According to **Emanuelli et al. (2019)**, COCs with stretched cumulus and partial cumulus (less than five cell layers) demonstrated reduced competence and greater levels of DNA fragmentation following IVM in comparison to healthy ones. He also concluded that these variations resulted from improved nuclear maturation brought about by COCs with complete cumulus covering maintaining MII block more effectively. It is best to pick COCs with many cumulus cell layers, compact and/or slightly enlarged, and with or without dark patches in the cumulus and oocyte **(Aguila et al., 2020)**.

2.2. *In-vitro* Maturation Medium Components

For the IVM of bovine oocytes, tissue culture medium-199 (TCM-199) is often utilized. It promotes high rates of nuclear maturation and has all 20 essential and non-essential amino acids. Conversely, bovine oocytes' nuclear maturation was not enhanced by a chemically defined protein-free medium that contained both essential and non-essential amino acids **(Bahrami et al., 2019)**.

Because follicle-stimulating hormone (FSH) is important for attracting follicles in vivo, it is frequently added to the maturation medium **(Gervásio et al., 2014).** FSH must be supplemented at levels that allow a cohort of oocytes to resume meiosis without resulting in chromosomal abnormalities **(Bahrami and Pauline, 2022).** Additionally, a complex combination of amino acids is added to the maturation medium; they function as an energy source to support nuclear maturation. An example of this is glutamine, which may be converted into different tri-carboxy acid cycle (TCA) intermediates that produce adenosine tri-

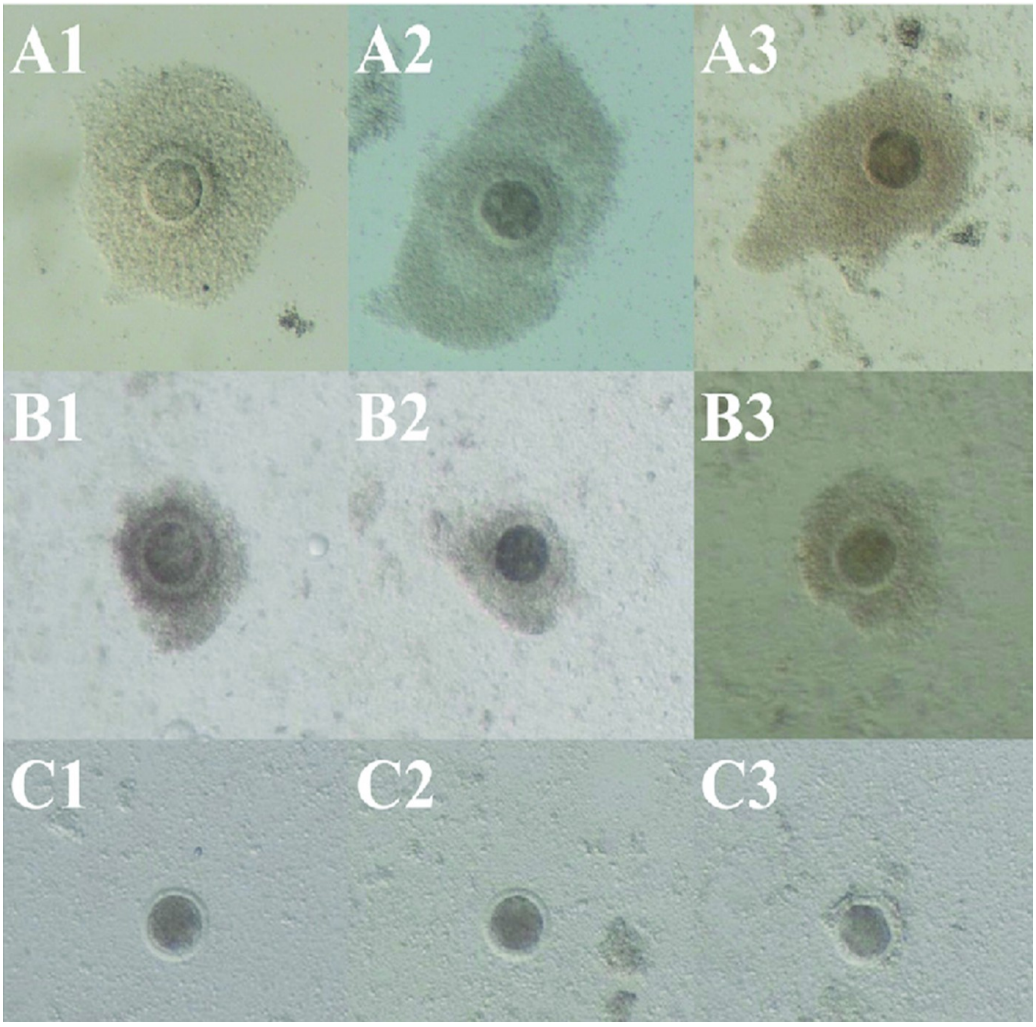

Figure 2: COC categorizing using training set A1-3 representative images: Good quality (category A), B1-3. Category B: Medium quality C1-3. Inferior quality (category C) (**Cavusoglu et al., 2023**).

phosphate (ATP), glucose, or pyruvate **(Bahrami and Pauline, 2022).**

Luteinizing hormone's (LH) potential to promote cumulus cell growth led to its inclusion in bovine IVM medium. Human chorionic gonadotropin (HCG) can attach to the luteinizing hormone/choriogonadotropin receptor (LHCGR), making it a replacement for LH. However, it does not raise the percentage of MII oocytes in bovine after IVM **(Bahrami and Pauline, 2022).**

Due to the fact that the contents of extracellular vesicles (EVs) may significantly affect oocyte maturation **(Lange-Consiglio et al., 2017),** the role of EVs in gamete maturation and embryo development has recently attracted more attention **(Machtinger et al., 2016).**

Increased cumulus expansion is seen in bovine **(Hung et al., 2015)** and murine **(Javadi et al., 2022)** COCs when EVs are added to an IVM system.

Researchers have looked at how adding follicular fluid (FF) to the maturation medium affects the rate of maturation and the development of the ensuing embryos in a number of studies **(Spacek and Carnevale, 2018).**

Bahrami et al. (2019) simplified the maturation medium composition by identifying the necessary fetal bovine serum (FBS), exogenous hormones, and particular amino acid groups for optimum IVM of bovine COCs.

2.3. Culture Conditions

Nuclear maturation is impacted by the incubation environment's internal atmosphere. Typically, oocytes are cultured at the core body temperature of their host species, with carbon dioxide (CO_2) concentrations between 5 and 6%, to maintain pH at normal values **(Zhou et al., 2016).** The bovine oocyte maturation rate is slowed down by low oxygen concentration as the main process that produces ATP is oxidative phosphorylation **(Zhang et al., 2017).** Con-

sequently, it's possible that bovine oocytes need more oxygen to produce ATP, although at greater quantities, it can cause a rise in ROS generation **(Wrenzycki and Stinshoff, 2013).**

The incubation temperature has an effect on the maturation of bovine oocytes *in-vitro*, in addition to the components of the culture medium. Traditional *in-vitro* bovine embryo production procedures, such as oocyte maturation, fertilization, and embryo culture, are normally performed at 38.5°C or 39°C, corresponding to cattle's core body temperature. Previous studies have suggested that when cow oocytes are cultivated *in-vitro* at a temperature lower than their core body temperature, oogenesis or maturation improves. This, in turn, could lead to improved development after fertilization **(Şen and Kuran, 2018). Şen and Kuran (2018)** examined how bovine oocyte maturation and embryo developmental competence were affected by incubation temperatures of 36.5°C and 38.5°C. The current investigation's findings showed that at 36.5°C, oocytes can finish their maturation. After IVF, their embryonic development is comparable since the oocytes developed at the standard culture temperature of 38.5°C.

The time it takes for oocytes to mature *in-vitro* and reach the MII can vary **(Ruiz et al., 2017)**. Therefore, the primary goal of **Ruiz et al. (2017)** study was to evaluate the impact of IVM time on the competence and nuclear maturation state of alpaca oocytes extracted from non-stimulated ovaries produced in an abattoir. According to the study's findings, the maximum rate of MII COCs was produced after 32 hours of IVM.

3. Oocyte and GC Expression of Genes Linked to Oocyte Maturation

Gene expression profiling in granulosa cells (GCs) and oocytes may serve as genetic markers for predicting oocyte maturation and competence **(Melo et al., 2016).** Key differentially expressed genes include members of the transforming growth factor beta (*TGFβ*) superfamily—growth differentiation factor-9 (*GDF9*), bone morphogenetic protein-6 (*BMP6*), bone morphogenetic protein-15 (*BMP15*)—and the phosphatase and tensin homolog (*PTEN*) gene.

B-cell leukemia/lymphoma 2 protein (*BCL2*) is essential for oocyte maturation and early embryonic development, according to **Huang et al. (2018).** When comparing buffalo oocytes in the GV stage to those in the MII stage, there was about a 2.5- fold increase in the amount of *BCL2* mRNA and protein.

According to **Uchime et al. (2016),** GCs' expression of genes linked to apoptosis, namely *BCL2* and the BCL-2-associated X protein (*BAX*) changed as pig oocytes matured.

Boldura et al. (2016) studied the expression of *BCL2* and *BAX* in cattle at 0, 24, and 48 hours after IVM of COCs. They found that *BCL2* mRNA was over-expressed at the 24-hour mark compared to the 0 and 48-hour time points. They attributed the low levels of *BCL2* expression at 0 hours to the fact that the stress associated with *in-vitro* culture had not yet reached its maximum, and the apoptotic process had not yet begun. They noted that the overexpression of *BCL2* after 24 hours suggests that the cells exposed to stress factors are trying to adapt to new conditions. Furthermore, they explained that the significant increase in *BAX* mRNA expression from 0 to 24 hours, peaking at 48 hours, indicates that the cells are entering the apoptotic process at that time.

4. The Simulated Physiological Oocyte Maturation (SPOM) System

Despite its various applications, *in-vitro* production (IVP) is still less efficient compared to in vivo embryo production **(Lonergan and Fair, 2014).** One of the main challenges in cattle IVP is replicating the processes that occur during the in vivo maturation of oocytes, which involves both nuclear and cytoplasmic changes **(Navarro et al., 2024).** These changes in the oocytes of the dominant follicle are referred to as oocyte capacitation or pre-maturation. They are believed to enhance oocyte competence and "prime" the oocyte for final maturation and subsequent development **(Razza et al., 2018).**

Because meiosis arrest depends on the oocyte's optimal concentration of cyclic adenosine monophosphate (cAMP), IVM mimics the final stage of oocyte development by mechanically removing CCs and FF, which causes an abrupt decrease in intracellular cAMP concentrations. This results in meiotic resumption, incomplete cytoplasmic maturation and oocytes with varying degrees of competence **(Ferré-Pujol et al., 2019).**

SPOM is an innovative ex vivo system designed to simulate physiological oocyte maturation. It employs a two-step maturation protocol that incorporates cAMP modulators, including forskolin, 3-isobutyl-1-methylxanthine, and

cilostamide **(Park et al., 2016).** Research has demonstrated that optimizing cAMP concentration during IVM can enhance oocyte competence **(Park et al., 2016).** Another approach to improve oocyte readiness for development is to extend the time for coordinating nuclear and cytoplasmic maturation. This can be achieved by blocking meiosis during a pre-maturation phase immediately after the oocytes are extracted from the follicles **(Adona et al., 2008).**

Navarro et al. (2024) investigated the effect of SPOM on cytoplasmic maturation by measuring the levels of stress-related genes and assessing mitochondrial activity and distribution as indicators of cytoplasmic maturation. Additionally, they examined the impact of cAMP treatment on nuclear maturation, cleavage, and blastocyst development. The findings suggest that using cAMP modulators during IVM leads to the production of competent oocytes, which, after fertilization, can develop into a higher quantity and quality of blastocysts compared to standard IVM conditions.

5. Some Antioxidants' Impacts on the Maturation of Bovine Oocytes *In-vitro*

Oxidative stress (OS) signifies a disruption in the delicate balance between the generation and clearance of certain molecules known as ROS **(Park et al., 2016).** ROS are molecules containing oxygen created by mitochondria and cellular metabolism which has a role in signaling and gene expression **(Halliwell and Gutteridge, 2015; Lepetsos et al., 2019).**

Physiological levels of ROS can enhance gamete function and development; however, excessive ROS generation beyond the oocyte's antioxidant capacity can result in OS **(Rakha et al., 2022**). Oocytes cultured *in-vitro* are inevitably affected by oxidative stress. Antioxidant enzymes in the follicular fluid quickly break down excess ROS, balancing their synthesis and removal in cells **(Park et al., 2016).** IVM-created oocytes lack antioxidant enzymes, leading to a breakdown in the equilibrium and increased ROS levels. OS negatively impacts oocyte maturation and contributes to poor oocyte quality **(Yu et al., 2019).**

There are various exogenous factors contribute to the production of ROS including exposure to high oxygen concentrations, visible light, pollutants, and certain components used during the IVM process (**Tiwari et al., 2016)**.

Light exposure can unbalance prooxidants and antioxidants, producing ROS and increasing hydrogen peroxide (H_2O_2) concentration. The greater the exposure duration, the more harmful the embryo's subsequent development **(Oh et al., 2007).**

High oxygen levels activate pro-oxidant oxidase, speeding up oxidation events and accumulating ROS, adversely impacting embryo development *in-vitro* **(Guerin et al., 2001).**

Changes in pH levels in culture media can significantly impact sperm motility, oocyte maturation, and embryo development **(Will et al., 2011).** In vitro culture systems that maintain moderate and consistent CO_2 concentrations help keep the pH within the normal range. Extremely high temperatures can lower pH levels, increasing the risk of OS and negatively affecting cellular processes **(Larkindale and Knight, 2002).**

The three main energy substrates necessary for oocyte maturation are pyruvate, lactate, and glucose **(Lin and Wang, 2020). Cekleniak et al. (2001)** suggested that increasing glucose levels in the culture media could elevate the production of oxygen radicals through glycolysis and oxidative phosphorylation, which could be harmful to IVM.

Serum is a source of proteins that nourish the COCs and aid in the oocyte's ongoing development. It can remove metal ions to prevent the medium from producing free radicals. Additionally, serum contains a variety of nutrients, growth factors, and other substances that may either directly or indirectly neutralize ROS generated in the culture environment **(Esfandiari et al., 2005).**

Over the past decade, the impact of antioxidants in IVM has been studied by many researchers. Because of their proven effect on ROS, it is essential to research antioxidants and their roles in this context **(Naspinska et al., 2023).**

5.1. Quercetin (QT)

Quercetin is a natural flavonoid which present in fruits, grains, teas, and vegetables. Plants including onions, apples, broccoli, and berries contain it **(Andreucci et al., 2018).** According to earlier research, QT antioxidant enzymes, scavenges free radicals, and eliminates oxidation products to avoid malfunctioning mitochondria **(de Oliveira et al., 2016). Silva et al. (2018)** investigated how QT, an antioxidant substitute for cysteamine (CIS), affected IVM; they observed that the QT group had a greater proportion of MII

oocytes than the CIS group.

In a study conducted by **Cao et al. (2020),** the impact of QT on oocytes from aged mice was investigated; the results revealed that treating oocytes from aged mice with 10 μM QT significantly increased the IVM rate. On the other hand, the QT group oocytes had significantly fewer abnormalities, indicating that some of the mitochondrial dynamics impairment associated with oocyte ageing can be mitigated by QT **(May-Panloup et al., 2016).**

5.2. Resveratrol

It is a natural polyphenol present in various plants and foods, including peanuts, mulberry, cocoa, Japanese knotweed roots, grapes, and red wine **(Wang et al., 2014).** Resveratrol is a white solid powder with a molecular weight of 228.25 g/mol. It has minimal water solubility and is photo/pH-sensitive **(Zabihi et al., 2019).**

The experiment of **Gutierrez-Castillo et al. (2023)** found that resveratrol administration reduced ROS levels, while resveratrol paired with Ethyleneglycol- bis(β-aminoethyl)-N,N,N',N'-tetraacetic Acid (EGTA) resulted in the lowest levels. Resveratrol not only reduced ROS levels, but also increased oocyte development competence during verification.

5.3. Ferulic Acid (FA)

It is a phenolic compound that is a metabolite of phenylalanine and tyrosine, commonly found in fruits and vegetables **(Lee and Hyun, 2017).** It's been demonstrated to possess characteristics that combat ageing. **(Neopane et al., 2023).** It is theorized that FA can prevent oxidative stress, which is commonly linked to aging **(Wang et al., 2021),** and so avoid oocyte aging thereby improving oocyte quality**.**

The effects of FA supplementation on the maturation of bovine oocytes and the development of embryos were investigated by **Wang et al. in 2023**. The results showed that, as compared to the control group, supplementing with 5 μM FA considerably boosted the maturation rates of bovine oocytes and the expansion of CCs. The results also showed that FA can sustain antioxidant levels (Glutathione "GSH", Superoxide dismutase "SOD", and catalase "CAT") in oocytes stable, reducing the OS brought on by H2O2.

Yin et al. (2023) examined the potential of FA to prevent the decline in bovine oocyte quality during *in-vitro* aging. Their findings indicated that adding 5 μM FA to bovine oocytes that had been aged *in-vitro* decreased the rate of abnormalities. Furthermore, by removing excess ROS and preserving intracellular GSH levels, together with the activity of antioxidant enzymes, the FA supplementation markedly increased antioxidant capacity.

5.4. Carvacrol

It is widely recognized for its many biological functions and is a prominent natural ingredient that is very prevalent in aromatic plants as an essential oil **(Imran et al., 2022).** Thyme yields 5–75% carvacrol oil by extraction, whereas hop marjoram and marjoram provide 50–70% oil. **(Ares et al.,2020**.

Carvacrol has been shown to have anti-inflammatory, anti-fungal, anti-cancer, hepato-protective, anti-spasmodic, vasorelaxant, and immunomodulatory properties in addition to its antioxidant capabilities **(Ezz-Eldin et al., 2020).**

The study conducted by **Morais et al. (2023)** aimed to ascertain the effects of carvacrol supplementation at different doses (0, 3, 12.5, and 25 μM) on bovine oocyte IVM. The degree of COC expansion and the evaluation of the nuclear maturation rate (first polar body and MII) are taken into consideration in the IVM of bovine oocytes. The pace of maturation was the same for all treatments. ROS levels were significantly greater in the Carv-25 μM treatment than in the other two.

5.5. *Nigella sativa* (NS)

The pharmacological characteristics and therapeutic potential of NS and its main constituent, thymoquinone (TQ), make them acknowledged as medicinal plants that support general health **(Goyal et al., 2017).** NS honey contains various antioxidant compounds, including flavonoids, chrysin, vitamin C, pinobanksin, catalase, and pinocembrin **(Fakhrildin & Alsaadi, 2014).** Additionally, a study on human sperm indicates that honey may have the potential to enhance sperm quality **(Fakhrildin & Alsaadi, 2014).**

The impact of different NS hydro-alcoholic extract (NSE) concentrations at 0, 1, 50, and 100μg/ml on the quality of oocytes in mice with polycystic ovarian syndrome (PCOS) during IVM was investigated by **Eini et al. (2020).** The findings demonstrated that oocyte maturation, OS, and epigenetic alterations were all improved by

an NSE concentration of 50μg/ml.

For the first time**, Kaabi et al. (2020)** evaluated how honey made from black seed (Nigella sativa) affected the IVM rate in sheep oocytes. The results indicated that 5% black seed honey is the optimal concentration for enhancing MII and GSH levels in matured sheep oocytes.

Kaabi et al. (2022) examined the effects of NS honey, Saudi Sider honey, and honeybee pollen on the IVM of sheep oocytes. According to the study, the oocytes' maturation rate, GSH levels, and gene expression were all boosted by the addition of natural honey and honeybee pollen at low quantities.

5.6. Melatonin

Melatonin is primarily created by the pineal gland from tryptophan, an important aromatic amino acid. Additionally, blood cells, the gastrointestinal tract's epithelium, and the retina and lens of the eye generate trace quantities **(Naspinska et al., 2023).**

Lima et al. (2022) used culture medium with different melatonin concentrations to investigate the effects of melatonin on cow oocytes that had developed under heat stress (10^{-12}, 10^{-9}, 10^{-6}, and 10^{-3} mol/L). Melatonin has been proven to be effective in minimizing the harmful effects of heat stress on oocytes and pre-implantation embryos **(Fernandes et al., 2019).** In mature bovine oocytes, a dosage of 1 μM of melatonin was observed to considerably reduce ROS levels **(Yaacobi-Artzi et al., 2020).**

5.7. Vitamin C

Vitamin C, also known as ascorbic acid, is an essential micronutrient primarily found in citrus fruits such as lemons and oranges, as well as in various vegetables. It is known for its antioxidant properties, but the exact mechanisms of its function at the cellular level remain unclear **(Santos et al., 2022).**

A study conducted by **Sovernigo et al. (2017)** found that adding 50 μg/mL of vitamin C to the medium reduced the levels of ROS. Additionally, vitamin C supplementation resulted in a higher percentage of blastocysts compared to the control group. Furthermore, embryos treated with vitamin C exhibited a greater total cell count.

According to **Al-Shimaa et al. (2017),** the most effective treatment for *in-vitro* embryo formation is 50 μM of vitamin C. When this is combined with 50 μM of cysteine, the effects are significantly enhanced. Additionally, a study conducted by **Husamaalden et al. (2020)** investigated the impact of vitamin C, as well as a combination of vitamin C and CIS, on bovine oocyte maturation, cleavage rates, and blastocyst formation. The findings revealed that a 200 mM dose of ascorbic acid improved oocyte maturation and blastocyst rates after *in-vitro* fertilization (IVF) compared to the control group, although it did not increase cleavage rates. In the second experiment, CIS was introduced to the culture media alongside vitamin C. The study revealed no substantial effect on oocyte maturation, although it did indicate a minor improvement in early cleavage and the generation of 2-celled embryos.

5.8. Vitamin A

Vitamin A is represented by several compounds, with the most significant being retinol (vitamin A1) and retinal (3-dehydroretinol or vitamin A2) **(Naspinska et al., 2023).** This vitamin plays a crucial role in various bodily processes, including cell differentiation and development, immune system function, vision, regulation of cell proliferation, and bone tissue growth **(Zasada et al., 2018).**

Gad et al. (2018) examined the effects of different vitamin A concentrations (5, 50, and 200 nM) on buffalo oocyte quality and maturation. The 5 nM treatment yielded the best results, with the highest rates of expansion and polar body formation, along with increased mitochondrial membrane potential and reduced ROS levels. Gene expression was also better in the 5 and 50 nM groups, while the 200 nM treatment produced the worst outcomes.

5.9. Nobiletein

Nobiletin is a polymethoxylated flavone found in citrus peel. It has gained popularity because it can be easily absorbed through cell membranes due to its structure and lipophilic nature **(Huang et al., 2016).** Nobiletin also exhibits various biological effects, including regulating the cell cycle **(Huang et al., 2016),** reducing apoptosis **(Liu et al., 2016),** and acting as an antioxidant **(Choi et al., 2007).** These properties are essential for the success of oocyte IVM.

The study conducted by **Cajas et al. (2020)** aimed to evaluate the protective effects of no-

biletin on the quality of matured bovine oocytes during IVM. COCs were supplemented with different concentrations of nobiletin: 10, 25, 50, or 100 μM. A control group was treated with 0.1% dimethyl sulfoxide (DMSO), which was used as the vehicle for diluting nobiletin. The results indicated that the groups treated with 25 μM (Nob25) and 50 μM (Nob50) of nobiletin had a higher percentage of matured oocytes in MII compared to the other groups. Additionally, the oocytes matured with 25 and 50 μM of nobiletin exhibited increased migration rates of cortical granules and enhanced mitochondrial activity while displaying lower levels of ROS and GSH compared to the other treatments. Regardless of the dose administered, nobiletin supplementation during the oocyte IVM process down regulated the expression of oxidative stress transcripts superoxide dismutase 2 (*SOD2*) and cytochrome-p450-family-51-subfamily-a-member-1(*CYP51A1*) while up regulating developmental-related genes like mitogen-activated protein kinase (*MAPK1*) and *BMP15*.

5.10. Kaempferol

It is a powerful flavonoid-containing antioxidant. Flavonoids' antioxidant potential is due to their ability to scavenge ROS, which in turn increases the production of intrinsic antioxidant enzymes such as SOD and GSH (**Nijveldt et al., 2001**).In buffalo, adding IVM medium with 10 μg/mL kaempferol increased oocyte maturation rates compared to the control group (**Bahgat et al., 2023**).

5.11. Biacalein

Baicalein, also known as 5,6,7-trihydroxyflavone, is a flavonoid that has been traditionally used in Chinese herbal medicine **(Fakruzzaman et al., 2020).** It is a major component of the plant Scutellaria baicalensis **(Kim et al., 2001).** Research has shown that baicalein possesses free radical scavenging and antioxidant properties **(Shieh et al., 2000).** Additionally, it is recognized for its antioxidant effects **(Chen et al., 2000)** and its role as an anti-inflammatory agent **(Lin and Shieh, 1996).**

Fakruzzaman et al. (2020) were the first to examine the effects of baicalein supplementation during IVM on bovine oocytes. Bovine oocytes, recovered from abattoir ovaries, were cultured in IVM medium with different concentrations of baicalein (0, 0.1, 1.0, and 10 μM). The study found that baicalein, particularly at 1 μM, acts as a potent antioxidant, enhancing developmental competence, increasing the hatching rate, and raising total blastocyst cell numbers while reducing apoptosis.

5.12. EMD-300® and EMP3-H200®

They are innovative antioxidant nanoformulations rich in flavonoids. In recent years, nanotechnology has been effectively utilized in assisted reproductive technologies (ART) to enhance oocyte maturation, fertilization, and *in-vitro* embryo development **(Hashem and Gonzalez-Bulnes, 2021).** Nanoparticles (NPs) possess unique physical properties that distinguish them from microparticles and bulk materials **(Jeevanandam et al., 2018).** These properties include a reduced size, larger surface area, higher purity, enhanced stability, and interactions at fluid interfaces. As a result, NPs are considered promising candidates for improving *in-vitro* embryo production (IVEP) **(Silva et al., 2021).**

Elsaka et al. (2025) investigated the effects of adding EMD-300® and EMP3-H200® to IVM medium on oocyte IVM and the expression of OS, apoptosis, and pluripotency genes in buffaloes. COCs from buffalo ovaries were cultured in IVM media with 0.5% or 1.0% EMD 300® or EMP3-H200® for 22 hours, respectively. Supplementing IVM medium with 0.5% EMD-300® or EMP3-H200® boosted buffalo oocyte nuclear maturation by more than 1.0%. Their findings show that these compounds have antioxidant characteristics, supporting their ability to protect oocytes from oxidative damage.

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