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The domestic cat oocyte: Strategies and challenges in IVM and cryopreservation

Oócito do felino doméstico: Estratégias e desafios na MIV e na criopreservação

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Abstract

The oocyte plays a pivotal role in the development of reproductive biotechnologies, as its developmental competence directly impacts embryo production. This review compiled the main findings on the cat oocyte intrinsic characteristics, the strategies used to optimize oocyte IVM, and the challenges and approaches employed in the oocyte cryopreservation protocols. Cat oocytes present a great lipid content, and the ones with a dark, finely granulated cytoplasm and multiple layers of cumulus cells are classified as top-quality, whereas those with light cytoplasm are associated with lower fertilization rates. Around 40-60% of oocytes submitted to IVM achieve the MII stage. Generally, they present an impaired glucose metabolism compared to their in vivo counterparts and can respond to cAMP modulators differently compared to other species. Moreover, they present a lipid accumulation during IVM, and lipid modulators can reduce their accumulation and improve cryosurvival, however, they also impact oxidative metabolism. Ascorbic acid, L-cysteine, L-carnitine, and resveratrol have been added before or during IVM with positive effects on oocyte oxidative parameters following IVM and on cryopreservation. Different strategies have been used to optimize oocytes' cryotolerance and improve survival rates, like delipidation before vitrification and the addition of the antifreeze protein I and extracellular vesicles in the cryopreservation solutions. The understanding of cat oocyte singularities beyond its behavior under in vitro conditions is essential to the development of strategies that improve IVM and the outcomes after cryopreservation.

Keywords: *antioxidants, lipid modulators, vitrification, feline*

Introduction

The domestic cat is an important research model for developing assisted reproductive technologies (ARTs) in conservation scenarios (Fastard et al., 2000; Singh et al., 2019). Since several members of the *Felidae* family are classified as vulnerable or threatened with extinction (IUCN, 2022), the acquisition of knowledge about the domestic feline reproductive physiology can optimize the development of techniques that are potentially applicable to domestic and non-domestic felids (Luvoni, 2006; Tharasanit et al., 2012).

Research involving reproductive technologies in the feline species has achieved oocyte maturation (Leal et al., 2024a) and *in vitro* fertilization (Rakhmanova et al., 2023*)*, as well as the cryopreservation of oocytes (Fernandez-Gonzalez et al., 2017; Oliveira et al., 2022; Leal et al., 2024b) and embryos (Pelican et al., 2006) beyond embryo transfer (ET) (Rakhmanova et al., 2023). Advances in this area have also contributed to the development of domestic cats derived from vitrified oocytes, followed by *in vitro* fertilization (Galiguis et al., 2014).

Bearing in mind that oocytes can be affected during *in vitro* processes, especially by oxidative stress, and that their intrinsic quality and developmental competence are essential factors for embryo development (Ramos Leal et al., 2018), it is understandable that the oocyte plays a protagonist role in the success of the mentioned reproductive biotechnologies.

Although these technologies have been successful in selected species or feline populations, the efficiency of the oocyte *in vitro* maturation (IVM), for example, still limits the application of the embryo *in vitro* production (IVEP) technology (Ramos Leal et al., 2018). Additionally, oocytes from domestic cats appear to exhibit physical characteristics that hinder the development of successful cryopreservation methods (Luvoni, 2006).

One of the characteristics that makes the feline oocyte a challenge for cryobiological research is its high lipid content, which is naturally present in it (Rakhmanova et al., 2023). Although intracellular lipids are responsible for providing energy for oocyte maturation and early embryonic development and

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exhibit several important cellular functions (Jin et al., 2017), studies in different species have already associated a decreased cryosurvival with an excessive lipid amount (Sudano et al., 2011).

In addition, it has already been established that IVM can alter oxidative status and lead to abnormal lipid accumulation in oocytes (Del Colado et al., 2015; Leal et al., 2018; Leal et al., 2024a). However, the exact mechanism through which this abnormal accumulation occurs is unknown, but it seems to be associated with abnormalities in energy metabolism (Sudano et al., 2011; Barreira et al., 2018).

Considering that cat oocytes submitted to *in vitro* conditions suffer impact in their oxidative status, allied to the fact they already present a high concentration of lipids that increases during IVM, and that the oocyte cryopreservation is an indispensable tool for long-term storage of endangered species germplasm, this review highlights the importance of the female gamete in the development of reproductive biotechnologies and compiled main findings on the (i) cat oocyte intrinsic characteristics, (ii) the strategies used to optimize IVM, and (iii) the challenges and approaches described in the oocyte cryopreservation protocols.

Cat oocyte specificities

Research on feline oocytes provides essential insights into their maturation, fertilization, and developmental potential, playing a vital role in both domestic cat reproduction and conservation efforts for endangered feline species. To achieve knowledge about these structures is crucial for the success of embryonic development and *in vitro* fertilization (Ochota et al., 2023). Despite the endocrinology of the feline estrous cycle being fully characterized nearly four decades ago (Shille et al., 1979; Wildt et al., 1981; Goodrove et al., 1989), studies on the ultrastructural patterns of feline oocytes remain scarce, particularly regarding their organelles, such as mitochondria, and lipid droplets (LDs).

Domestic cat oocytes have an average diameter of approximately 161 µm and are classified into two types based on cytoplasmic coloration. Oocytes with a dark, finely granulated cytoplasm and multiple layers of cumulus cells are classified as top-quality and exhibit a higher fertilization potential under *in vitro* conditions, whereas those with light cytoplasm are associated with lower fertilization rates. The dark coloration results from a high lipid concentration, which plays a crucial role in oocyte metabolism and developmental competence (Kochan et al., 2020; Młodawska et al., 2024).

Recently, our team identified 8 lipid species in immature cat oocytes using high-performance thin-layer chromatography: esterified sterol (ES), triacylglycerol (TAG), fatty acids (FA), diacylglycerol 1,3 + cholesterol, diacylglycerol 1,2 + cholesterol, oxysterol, monoacylglycerol (MAG), and phospholipid (Vasconcelos et al., 2024). Although the total lipid content is smaller in immature cat oocytes compared to the ones submitted to IVM (Leal et al., 2024a), these oocytes present a higher expression of the *FABP3* gene, suggesting a triggering of lipid reserves still in the immature stage (Vasconcelos et al., 2024). Understanding the mechanisms that govern the behavior of these lipid species during IVM and cryopreservation is fundamental for optimizing these processes (Leal et al., 2024a).

Beyond cytoplasmic characteristics, the functionality and behavior of oocyte organelles are fundamental to their maturation and developmental competence. Mitochondria, for instance, are essential for oocyte development, playing a key role in energy production, apoptotic regulation, and calcium (Ca^{2+}) homeostasis. It was already reported that in feline oocytes, mitochondria exhibit a peripheral distribution pattern, especially in mature oocytes – a distinctive feature that contrasts with the more dispersed distribution observed in other species (González et al., 2012). This spatial arrangement likely reflects the high energy demand in the cortex, as oocytes rely on *cumulus* cells to supply energy substrates via gap junctions (Eppig and O'Brien, 1996; Songsasen et al., 2001).

In addition to mitochondria, other organelles contribute significantly to oocyte function. The endoplasmic reticulum, for example, plays a crucial role in Ca^{2+} regulation, acting as the primary intracellular reservoir of this ion. It regulates essential processes in oocyte maturation, fertilization, and activation by controlling Ca^{2+} release and triggering key molecular pathways (Wakay et al., 2019). Another critical component of feline oocytes is the zona pellucida (ZP), a glycoprotein-rich extracellular matrix that mediates sperm binding and the acrosome reaction. In cats, the ZP is composed of four glycoproteins (ZP1, ZP2, ZP3, and ZP4), a structural composition that is essential for species-specific fertilization mechanisms (Stetson et al., 2015).

From a biochemical and molecular perspective, studies indicate that glucose-6-phosphate dehydrogenase (G6PDH) activity is strongly associated with the maturational competence of feline oocytes. Additionally, the expression of genes such as CC motif chemokine receptor 2 (CCR2) and its ligands has been identified in feline *cumulus*-oocyte complexes, suggesting a role in the ovulatory cascade and oocyte

maturation (Rojo et al., 2018a; Jaworski et al., 2020). Moreover, the SDF1-CXCR4 signaling pathway enhances the expression of key periovulatory genes, further supporting its involvement in the ovulatory process (Rojo et al., 2018b).

Acquaintance with the intrinsic characteristics of feline oocytes is essential, as advancements in assisted reproduction for this species can directly contribute to the conservation of wild felines. Notably, the high lipid content of these oocytes plays a crucial role in mammalian gamete cryobiology, offering valuable insights for refining preservation methods and enhancing *in vitro* maturation and fertilization techniques.

Cat Oocyte *in vitro* Maturation (IVM)

One of the main challenges for IVEP is to mimic *in vitro* the events of *in vivo* oocyte maturation, as it is a complex and delicate process where the oocyte is prepared to support fertilization and the initial development of the embryo until the activation of the embryonic genome (reviewed by Ramos Leal et al., 2018). Evidence already proves that *in vivo* cat oocytes present a different metabolism from those submitted to IVM. Although glycolysis naturally increases with the resumption of oocyte meiotic maturation, IVM oocytes present impaired glucose metabolism compared to their *in vivo* counterparts (Spindler and Wildt, 1999).

It is important to highlight that the most common source of oocytes for research comes from ovaries obtained in elective spay surgeries (reviewed by Colombo et al., 2021). Although the ovaries can be very heterogeneous when it comes to the differences among stages of the reproductive cycle, it has already been reported that the reproductive stage (inactive, follicular, or luteal) did not affect maturation or fertilization rates. Even so, it can impact oocyte developmental competence following *in vitro* fertilization (IVF), presenting, surprisingly, lower rates in the follicular stage (Karja et al., 2002).

During IVM, the oocyte goes through nuclear and cytoplasmic changes that include the resumption of meiosis and reorganization of organelles. Despite the conduct of several studies aiming to improve IVM, the oocytes that achieve metaphase II (MII) in cats are still around 40-60% (Luvoni et al., 2018; Fastard, 2000), which is low compared with farm animals, for example (reviewed by Fastard et al., 2000). In addition, it is well documented that the removal of COCs from antral follicles leads to a precocious oocyte meiotic resumption, creating an asynchrony between nuclear and cytoplasmic maturation (Gilchrist and Thompson, 2007; Ramos Leal et al., 2018).

This asynchrony has been extensively studied in several species (Albuz et al., 2010, Rose et al., 2013; Zheng et al., 2013; Metcalf et al., 2020; Suresh et al., 2021), and although the exact mechanism of oocyte maturation was not quite elucidated, it was already established that cAMP plays a pivotal role in maintaining the meiotic arrest in mammalian oocytes (Gilchrist et al., 2016). Some cAMP modulators have been employed during the IVM of several species, aiming to recover the synchrony between nuclear and cytoplasmic maturation by prolonging meiotic arrest (Leal et al., 2022). This modulation encompasses the use of substances related to its synthesis, through adenylate cyclase (AC) stimulation, or its degradation, by phosphodiesterase (PDE) inhibition, aiming to keep its high levels (Ramos Leal et al., 2018; Leal et al., 2022).

Herrick et al. (2014) used the widely known cAMP modulators forskolin, an AC stimulator, and Isobutyl-1-methylxanthine (IBMX), a PDE inhibitor, during cat IVM and found that IBMX was able to reversibly inhibit spontaneous maturation, keeping the effect observed in other species. However, forskolin presented the opposite pattern, stimulating nuclear maturation (Herrick et al., 2014; Leal et al., 2024c). Besides, it did not affect nuclear rates at 24 h IVM (Leal et al., 2024d), demonstrating that forskolin did not stimulate AC in cat oocytes, since it induced maturation by accelerating the meiotic progression, which was the opposite behavior observed in other species (Leal et al., 2024c). Moreover, forskolin did not impact the expression of developmental competence-related genes when added to IVM (*unpublished data*).

Another relevant aspect to be considered in cat IVM success is the presence of *cumulus* cells. In a study performed by Sowinska et al. (2017), it was evaluated the effect of IVM with COCs, denuded oocytes (DO), and the co-culture of denuded oocytes with *cumulus* cells (CC) or with COCs on maturation rates. Their results showed that the oocyte maturation decreased in all groups except the IVM with COCs, indicating that CC connected with an oocyte into a COC represents a better strategy for IVM in cat oocytes.

In previous studies, there were reports that cat oocytes needed about 40 h of IVM to reach MII stage, which is a similar period to the time from mating to ovulation in the queen (Goodrowe et al., 1989; Fastard et al., 2000). However, more recent studies demonstrated that oocytes could reach MII in the first 24 h of IVM (Leal et al., 2024a,b). In addition to the IVM duration, another important issue is the

supplementation used in the IVM medium. To make it as close as possible to the physiological environment, several substances have been tested over the years (Luvoni et al., 2018). A leading example is the consensus that bovine serum albumin (BSA) is a better protein source in cat IVM compared to fetal bovine serum (FBS) since the last can inhibit oocyte maturation (Johnston et al., 1993; Luvoni et al., 1993; Wood et al., 1995).

In this sense, chemical components of IVM medium present differences among labs, and studies give insights about the addition of substances that can reflect in a beneficial outcome for cat IVM (reviewed by Colombo et al., 2021). Supplementation of hormones, growth factors, and antioxidants are employed to improve oocyte developmental competence. Although some of them are not always present as standard supplementation in the IVM medium, they can be added to optimize the results (Comizzoli et al., 2003; Cochia et al., 2019, Colombo et al., 2021). Given this, knowledge regarding the specificities of cat oocytes, such as their ultrastructural patterns and organelles behavior, can be fundamental to the understanding of how these oocytes respond in *in vitro* environments.

Impact and behavior of lipids in IVM

A physiologically greater amount of lipids is found in the oocytes of some species such as cattle, pigs, and cats (Guraya et al., 1965; McEvoy et al., 2000). Although these lipids are related to physiological metabolic functions, it was already established that the oocyte lipid content increases during IVM in cats (Leal et al., 2024a). Besides the cat, the accumulation in lipid content during IVM is a phenomenon described in other species like cattle and sheep (Leal et al., 2018; Barrera et al., 2018). In cattle, for example, the increase in this content is considered abnormal since oocytes matured *in vivo* do not show this accumulation (Del Collado et al., 2015, 2017; Leal et al., 2018), however, information about this lipid accumulation in cat oocytes is still scarce.

Sowinska et al. (2025) observed that the lipid droplets (LD) change their distribution during IVM: in immature oocytes, LDs are found in a diffuse, peripheral, and irregular pattern, but when the oocyte achieves MII status, the diffuse pattern is dominant. In addition to that, the number and area of LD also increase while their diameter decreases following IVM. Moreover, the LD in the *cumulus* cells is also affected during IVM, increasing in number, area, and percentage of occupied area (Sowinska et al., 2025).

Immature cat oocytes have a lower content of ES and MAG when compared to those undergoing IVM (Fig.1). However, the TAG levels are similar between immature and matured oocytes (Vasconcelos et al., 2024) (Fig.1). In a study that aimed to understand the course of lipid content, our team evaluated the total lipid content of cat oocytes and observed a lipid accumulation starting at the timepoint of 28 h of IVM (Leal et al., 2024a). Considering that lipid accumulation reflects a greater sensitivity to cryopreservation (Cañon-Beltran et al., 2020), the use of lipid modulators during IVM represents a strategy to reduce the lipid content of oocytes.

Leal et al. (2024a) also tested the effect of different lipid modulators during IVM on lipid content, and the conjugated linoleic acid t10, c12 (CLA) was able to reduce the lipid amount in cat oocytes when evaluated at 28 h of IVM. Other lipid modulators such L-carnitine (LC) and Forskolin (FK) were also evaluated, but they did not reduce the oocyte lipid amount. However, when the three modulators were added to the IVM, the reduction in the lipid content of cat oocytes was even greater than with the single CLA use. Even so, the modulators did not seem to interfere with the nuclear maturation rates (Fig.1) when used isolated or in combination (Xavier et al., 2023; Leal et al., 2024a,d,e), but in combination, they were able to improve the cryosurvival of cat oocytes (Leal et al., 2024a).

Despite the use of these lipid modulators in combination proven to be beneficial to cryopreservation and did not impact the MII rates, they seem to affect the oocyte metabolism and gene expression. The three modulators (CLA+FK+LC) increased reactive oxygen species (ROS) and glutathione (GSH) levels despite keeping a lower redox balance (ROS/GSH) (Leal et al., 2024a). The combined use of lipid modulators also showed a lower expression of the sirtuin 1 (*SIRT1*) gene, which is associated with mitochondrial biogenesis in conditions of energy deficiency and is involved with the replacement of damaged mitochondria (Tang et al., 2016), indicating that the lipid cocktail was able to maintain a better metabolism balance during IVM despite of the difference in the levels (Leal et al., 2024a).

The lipid modulators (CLA+FK+LC) during IVM also provoke an increase in TAG levels when compared to immature oocytes, but the levels are similar to the *in vitro* matured oocytes without lipid modulation. On the other hand, despite being a byproduct of TAG breakdown, MAG increases in IVM with or without the presence of modulators (CLA+FK+LC), which suggests a higher metabolic expense in the cat IVM stage regardless of lipid modulation. Also, besides ES increasing during IVM, the combination

use of lipid modulators (CLA+FK+LC) can keep the ES levels similar to the immature oocytes (Vasconcelos et al., 2024).

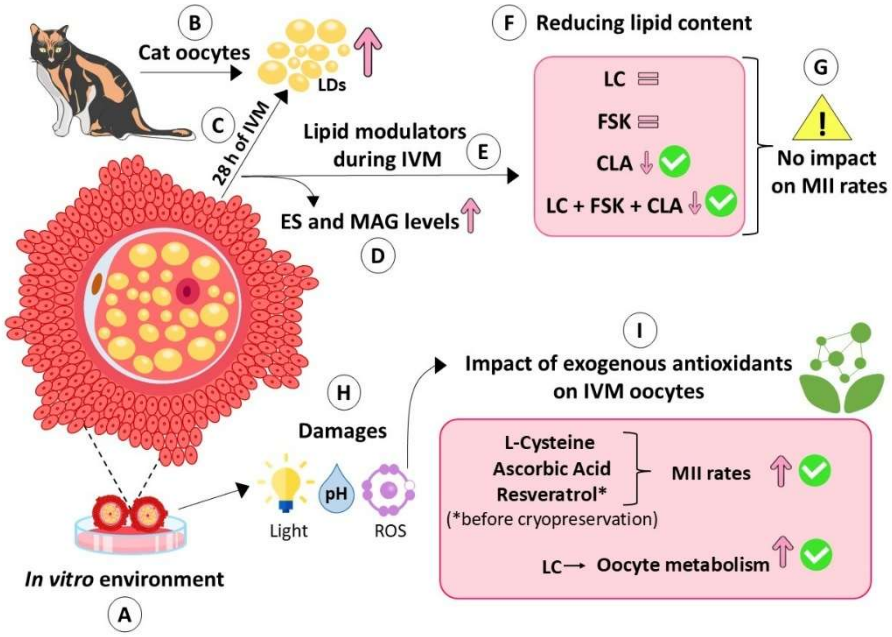

Figure 1: The *in vitro* environment promotes lipid accumulation in oocytes (A). This accumulation occurs naturally in feline oocytes (B) and becomes more pronounced after 28 hours of *in vitro* maturation (IVM) (C). During this period, increased levels of esterified sterol (CE) and monoacylglycerols (MAG) are observed in feline oocytes (D). Strategies to reduce excessive lipid content include the use of lipid modulators during IVM (E). L-carnitine (LC) and forskolin (FSK) did not significantly reduce lipid levels, Conjugated Linoleic Acid (CLA), and a combination of these modulators, effectively decreased intracellular lipid content (F), and did not impact MII rates. Additionally, the *in vitro* environment can compromise oocyte quality due to light exposure, pH fluctuations, and the generation of reactive oxygen species (ROS) (H). Supplementation with exogenous antioxidants—such as L-cysteine, ascorbic acid, and resveratrol—lowers ROS levels and enhances oocyte maturation rates to the metaphase II (MII) stage (I). L-carnitine supports the improvement of energy metabolism in feline oocytes.
LDs = Lipid droplets.

In addition to that, although fatty acid binding protein 3 (*FABP3*) transcripts are increased in immature cat oocytes compared to the ones matured *in vitro*, the presence of the lipid modulators in combination during IVM increases these transcripts even more, suggesting enlarged lipid traffic (Vasconcelos et al., 2024) since this protein is related to lipid transport to organelles (Ockner, 1991). According to Sowinska et al. (2025), a significant increase is also observed in the mRNA level of perilipin 2 (*PLIN2*) gene in the oocyte and the *cumulus* cells after IVM, once this gene is associated with the perilipin-2 protein localized on the surface of lipid droplets. Furthermore, the addition of lipid modulators during IVM also generates a higher expression of *PLIN2* compared to immature oocytes (Vasconcelos et al., 2024). This gene is associated with the prevention of lipid degradation (Bickel et al., 2009), since it participates in LD formation and stabilization, lipid uptake, and interacts with lipases. Its higher expression in the presence of lipid modulators could be a response from the oocyte to their action in the attempt to keep the lipid reserve, since it is important for several cellular processes (Vasconcelos et al., 2024).

After all, our recent study demonstrated that the combined use of these three modulators disrupts the lipid dynamics of cat oocytes in a manner not yet fully elucidated (Vasconcelos et al., 2024). Although the impact of lipid accumulation on the cryotolerance of oocytes is not completely clear, the amount and composition of intracellular lipids are essential factors since the lipid phase transition can change and disrupt intracellular processes and for this reason, the understanding of the changes caused by lipid modulation during IVM on the oocyte developmental competence need to be deeper investigated.

Impact of the antioxidant use on IVM

In addition to lipid-related alterations, another challenge in IVM is to preserve the physiological oxidative status and developmental competence of oocytes submitted to *in vitro* conditions, which can lead to excessive accumulation of intracellular ROS due to all manipulations and experimental environments. Microscopy and visible light, as well as higher pH gradients and oxygen partial pressures, are some of the factors that can cause an increase in intracellular ROS (Fig.1), disrupting redox homeostasis and leading to cell quality loss, which consequently results in reduced maturation rates and embryo development (Piras et al., 2020; Cao et al., 2022).

Aiming to mitigate ROS-induced damage, in recent years, studies with supplementation with a wide variety of antioxidants in IVM medium are being conducted as a strategy to protect the cells from the deleterious effects generated by excess ROS, by enhancing intracellular redox regulation. Adding exogenous antioxidants during IVM has positively impacted and increased the rate of oocytes achieving nuclear maturation in many species, such as the addition of nobiletin in bovine *in vitro*-maturated COCs (Cajas et al., 2020), β-cryptoxanthin in porcine oocytes (Park et al., 2018), and melatonin and tretinoin for mice (Aghaz et al., 2021), e.g.

In cats, the supplementation of ascorbic acid and cysteine in IVM medium has improved maturation rates (Fig.1) with a beneficial impact on embryo development *in vitro* through regulation of GSH balance and synthesis (Comizzoli et al., 2003), and, as a result of promising findings, L-cysteine and its products have already been included as a standard supplementation in IVM media for oocyte maturation in domestic cats (Cocchia et al., 2019, Leal et al., 2024a). L-cysteine promotes beneficial effects on early chromatin remodeling (Comizzoli et al., 2003) and can improve oocyte developmental competence via the expression of genes related to growth factors, like bone morphogenetic protein 15 (*BMP-15*) and growth differentiation factor 9 (*GDF-9*) (Elgebaly et al., 2022).

Recently, our research group has been conducting studies on the addition of resveratrol and LC to the IVM process, aiming, among other outcomes, to reduce oxidative stress (Fig.1) and its possible negative consequences. Resveratrol was able to promote higher MII-stage rates when COCs were pre-exposed to the antioxidant for 90 minutes before cryopreservation (Oliveira et al., 2023), however, it did not seem to impact MII rates when added during or before IVM (Cupello et al., 2023). On the other hand, LC could manage the oocyte antioxidant response higher than ROS production, positively impacting oocyte metabolism after IVM (Leal et al., 2024e). Resveratrol improves oocyte quality and can reverse the effects of oxidative stress through modulation of sirtuins, which are directly related to mitochondrial metabolism, oxidative phosphorylation, and mitochondrial biogenesis (Grzeczka and Kordowitzki, 2022). Conversely, LC promotes β-oxidation and antioxidant defenses by stimulating lipid metabolism and acting as a free radical scavenger that shields antioxidant enzymes from oxidative damage (Dunning and Robker, 2017; Leal et al., 2024e). Finally, antioxidant supplementation in IVM medium is an interesting strategy to improve protocols to enhance oocyte *in vitro* outcomes, aiming at improving *in vitro* embryo production. Several studies have been conducted in this field, and since oxidative stress is a major cause of poor oocyte quality (Aghaz et al., 2021), the use of antioxidants is a viable alternative to potentialize desired outcomes of this assisted reproductive technology and improve the oocyte resistance to cryopreservation through a better oxidative status achievement (Oliveira et al., 2023).

Cat oocyte cryopreservation and challenges

Cryopreservation represents a fundamental technique in assisted reproduction programs, as it enables the long-term storage and preservation of cells and tissues, to establish and maintain cryobanks (Gao and Crister, 2000). There are two main techniques for oocyte cryopreservation: slow freezing and vitrification. These methods differ primarily in the concentration of cryoprotectants required and the rate at which the cooling/freezing curve occurs (Luvoni and Colombo, 2020).

In slow freezing, the cooling rate is more tightly controlled, as the process occurs gradually. Additionally, it requires lower concentrations of cryoprotectants, resulting in reduced toxicity and allowing for proper cellular dehydration. Vitrification, in contrast, is characterized by ultra-rapid freezing, in which the sample transitions from a liquid to a glass-like state without the formation of ice crystals (Rajan and Matsumura, 2018).

Despite both methods showing similar proportions of cat oocytes with normal morphology after culture (Aparicio et al., 2013), vitrification is considered the most suitable technique for oocyte cryopreservation (Mogas et al., 2019; Luvoni and Colombo, 2020). Even so, vitrification increases

apoptotic markers in cat oocytes (Colombo et al., 2020), and, in general, the cryopreservation of the female gamete remains a significant challenge since during cryopreservation, the oocyte is exposed to several non-physiological stressors, including osmotic changes, exposure to low temperatures, and cryoprotectant toxicity, all of which can cause significant cellular damage and, consequently, lead to cell death (Smith et al., 2011; Arav and Natan, 2013). In addition, there are also intrinsic cellular factors that influence the success of the process. Oocytes are cells more susceptible to cryoinjuries due to their larger size and cellular complexity (Comizzoli and Holt, 2014), the presence of zona pellucida and *cumulus* cells surrounding oocytes, preventing the transmembrane movement of cryoprotectants (Colombo and Luvoni 2020). Moreover, the permeability of oocytes to water and cryoprotectants may vary, as each animal species presents differential expression levels of water and cryoprotectant channels in the membrane (Jin et al., 2011; Kim et al., 2017).

Cryoprotectants added to cryopreservation solutions are intended to enhance cell survival and are classified based on their mechanism of action. They can be permeable to the cell membrane, allowing them to replace intracellular water; these are referred to as intracellular or permeable cryoprotectants, with dimethyl sulfoxide and ethylene glycol being the most commonly used (Bojic et al., 2021). Although both are often used together, Luvoni and Pelizzari (2020) demonstrated that cryopreserved cat oocytes can be fertilized successfully and that their development *in vitro* increases when mature oocytes are frozen only with ethylene glycol. In contrast, some cryoprotectants may remain in the extracellular environment, where they promote water efflux from the cell via osmosis and contribute to membrane stabilization. These are known as extracellular or impermeable cryoprotectants, with sucrose serving as a representative example (Elliot et al., 2017). Regardless of their classification, the primary function of all cryoprotectants is to remove intracellular water and depress the freezing point of the solution, thereby preventing or minimizing damage caused by ice crystal formation (Chang and Zhao, 2021).

Although Sowinska et al. (2020) demonstrated that the meiotic status during cryopreservation did not affect the oocyte developmental competence, for some authors, the oocyte developmental stage also influences success and can be a key element in improving cat oocyte cryopreservation technique (Luvoni and Pellizzari, 2000). Immature oocytes have the advantage of presenting a surrounding envelope around their genetic material, offering protection against potential damage (Luciano et al., 2009), and they also present an interesting hyperosmotic tolerance that can be increased by supplementation with cytochalasin B (Comizzoli, 2008). Comizzoli et al. (2015) evidenced that epigenetic modifications associated with germinal vesicle (GV) chromatin compaction induced by resveratrol are reversible and beneficial for cat oocyte cryosurvival. Even though both slow freezing and vitrification impaired intercellular junctions, slow freezing represents a suitable method for maintaining functional coupling with *cumulus* cells in the GV stage after thawing (Luciano et al., 2009). Furthermore, from a conservation perspective, cryopreserving cat oocytes at this developmental stage is often more advantageous and applicable, especially in situations where *in vitro* maturation is not feasible (Luvoni and Pellizzari, 2000). On the other hand, the use of *in vitro* matured oocytes for vitrification seems to present better results in some species, since cat oocytes are considered even more challenging to preserve, since the success of the process depends on the maintenance of the ability to undergo IVM after thawing (Luvoni et al., 2006; Comizzoli et al., 2008).

Another important factor to consider is the relationship between the lipid content and the cryoprotectants. Feline oocytes exhibit a high concentration of lipids in their cytoplasm, which can negatively affect oocyte permeability to cryoprotectant solutions and contribute to intracellular ice formation, potentially leading to alterations during the freezing/thawing process (Comizzoli and Holt, 2014). To enhance oocyte cryotolerance, some substances can be incorporated into cryopreservation solutions, including antioxidants, delipidating agents, and cytoskeletal stabilizers, among others. These additives help to mitigate oxidative stress by reducing ROS, inhibiting cellular apoptosis, and regulating both membrane lipid composition and intracellular lipid content. These are some key factors that impact the oocyte's ability to withstand freezing and thawing processes (Olexiková et al., 2024).

The impact of lipid modulation on cryopreservation

Difficulties regarding the cryopreservation of oocytes and embryos with high lipid content have been described and explored for more than four decades (reviewed by Amstislavsky et al., 2019). Although there is still no consensus on the exact mechanisms by which lipids affect cryosurvival, it is well known that the high intracellular lipid content makes oocyte cryopreservation more complex and is associated with cell damage and low tolerance to the process (Sudano et al., 2011; Barreira et al., 2019). In addition, excess lipids can also cause an imbalance in energy metabolism and trigger processes such as oxidative stress,

besides the unwanted activation of a series of signaling pathways due to the disordered breakdown of lipid droplets with the release of lipid mediators (Danielli et al., 2023).

Lipids are usually distributed in membranes and cytoplasmic LDs in a cell (Giugliarelli et al., 2016). Evidence suggests that thermotropic lipid phase transitions (LPTs) are considered one of the events responsible for the low rates after thawing, presenting the potential to modify and disrupt intracellular processes (Oktrub et al., 2018). It seems that the balance between coexisting lipid phases plays a fundamental role in cell regulation (Van Meer et al., 2008). The decrease in temperature in cryopreservation protocols changes this balance, disrupting the membrane function (Quin, 1985). Cell cooling can lead to the LPT that modifies the membrane properties by altering the functioning of membrane proteins, spatial lipid distribution, and membrane transport (Fig.2) (Papahadjopoulos et al., 1973; Quin, 1985; Van Meer et al., 2008). These modifications occur not only due to temperature but also to the effect of water solutes and ice nucleation. Changes in membrane properties explain part of the cell injuries in oocytes (Arav et al., 1996; Ghetler et al., 2005), but problems in cryopreservation are also related to oocytes rich in LDs, and although the damaging mechanism associated with LDs is still unknown, Oktrub et al. (2018) demonstrated that the crystallization of LDs occurs differently for different cell types. In mature oocytes, lipid crystallization occurs gradually during freezing while in COCs undergoes a sharp transition. The role and behavior of lipid redistribution after heating above the onset of the LPT remains not elucidated. After the melting of ordered phases, most lipids inside the LD appear mixed in a uniform mixture, and Mokrousova et al. (2020) suggest that aspects of lipid redistribution can be related to cell survival and lipid metabolism after thawing.

The impact of reducing lipid content to improve cryopreservation has been widely studied in several species over the years (reviewed by Vasconcelos et al., 2024b), however, this effect in cat oocytes still needs to be better investigated. More recently, some studies have focused on investigating how the lipid content in the oocyte and embryo affects cryotolerance and how the reduction of these lipids can increase cryosurvival rates (Galiguis et al., 2014; Oktrub et al., 2018; Rakhmanova et al., 2023; Leal et al., 2024a). Simple exposure to the *in vitro* environment is one of the factors responsible for the abnormal accumulation of lipids in oocytes. Leal et al. (2024a) showed that a high accumulation of lipids is observed in the oocyte after 28 h of *in vitro* maturation in cats and that this content can be reduced with the supplementation of lipid modulators during IVM, leading to a higher oocyte viability after cryopreservation (Fig.2). Galiguis et al. (2014) demonstrated that the removal of intracellular lipids in oocytes before vitrification improved both the development and cryotolerance of feline embryos (Fig.2). However, considering the variety of important cellular functions developed by lipids (Jin et al., 2011) and their role in oocyte maturation (Dunning et al., 2014), the effect of the reduction in lipid content of cat oocytes still needs to be deeper studied and understood once it can reflect in the metabolism of oocytes and the further embryos.

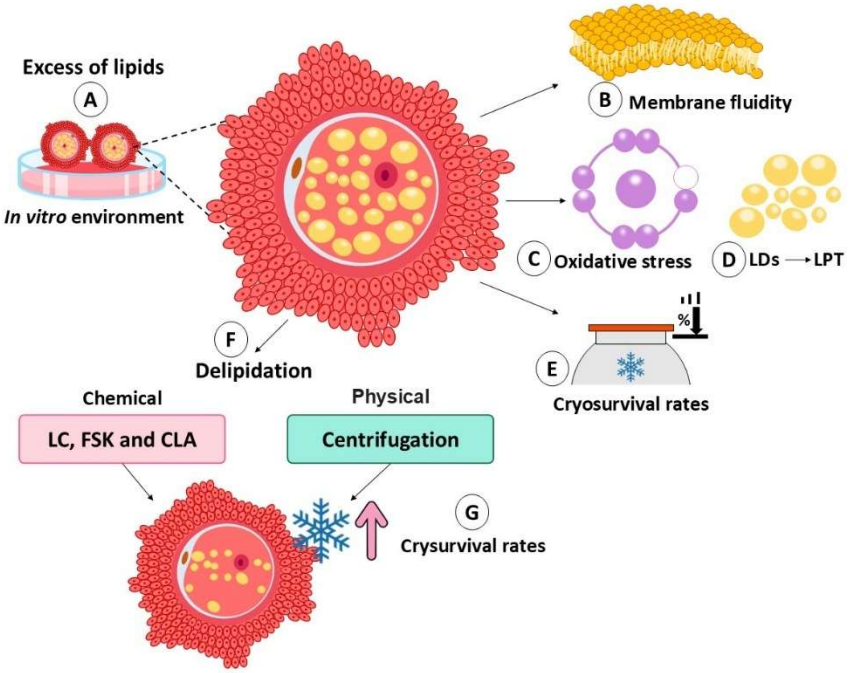

Figure 2: Excess lipids caused by the *in vitro* environment (A) cause damage such as altered membrane fluidity (B), oxidative stress (C), alteration in lipid phase transition (LPT) of lipid droplets (LDs) (D), and decreased cryosurvival rates (E). Chemical and physical delipidation are strategies to reduce the lipid content of feline oocytes (F) and improve cryotolerance rates (G).

Over the years, several strategies have been tested and applied in an attempt to improve cat oocyte cryopreservation (Table 1). Besides the lipid issue, oxidative damage is one of the major concerns in oocyte cryopreservation. High concentration of cryoprotectants allied to different osmolality between vitrification and warming solutions causes changes in intracellular ionic solute concentrations, decreasing GSH content while increasing H_2O_2 concentrations and ROS activity. All these factors directly impact on post-cryopreservation viability of vitrified/warmed oocytes, jeopardizing developmental competence in *in vitro* culture and future embryo production (Ahmadi et al., 2019), once supraphysiological ROS level can cause membrane disruption through lipid peroxidation, protein aggregation, and DNA damage (Soto-Heras and Paramio, 2020).

In this scenario, seeking to mitigate excessive ROS production and contribute to redox balance, the use of antioxidants during vitrification and warming processes has been implemented to reduce oxidative stress and enhance oocyte competence after cryopreservation (Colombo et al., 2022), but, as oocyte vitrification is still considered an experimental technique in domestic cats (Nowak et al., 2020), antioxidants have not been included as a standard supplementation in vitrification/warming solutions.

Studies have been undertaken to explore the benefits of antioxidant supplementation in cryopreservation solutions, including pre- and post-exposure of oocytes to antioxidant molecules, aiming to minimize oxidative stress and its detrimental effects on oocytes during the process. Our group has tested the effects on nuclear maturation and gene expression of feline COCs exposure to resveratrol before or after vitrification, in order to determine the optimal timing for antioxidant exposure during the process and establish the best outcomes in terms of redox balance and oxidative response generated by vitrification. It was shown that exposing oocytes for 90 minutes before vitrification enhances the expression of genes related to developmental competence and metabolism after warming, such as zygote arrest 1 (*ZAR-1*), *GDF9*, and *BPM15*, although there were no differences on MII-stage rates among groups considering the moment of resveratrol exposure (Oliveira et al., 2023). Concerning the use of LC, another study of our team demonstrated that its addition to equilibration/vitrification solutions was able to increase oocyte mitochondrial activity after warming, and according to gene transcription analyses, it did not provide benefits to improve oocyte cryotolerance in domestic cats. However, although LC does not affect feline oocyte nuclear maturation, its supplementation during IVM supports oxidative metabolism and seems important for preserving morphological and metabolic characteristics after cryopreservation. Nonetheless, its addition to cryopreservation solutions does not provide further advantages to feline oocyte cryotolerance (*unpublished data*). Given the scarcity of results in the literature regarding the effects of antioxidant supplementation in cryopreservation stages, further investigations are required to understand the oxidative stress response and the role of antioxidants on redox balance.

In addition to the oxidative damage caused by cryopreservation, this technique can also cause morphological and physiological damage involving the membrane, cytoskeleton, and mitochondria, creating even more oxidative changes and reducing oocyte developmental competence (Lei et al., 2014; Monteiro et al., 2017). In this sense, the use of molecules aiming to mitigate the damage inherent to the cryopreservation process emerges as an important strategy to improve the outcomes of this biotechnology. Considering this, the antifreeze proteins (AFPs), naturally found in organisms that live at glacial temperatures, have been used in the cryopreservation of several species with satisfactory results regarding the preservation of membrane structure, reduced oxidative stress, and fertilization rates (reviewed by Correia et al., 2021). Recently, our team tested, for the first time, the effect of AFP I in the cryopreservation of immature cat oocytes and we observed better morphological integrity and quality and improvement in the maturation rates. Moreover, AFP I also kept the expression of cryopreserved oocytes and the mitochondrial expression similar to fresh ones, mitigating some of the impact of cryopreservation and improving the cryosurvival of cat oocytes (Leal et al., 2024b).

Regarding the cryopreservation technique, for conducting vitrification, there are a wide variety of devices that can be used. Among the most used, are the open-pulled straw (OPS) (Vajta et al., 1998), Cryoloop (Lane et al., 1999), Cryotop (Kuyanama et al., 2005), nylon mesh (Matsumoto et al., 2001) and the conventional straw (Naik et al., 2005). Although the Cryotop method has been considered efficient for cat oocyte cryopreservation (Alves et al., 2012; Oliveira et al., 2023; Leal et al., 2024a,b), its use added to

strategies such as the addition of follicular extracellular vesicles in the vitrification and/or thawing media and culture with granulosa cells have increased the maturation rates demonstrating an enhanced ability of frozen-thawed oocytes to resume meiosis (Colombo et al., 2020b; Ferraz et al., 2020). The blocking of the connexin channels during the vitrification process is also an alternative to improve the maturation rates of vitrified cat oocytes but it seems to only slightly impact embryo production (Snoerck et al., 2018).

Table 1. Overview of the strategies applied for cat oocyte cryopreservation improvement over the last 15 years.

Reference	IVM	Objective	Cryo Method	Evaluation	Results	IVF	Strategy	Results
Nowak et al., 2024	Pre-cryo	Evaluate the effect of different concentrations of resveratrol (0.2, 2, 20 µM) after vitrification	Vitrification	Viability (FDA/EtBr staining), ROS levels (fluorescence analysis after staining) and embryo development after IVF	Highest viability with 0.2 µM resveratrol (68.89 %)	Yes	Insemination after 2-h incubation with the different concentrations of resveratrol	Highest cleavage rate (88.34 %) and more blastocyst formation with 0.2 µM resveratrol
Leal et al., 2024a	Post-cryo	Evaluate supplementation with 0 µg/mL, 0.5 µg/mL, 1 µg/mL of antifreeze protein I (AFPI) on vitrification solution	Vitrification	Actin integrity, mitochondrial activity and mass, ROS and GSH levels, nuclear maturation, expression of genes related to oocyte quality and metabolism and ultrastructure analysis	AFP I improved maturation rates, morphological quality, and actin integrity. AFP I (1 µg/mL) maintained mitochondrial activity, reduced mitochondrial mass, increased ROS levels, and had the gene expression more similar to fresh group	No	No	No
Leal et al., 2024b	Post-cryo	Investigate lipid accumulation during IVM and assess the effects of lipid modulators (CLA, forskolin, L-carnitine) on lipid content, oxidative stress, mitochondrial activity, gene expression, and cryosurvival	Vitrification	Lipid content (Oil Red O staining), nuclear maturation, mitochondrial activity, ROS and GSH levels, gene expression, viability after vitrification (morphological and Neutral Red staining)	Lipid content increased after 28 h IVM with any lipid modulator. MIX group showed the greatest reduction in oocyte lipid content (28 h of IVM). Mitochondrial activity and MII rate in CONTROL (45%) vs MIX (41%) groups did not differ. After	No	No	No

					vitrification, MIX (74%) presented a higher viability vs control (53%).			
Nowak et al., 2022	Pre-cryo	Compare different commercial microvolume techniques (Kitazato, Cryotech, and Vitrolife) of vitrification	Vitrification	Viability (FDA and EtBr staining), cleavage rates after IVF, development to morula and blastocyst stages	No differences among the three vitrification groups. Viability with Vitrolife (84.5%), similar to Cryotech (84%) and Kitazato (79.6%)	Yes	No	It was observed 35% of embryonic divisions in the Vitrolife and Kitazato groups and 45% in the control group
Colombo et al., 2020a	Post-cryo	Assesse whether vitrification may trigger two apoptotic markers (DNA fragmentation and caspase activity). Effects of Z-VAD-FMK on the same markers and on vitrified oocytes in vitro development	Vitrification	DNA fragmentation (TUNEL assay), caspase activity (fluorescence staining), nuclear maturation, fertilization rate, embryo cleavage and development to morula and blastocyst stages	Vitrification induced DNA fragmentation and increased caspase activity. Addition of Z-VAD-FMK reduced DNA fragmentation and caspase activity, and improved maturation rates close to fresh oocytes.	Yes	No	Z-VAD-FMK brought no improvement in embryo development (cleavage stage). No blastocyst formation in vitrified groups
Colombo et al., 2021	Post-cryo	Compare the yield and developmental competence of vitrified oocytes collected and cryopreserved right after ovary excision or after 24 h cooled ovary transport	Vitrification	Nuclear maturation, fertilization rate, cleavage, embryo development (morulae and blastocysts)	Maturation rates were similar for both vitrified groups	Yes	No	Fertilization rates were similar between vitrified groups, but there was a difference in cleavage between the oocytes vitrified right afer surgery (25.6%) and 24 h collected (14.5%). No differences in further embryo development

Sowinska et al., 2020	Pre and Post-cryo	Assess the potential toxicity of the vitrification solution. Investigate whether the meiotic stage (immature vs. in vitro matured) affects the developmental competence after vitrification	Vitrification	Maturation rates, fertilization (cleavage) rates after ICSI, development to morula and blastocyst stages. Suitability for ICSI	Vitrification procedure reduced the meiotic competence of oocytes	Yes	ICSI	No difference in fertilization and morula rates between immature vs. mature. Immature vitrified oocytes had lower suitability for ICSI compared to matured oocytes. No blastocyst formation from vitrified oocytes
Nowak et al., 2020	Pre-cryo	Evaluate the applicability of the Cryotech technique for the vitrification of domestic cat oocyte	Vitrification	Viability after exposure to vitrification media (toxicity test). Viability after vitrification. Parthenogenetic activation: cleavage, morula, blastocyst formation	Exposure of oocytes to Cryotech media (without vitrification) resulted in a reduction of viability to 71%, whereas the survival rate of oocytes in the vitrified group was 62%	No	Parthenogenetic activation	No differences in cleavage rate or embryo development were observed between the experimental and control groups. No embryos reached the blastocyst stage in either group
Ferraz et al., 2020	Post-cryo	Characterize protein content of cat follicular fluid extracellular vesicles (ffEVs) and determine the influence of ffEVs on oocyte cryosurvival and the ability to undergo in vitro maturation	Vitrification	Proteomics of ffEVs, oocyte viability (morphology), meiotic resumption and nuclear maturation (GVBD to MII), oocyte uptake of ffEVs (fluorescent labeling)	Vitrification compromised meiotic resumption (21 ± 14.1%). The presence of ffEVs before and/or after vitrification improved meiotic resumption (28.3 ± 13.1% vs 8.6% without ffEVs). Only vitrified oocytes with ffEVs reached MII (17.9%).	No	No	No

Colombo et al., 2020b	Post-cryo	Verify the functionality and efficiency of domestic cat ganulosa cells (GCs; 3D follicle-like structures vs 2D monolayer condition) and whether vitrified oocytes could benefit from the presence of GCs during in vitro maturation	Vitrification	Estradiol and progesterone production by granulosa cells. Viability (FDA/PI staining) of vitrified oocytes, meiotic resumption and full maturation after IVM. Degeneration rates	90.27% of vitrified oocytes were viable and matured in 3D and 2D conditions. GCs maintained their functionality in both the conditions. Vitrified oocytes resumed meiosis at higher rates when cultured with 2 days GCs	No	No	No
Snoek et al., 2018	Post-cryo	Evaluate whether inhibiting connexin channels (Cx37 and Cx43) with peptide Gap26 during vitrification and warming may improve maturation and blastocysts development	Vitrification	Maturation rate (PolScope™ microsope). Cleavage after parthenogenetic activation, and evelopment to blastocyst stage	49% of the oocytes in the control group matured, compared to 8% and 19% of vitrified and vitrified-peptide groups, respectively. Comparing both, the vitrified-peptide group had larger maturation rates	No	Parthenogenetic activation	38% of the oocytes in the control group had cleaved, versus 3% and 9% in the vitrified and vitrified-peptide group, respectively. No blastocysts were detected at day 8 in the vitrified group, while 2% and 13% of the oocytes developed to blastocysts at day 8 in the vitrified-peptide and control non vitrified group, respectively
Fernandez-Gonzalez and Jewgenow, 2017	Post-cryo	Compare the efficiency of two commercial vitrification kits (Vit Kit® and Kitazato®) and a three-step protocol (IZW) for cat oocytes, and evaluate the effect of slush nitrogen (SN_2)	Vitrification	Nuclear maturation percentage, cleavage after ICSI and morula formation after ICSI	Vit Kit® showed the lowest proportion of matured oocytes (10.1%). Maturation percentage differed between IZW (24.5%) and Kitazato® (38.7%)	Yes	ICSI	There were no difference in the cleavage and the morula percentage between IZW and Kitazato® protocols. Application of SN2 did not result in any improvement of

		on survival and development						oocytes' cryopreservation
Galiguis et al., 2014	Pre-cryo	Evaluated whether lipid polarization before vitrification enhances cryosurvival and whether embryos derived from lipid polarized/vitrified oocytes can produce live kittens	Vitrification	Oocyte degeneration rates, cleavage and blastocyst rates, blastocyst cell number, pregnancy and live birth after embryo transfer	Lipid polarization before vitrification reduced oocyte degeneration and enhanced cryosurvival.	Yes	No	Cleavage rates higher in centrifuged oocytes (44.7%) vs non-centrifuged (30.0%). Blastocyst rates higher in centrifuged oocytes, but lower for vitrified oocytes (3.9%) compared to fresh (28.6%). One live kitten was born from partially polarized vitrified oocytes after embryo transfer. Fully polarized embryos implanted but were reabsorbed
Mikołajewska et., 2012	Pre and Post-cryo	Evaluate the effect of different vitrification protocols (cryoloop vs. cryotop; with and without Ficoll PM-70) on viability and subcellular structure integrity	Vitrification	Cell viability (FDA/PI staining), cytoskeleton organization ((Anti-a-Tubulin–FITC antibody, Phalloidin-TRITC), nuclear configuration (DAPI staining), classification of structure integrity (normal, slightly abnormal, abnormal)	52% and 41% of live mature and immature oocytes, respectively and until 32% of microtubules, 28% of nuclear configuration and 36% of microfilaments in the normal pattern can be obtained with the protocols. Ficoll	No	No	No

					PM-70 essentially improves the oocytes survival upon vitrification			
Alves et al., 2012	Post-cryo	Compare viability and resumption of meiosis after warming and culture (24 and 48 h), of ex situ (isolated) and in situ (enclosed in the ovarian tissue) oocytes vitrified with DAP 213 in cryotubes or Cryotop method	Vitrification	Oocyte and cumulus cells viability (FDA–PI staining), resumption of meiosis (Hoechst staining after 24 h and 48 h IVM)	After 48 h of culture, ex situ oocytes vitrified with Cryotop achieved the rates of meiosis resumption similar to fresh oocytes (53.8% vs 67.5%) and ex situ and in situ oocytes vitrified with DAP 213 showed similar rates of resumption of meiosis. No differences were observed comparing ex situ and in situ oocytes vitrified by the same method. Cumulus cells were highly susceptible to vitrification	No	No	No

Pope et al., 2012	Pre-cryo (In vivo and in vitro matured)	Evaluated the cleavage rate after IVF or ICSI of in vivo- and in vitro-matured oocytes after vitrification and fetal development after transfer of ICSI-derived embryos	Vitrification	Oocyte morphology after warming, cleavage rates after IVF/ICSI, blastocyst formation, pregnancy and live birth after embryo transfer	93% (in vivo) and 97% (in vitro) oocytes appeared morphologically normal after warming.	Yes	IVF and ICSI	Cleavage rates were similar between fresh and vitrified oocytes. Blastocyst development only from vitrified in vitro-matured oocytes fertilized by IVF. In vivo viability of zygotes and/or embryos produced via ICSI of vitrified oocytes was established by birth of live kittens
Tharasanit et al., 2011	Post-cryo	Assess different cryoprotectants (DMSO, EG) and exposure techniques (2-step vs 4-step) for vitrification of immature oocytes, and evaluate embryo development and pregnancy after embryo transfer	Vitrification	Nuclear maturation (GVBD, MI, MII), cleavage and blastocyst formation after IVF, in vivo development after embryo transfer (pregnancy)	Vitrification reduced significantly the meiotic and developmental competence of immature cat oocytes compared with the non-vitrified controls. 4-step EG vitrification yielded highest MII rates (37.6%)	Yes	No	Vitrification reduced cleavage (24.8%) and blastocyst rates (30.2%) compared to non-vitrified controls (62.5% and 49.3%). Pregnancy was detected after transfer of vitrified-derived embryos, but fetuses were resorbed. Live kittens were born from non-vitrified embryo transfer controls
Cocchia et al., 2010	Post-cryo	Evaluate survival and developmental competence of immature domestic oocytes vitrified in open pulled straws (OPS) using a mixture of EG and DMSO	Vitrification	Viability of cumulus oocyte complexes (cFDA/Trypan blue staining), cleavage rate, morula and blastocyst formation after IVF	Percentage of non-viable was higher in vitrified oocytes than in control (11% vs 54.5%).	Yes	No	Cleavage rate and blastocyst formation were lower for vitrified oocytes (18.6% and 4.3%) than control ones (48.2% and 20.6%), respectively. First report of blastocyst development from vitrified GV-stage cat oocytes

Comozzoli et al., 2009	Post-cryo	Examine whether in vitro compaction of GV chromatin using resveratrol improves survival, meiotic competence, and embryo development of vitrified cat oocytes	Vitrification	GV chromatin acetylation and compaction, nuclear maturation, fertilization, cleavage and embryo development after IVF	Vitrification exerted an overall negative influence on oocyte meiotic and developmental competence	Yes	Parthenogenetic activation for control	The bility to reach MII, achieve early first cleavage, and develop to an advanced embryo stage (8–16 cells) was improved in vitrified oocytes previously exposed to 1.0 mmol/l Res compared to all counterpart treatments
Luciano et al., 2009	Post-cryo	Compare slow freezing and vitrification methods for preserving the functional coupling with cumulus cells as well as nuclear and cytoplasmic competence after warming of immature oocytes	Slow freezing and vitrification	Cytoskeleton organization by fluorescence staining (microtubules: anti-tubulin antibody + FITC=green; microfilaments: phalloidin conjugated with TRITC=red; DNA: DAPI= blue), gap junction functionality (lucifer yellow microinjection) and meiotic competence after IVM	Slow freezing achieved better preserved cytoskeleton (80% normal) vs vitrification (16.6% normal), preserved more functional gap junctions (37.2% open or partially open) vs vitrification (19%), and higher matural capability (32.5%) vs vitrification (14.1%)	No	No	No
Merlo et al., 2008	Pre-cryo	Evaluate the efficiency of vitrification using the cryoloop technique and assess embryonic development after IVF	Vitrification	Oocyte survival (degenerated oocytes post-warming), cleavage rate, morula and blastocyst development up to day 10, hatched blastocyst rate	Higher degeneration in vitrified oocytes (49.8%) vs controls (17.6%)	Yes	No	Cleavage (32.2%) and blastocyst rate (11.8%) were lower than controls (51.2% and 28.2%, respectively). Hatched blastocyst rate was higher for vitrified embryos (44.4%) than controls (18.9%). First study to report hatched

								blastocysts in vitro from vitrified cat oocytes
Murakami et al., 2004	Pre-cryo	Evaluate the effect of sucrose incubation time during cryoprotectant dilution on survival and development of vitrified cat oocytes	Vitrification	Survival after warming (morphology), cleavage rate (2-cell stage), development to morula and blastocyst stages, total cell number in blastocysts, parthenogenetic activation check	Best survival with 0.5 min in 0.5 M sucrose after warming	Yes	Parthenogenetic activation for control	Only oocytes incubated for 0.5 min in sucrose after warming developed to morula (4.2%) and blastocyst (3.7%). Non-vitrified had much higher blastocyst rates (~34.4%). No parthenogenetic activation was observed
Luvoni and Pellizzari, 2000	Pre and Post-cryo	Evaluate the ability of cat oocytes at different maturation stages to survive cryopreservation and develop after IVM and IVF	Slow freezing	Resumption of meiosis (GVBD and MII rates), morphological integrity after thawing. Cleavage after IVF and embryonic development beyond 8-cell stage	Resumption of meiosis occurred in all treatments, highest rates were achieved after exposure to 1.5 M of EG and DMSO. Morphological integrity after thawing was superior in oocytes frozen at the MII rather than the GV-stage using either DMSO or EG	Yes	No	After fertilization, mature oocytes frozen in EG cleaved in better proportions (38.7%) than immature oocytes (6.8%). Only mature oocytes frozen with EG developed beyond the 8-cell stage (11.3%)

Altogether, the ultimate goal of all approaches mentioned in this review topic is to improve COC viability and preserve its developmental competence by establishing reliable protocols for feline oocyte cryopreservation.

Final Considerations

The oocyte plays a protagonist role in the development of reproductive biotechnologies, and the understanding of the cat oocyte singularities and specificities beyond its behavior under *in vitro* conditions is essential to the development of strategies that improve IVM and the outcomes after cryopreservation. Over the years, several studies have demonstrated that incorporating molecules with antioxidants and cryoprotective properties into IVM and cryopreservation protocols can indeed be a strategy to enhance desired outcomes and increase the effectiveness of these processes. Furthermore, the improvement of these biotechnologies in the domestic cat can be a key strategy to preserve endangered wildlife.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

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