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IMPROVING THE EFFICIENCY OF OOCYTE CRYOPRESERVATION IN VETERINARY MEDICINE

Presentata da: **Dott.ssa Penelope Maria Gugole**

Coordinatore Dottorato
Prof.ssa Carolina Castagnetti

Supervisore
Prof.ssa Barbara Merlo

Co-supervisore
Prof.ssa Eleonora Iacono

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ALMA MATER STUDIORUM UNIVERSITY OF BOLOGNA
Department of Veterinary Medical Sciences

Abstract

Ph.D. in Veterinary Clinical Sciences
Improving the efficiency of oocytes cryopreservation in Veterinary Medicine

by Penelope Maria Gugole

Oocyte vitrification represents one of the most widely applied cryopreservation techniques, but several biological and technical challenges remain unresolved, particularly in veterinary medicine. This thesis investigated how vitrification influence oocyte quality and survival, focusing primarily on the equine and bovine species. The first study evaluated the effect of naloxone, an opioid antagonist with antioxidant properties, and the impact of an uncontrolled room-temperature holding period prior to vitrification on immature equine oocytes. Vitrification increased oxidative stress, apoptosis, and the proportion of mitochondria with high mitochondrial membrane potential. Overnight holding reduced oxidative stress but also lowered GSH levels after vitrification, potentially impairing developmental competence and altering the oocyte's response to naloxone. Naloxone improved meiotic competence only in oocytes vitrified immediately after collection. Moreover, it showed antioxidant activity in oocytes vitrified after holding but an opposite effect in those vitrified immediately. The second study extended this investigation to held mature equine oocytes, using bovine oocytes as a comparative model. Overall, naloxone provided no meaningful protection during equine oocyte vitrification, although a slight reduction in ROS indicates limited antioxidant activity. Comparative experiments with bovine oocytes confirmed their value as a preliminary model but highlighted species-specific differences, particularly regarding naloxone's interaction with the holding phase. The final study assessed a shortened, DMSO-free vitrification commercial protocol using bovine oocytes as a model for human applications. The modified protocol ensured high post-warming survival and stable redox balance across both closed and semi-open vitrification systems, with an observed increase in mitochondrial activity potentially linked to post-warming recovery processes. Collectively, these findings highlight the complexity of oocyte physiology and the influence of species-specific features, maturation stage, and protocol design on vitrification outcomes. The results contribute to refining current cryopreservation strategies and provide a foundation for developing safer, more efficient protocols applicable to animal assisted reproduction.

Sommario

La vitrificazione rappresenta la più moderna e diffusa tecnica di crioconservazione, una metodica che consente di conservare per un periodo di tempo virtualmente illimitato i gameti, sia maschili sia femminili, a temperature inferiori allo zero. La vitrificazione dei gameti femminili, gli ovociti, ha raggiunto buoni livelli di successo nella specie umana, sebbene con margini di miglioramento, e i risultati possono considerarsi complessivamente soddisfacenti. Al contrario, in medicina veterinaria la crioconservazione dei gameti femminili rappresenta ancora una sfida. L'ampia varietà di specie di interesse veterinario e le differenze tra queste ultime in termini di caratteristiche dei rispettivi gameti rendono impossibile l'applicazione di un protocollo di congelamento universalmente valido.

Lo scopo di questo lavoro è stato quello di indagare come la vitrificazione influisca sulla salute degli ovociti e di valutare se modifiche al protocollo standard possano determinare effetti positivi sulla qualità cellulare. La ricerca si è focalizzata sul modello equino, una delle specie più complesse per la crioconservazione dei gameti, nella quale il patrimonio di conoscenze è ancora limitato e la letteratura riporta risultati spesso discordanti e generalmente insoddisfacenti. Parte del lavoro si è concentrata sullo studio del protocollo di vitrificazione in questa specie, valutando lo stato di maturazione ovocitaria, la possibilità di conservare i gameti a temperatura ambiente per un periodo prolungato ("holding overnight") e l'effetto del naloxone, una molecola con riconosciuta azione antiossidante, già valutata in precedenti studi preliminari con risultati promettenti. Il progetto ha portato alla realizzazione di due lavori sperimentali distinti, successivamente pubblicati su riviste internazionali del settore. Entrambi hanno indagato l'effetto della vitrificazione su ovociti equini, differendo però per lo stadio di maturità ovocitaria al momento del trattamento: nel primo studio gli ovociti erano immaturi, nel secondo erano stati precedentemente maturati in vitro. Nel primo studio, i risultati hanno evidenziato che l'azione del naloxone varia in base alla presenza o meno della fase di holding prima della vitrificazione. La maturazione in vitro è risultata più efficiente nel gruppo trattato con naloxone ma non sottoposto a holding, mentre lo stress ossidativo si è ridotto in presenza di entrambi i trattamenti, senza tuttavia differenze significative nei livelli di glutatione o nel potenziale di membrana mitocondriale tra i gruppi sperimentali. L'effetto del naloxone è invece risultato significativo in relazione ai processi apoptotici, con un aumento della percentuale di ovociti in apoptosi precoce nei gruppi in cui la molecola era presente, suggerendo che la sua azione possa dipendere dalle condizioni metaboliche e dal momento di applicazione del

trattamento. Nel complesso, i risultati ottenuti indicano che la combinazione di un periodo di holding controllato e di modifiche mirate al protocollo di vitrificazione può contribuire a migliorare la qualità ovocitaria post-scongelamento. Tuttavia, ulteriori studi saranno necessari per chiarire in modo definitivo il ruolo protettivo del naloxone e dell'holding nel modello equino. Il secondo lavoro, anch'esso focalizzato sulla vitrificazione di ovociti equini in presenza di naloxone, ha utilizzato il bovino come modello comparativo. Nonostante le differenze fisiologiche tra le due specie, il bovino rappresenta un modello preliminare utile per la standardizzazione dei protocolli sperimentali, permettendo di ridurre l'utilizzo di ovociti equini, più difficili da reperire e manipolare. Questo approccio comparativo ha consentito di ampliare la comprensione dei meccanismi cellulari coinvolti nella vitrificazione e di valutare l'effetto del naloxone in due contesti biologici differenti. Nel modello bovino, gli oociti trattati con naloxone non hanno ottenuto risultati significativamente diversi dal controllo, né allo stadio immaturo che maturo, al contrario, nel cavallo gli ovociti maturi hanno evidenziato risultati opposti, con una riduzione della vitalità post-scongelamento. Ciò suggerisce che lo stadio meiotico rappresenti un fattore cruciale nell'influenzare la risposta cellulare al naloxone e al processo di vitrificazione, e che il modello bovino non possa riprodurre pienamente la risposta equina. Nonostante l'assenza di attivazione dei geni pro-apoptotici e una lieve riduzione nella produzione di specie reattive dell'ossigeno, la bassa vitalità degli ovociti equini maturi sottoposti al trattamento, unita ai bassi tassi di fecondazione e sviluppo embrionale, suggerisce che gli ovociti maturi equini non siano più resistenti di quelli immaturi in queste condizioni sperimentali. Nel complesso, i risultati indicano che la risposta al trattamento con naloxone e alla vitrificazione è specifica di specie e di stadio meiotico, confermando la necessità di ulteriori studi volti a chiarire i meccanismi cellulari coinvolti per sviluppare protocolli più efficaci di crioconservazione ovocitaria nel cavallo. Il terzo e ultimo studio si distingue dai precedenti per soggetto e finalità. L'obiettivo principale è stato quello di valutare un protocollo commerciale di vitrificazione modificato nei tempi di esposizione alle soluzioni crioprotettive e, contemporaneamente, di analizzare l'effetto di due diversi sistemi di supporto al congelamento**,** definiti rispettivamente semi-aperti e chiusi sulla base di un contatto o meno con l'azoto liquido, necessario per il congelamento ultrarapido tipico della vitrificazione. Il modello sperimentale utilizzato è stato quello bovino, scelto come riferimento per proporre un possibile modello animale per la riproduzione umana, in particolare per lo studio della crioconservazione dei gameti femminili. I risultati non hanno evidenziato differenze statisticamente significative tra i due sistemi di vitrificazione in termini di vitalità e recupero post-scongelamento. Per quanto riguarda il protocollo, i dati ottenuti sono stati positivi, mostrando buoni livelli di sopravvivenza

e assenza di variazioni significative nei parametri di stress ossidativo e contenuto di glutatione. Tuttavia, è stato osservato un aumento del potenziale di membrana mitocondriale, che potrebbe riflettere un incremento della domanda energetica cellulare durante la fase di recupero post-scongelamento o, alternativamente, rappresentare un evento precoce associato a processi apoptotici iniziali. Nel complesso, il lavoro ha dimostrato che il protocollo modificato garantisce ottimi risultati post-scongelamento, riducendo l'esposizione ai crioprotettori e mantenendo una buona qualità cellulare. Inoltre, entrambi i sistemi di vitrificazione testati si sono rivelati ugualmente efficaci e applicabili. Ulteriori studi saranno tuttavia necessari per valutare la competenza funzionale degli ovociti vitrificati mediante questo protocollo, in particolare in termini di fecondazione e sviluppo embrionale.

In conclusione, i risultati ottenuti nei tre studi confermano la complessità dei meccanismi che influenzano la vitrificazione ovocitaria, evidenziando come le differenze interspecie e intraspecie possano incidere in modo significativo sugli esiti del processo. Le informazioni raccolte contribuiscono ad ampliare la conoscenza in un ambito ancora poco esplorato e sottolineano la necessità di proseguire gli studi in questa direzione. Ogni nuovo dato rappresenta un passo avanti verso la definizione di un quadro più completo e coerente, fondamentale per lo sviluppo di protocolli di vitrificazione sempre più sicuri ed efficaci nelle diverse specie animali.

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List of abbreviations

aART	**a**dvanced **A**ssisted **R**eproductive **T**echnologies
ART	**A**ssisted **R**eproductive **T**echnologies
COCs	**C**umulus–**O**ocyte **C**omplexe**s**
CPAs	**C**ryo**p**rotective **A**gent**s**
CR	**C**orona **R**adiata
DMSO	**D**i**m**ethyl**s**ulf**o**xide
DPBS	**D**ulbecco's **P**hosphate–**B**uffered **S**aline
EG	**E**thylene **G**lycol
ET	**E**mbryo **T**ransfer
FBS	**F**etal **B**ovine **S**erum
FCS	**F**etal **C**alf **S**erum
GLU	**Glu**tathione
GLY	**Gly**cerol
GV	**G**erminal **V**esicle
HSOF	**H**epes–buffered **S**ynthetic **O**viduct **F**luid
ICSI	**I**ntracytoplasmic **S**perm **I**njection
IETS	**I**nternational **E**mbryo **T**echnology **S**ociety
IVEP	**I**n **V**itro **E**mbryo **P**roduction
IVC	**I**n **V**itro **C**ulture
IVF	**I**n **V**itro **F**ertilization
JC1	**J**–Complex 1
LN_2	**L**iquid **N**itrogen (N_2)
MI	**M**etaphase **I**
MII	**M**etaphase **II**
NX	**N**alo**x**one
OPS	**O**pen **P**ulled **S**traw
PG	**P**ropylene **G**lycol
ROS	**R**eactive **O**xygen **S**pecies
TCM 199	**T**issue **C**ulture **M**edium 199

PART I
Background and Literature Review

1. General Introduction

1.1. The importance of fertility preservation

The development of fertility cryopreservation for sexual gametes can be traced back to the 18th century with Spallanzani's studies on the effects of temperature on equine spermatozoa, earning him the title of “father of cryobiology.” However, it was only in the 20th century, following decades of research by physicists and biologists, including the discovery of glycerol as a cryoprotectant by Polge, that the field truly advanced, culminating in the first live offspring from mouse embryos cryopreserved in 1972 (Whittingham et al., 1972) to the first birth from a cryopreserved human oocyte in 1983 (Trounson & Mohr, 1983). Since then, cryopreservation techniques have evolved exponentially, achieving remarkable results in just over a century of scientific progress. To date, fertility preservation through the cryopreservation of male and female gametes (spermatozoa and oocytes respectively) has become a key tool with significant medical, social, and ethical implications.

This procedure, implemented through various techniques, allows for the preservation of cells in liquid nitrogen for an indefinite period and can be applied in both human and veterinary medicine, with specific adaptations and considerations for each field.

The use of cryopreservation allows us to overcome fertility limitations caused by reproductive pathologies or by medical treatments with gonadotoxic effects that impair an individual’s reproductive potential. It also addresses increasingly common social factors that lead to delayed parenthood, as well as the desire to preserve one's genetic and reproductive material during the most fertile stage of life, the so called “elective oocyte freezing” (Cascante et al., 2023). This technology offers women the possibility to plan a future pregnancy using their own gametes retrieved at a biologically more favourable age, thus bypassing the natural age-related decline in fertility. The social implications of cryopreservation are manifold and vary significantly depending on national legislation and the socioeconomic context of each country.

In the veterinary field, fertility preservation has enabled not only the global distribution and commercialization of gametes, particularly semen, but also substantial genetic improvement in livestock species and sport animals (Huang et al., 2019) . Lastly, research using animal models continues to represent a valuable opportunity for the development of new techniques and protocols translatable to human medicine, contributing to the advancement of knowledge in this emerging and fascinating area of assisted reproduction (S. Aljaser, 2022). Cryopreservation

represents a key step within the landscape of assisted reproductive technologies (ART), intersecting multiple disciplines. Its continuous development and study provide significant benefits to the entire field of in vitro science.

1.1. Aims and scope of the thesis

This thesis aims to explore the current state and potential of female gamete cryopreservation within the field of veterinary medicine, with particular emphasis on its application to species of economic and zootechnical relevance. Among these, the equine species represents a significant and challenging species of interest due to its high reproductive and economic value and the considerable difficulties still encountered in achieving consistent cryopreservation outcomes. Despite remarkable progress in cryobiology over the past decades, the cryopreservation of oocytes, especially in large domestic animals, remains an area of limited efficiency, with outcomes that are often highly variable and species dependent. In the equine in particular, post-warming viability and developmental competence of oocytes remain unsatisfactory when compared to those achieved in other species or in male gametes. The present research focused primarily on the refinement of vitrification protocols for equine oocytes, with the goal of minimizing the cellular damage typically induced by the cryopreservation process, while the bovine model has been used as a reference for improving human oocyte vitrification protocols. In addition to the optimization of technical procedures, the work also explored how vitrification affects key aspects of oocyte health, including membrane integrity, mitochondrial function, oxidative stress, and gene expression related to cell survival and apoptosis.

By integrating these levels of investigation, the thesis seeks to contribute to a deeper understanding of how to preserve the developmental competence of oocytes. This is essential not only for improving reproductive efficiency and genetic resource management in animal production systems, but also for supporting biodiversity conservation strategies and advancing translational applications in assisted reproduction.

2. Cryopreservation

Cryopreservation encompasses a group of techniques used to freeze and store cells, typically in liquid nitrogen at −196 °C, with the aim of preserving their viability and functional integrity upon thawing. In veterinary medicine, cryopreservation plays a pivotal role in the conservation of animal genetic resources, the optimization of ART, and the preservation of endangered or economically valuable species. In particular, the cryopreservation of oocytes and embryos has become increasingly relevant in the management of domestic and wild animal reproduction, contributing to biodiversity preservation, genetic selection programs, and the development of advanced research models.

Historically, embryo cryopreservation preceded the successful cryopreservation of oocytes. The first report of cryopreserved mouse embryos dates to 1972 (Whittingham et al., 1972), followed by successful applications in several species including cattle (Wilmut & Rowson, 1973), rabbits (Bank & Maurer, 1974), rats (Kasai et al., 1982), horses (Slade et al., 1985), and non-human primates (Balmaceda et al., 1986). These early achievements relied on the slow-freezing method, as reviewed by Estudillo et la. (Estudillo et al., 2021). In the veterinary field, bovine embryo cryopreservation rapidly became a routine practice, significantly improving the efficiency of artificial insemination and embryo transfer programs worldwide.

Although oocyte cryopreservation was initially more challenging, notable progress has been made across species. The first successful human oocyte cryopreservation was reported in 1988 (C. Chen, 1986), following earlier studies in mice (Parkening et al., 1976). In the veterinary domain, oocyte cryopreservation has been attempted in bovine (Otoi et al., 1995), porcine (Somfai et al., 2014) and equine (De Coster et al., 2020) among others mammals. This highlights the interest on the applicability of oocyte cryopreservation in animal breeding, genetic banking, and experimental research.

While in human medicine cryopreservation is widely employed for fertility preservation, whether for medical, social, or oncological reasons (Pomeroy et al., 2022), its utility in veterinary contexts is broader and often more strategic, encompassing population management, species conservation, and the refinement of reproductive biotechnologies.

2.1. Cryopreservation Techniques

The idea of preserve biological material as samples, cells, tissue, embryos, and gametes is deep accepted in the scientific community. Cryopreservation makes possible preserving biological

material in low volumes and costs, is largely used in the fields of reproduction, in human and in veterinary, where the procedures are evolving to gain the maximum result and the minimum loss of oocytes, in terms of quality and numbers. Two main techniques evolved until nowadays: *slow freezing* and v*itrification*.

Slow freezing is done employing moderate cooling temperature and low concentration of cryoprotectants. Vitrification is the last point of the advance cryopreservation techniques; it minimizes the volume and maximizes the temperature and the concentration of cryoprotectants. Although slow-freezing protocols are still employed in specific contexts, vitrification has become the most widely adopted technique for oocyte cryopreservation due to its superior outcomes in terms of survival and developmental competence. Other cryopreservation strategies, such as ultra-rapid freezing, controlled-rate freezing, or encapsulation-based methods, are used for various cell types and tissues; however, they will not be discussed here as they are not currently applied to oocytes.

2.1.1. Slow freezing

The earliest attempts to cryopreserve mouse oocytes date back to 1972, when Whittingham and colleagues employed dimethyl sulfoxide (DMSO) as a cryoprotective agent and applied a slow-freezing protocol initially developed for embryos (Whittingham et al., 1972). Although oocyte survival rates were moderate, developmental competence and implantation outcomes were poor. Similarly unsatisfactory results were reported in other species. It was not until the mid-1980s that the first pregnancies following oocyte cryopreservation were achieved (C. Chen, 1986; van Uem et al., 1987). Due to the limited success of these early studies, interest in oocyte cryopreservation remained low for several years.

The conventional slow-freezing approach involves the gradual reduction of temperature in the presence of cryoprotectants. Between 5 °C and −15 °C, cells undergo osmotic dehydration as water exits the oocyte, leading to extracellular ice formation. This results in a rise in extracellular solute concentration, which further enhances osmotic gradients and promotes continued water efflux. Consequently, the oocyte progressively shrinks due to cellular dehydration. Two dehydration steps are typically included before cells are cooled below freezing temperatures (Zacà, 2024). The first step involve use of cryoprotective agents (CPAs) enables the removal of intracellular water to prevent ice crystal formation. The presence of CPAs generates an osmotic gradient that draws water out of the cell. Once dehydrated and

shrunken, the cell gradually returns to its original size as the cryoprotectants permeate through the membrane.

The second step consist in the addition of non-permeating cryoprotectant to the solution to minimize cellular shrinkage. The most used agents are sugars such as sucrose and trehalose (Ashwood-Smith, 1987; Eroglu et al., 2002; Wright et al., 2004). These compounds induce a second-phase dehydration, during which the cell undergoes a more rapid volume reduction prior to freezing. This accelerated shrinkage further reduces the likelihood of intracellular ice crystal formation.

This cooling phase must be prolonged enough to ensure sufficient water removal, minimize supercooling of the cytoplasm, and limit the formation of intracellular ice crystals.

To initiate controlled extracellular ice formation and reduce intracellular ice nucleation, a process known as seeding is employed, typically at −7 °C to initiate extracellular ice formation. This is commonly performed by touching the straw wall with forceps pre-cooled in liquid nitrogen. The purpose of seeding is to nucleate the first ice crystal in the solution at a distance from the oocytes, thereby preventing supercooling of the entire medium (Jain & Paulson, 2006). The resulting extracellular crystallization facilitates gradual dehydration of the oocyte.

During warming, small intracellular ice particles that may have formed can coalesce into larger crystals, a phenomenon known as recrystallization, which can damage cellular structures. Although the exact mechanisms by which warming rates affect cell survival remain unclear, rapid warming is considered beneficial as it reduces intracellular ice formation and associated cryoinjury. Cryoprotectants must be removed progressively during thawing to avoid osmotic shock (Porcu et al., 2012).

Automatic temperature control is achieved through a computerized freezing system, which consists of a cooling chamber into which liquid nitrogen is injected according to a preset program managed by the system.

Despite the significant scientific impact it achieved upon its introduction for gamete cryopreservation, the slow freezing technique has been almost entirely replaced by vitrification. The adoption of vitrification has simplified the cryopreservation process, both in terms of equipment requirements and manual handling. For these reasons, vitrification is now regarded as the preferred method for gamete cryopreservation in both human and veterinary medicine.

2.1.2. Vitrification

Nowadays, vitrification is the best strategy for human cryopreservation from mature or immature oocyte to the blastocyst stage (Da Luz et al., 2022).

The development of the vitrification technique and its application in the field of reproduction are credited to Rall and Fahy, who, after years of work, successfully vitrified mouse embryos in 1985 (Rall & Fahy, 1985). Their achievement marked a turning point, as the technique yielded results comparable to those obtained with conventional slow freezing.

The first child born from vitrified human oocytes was reported in 1999 (Kuleshova et al., 1999), using the open pulled straw (OPS) system introduced by Vajta in 1997 (Vajta et al., 1997), which yielded better outcomes compared to conventional straws employed in slow freezing (Vajta et al., 1998, 1999). Following the development of new devices, improved cryoprotectant solutions, and the introduction of commercial kits, vitrification has progressively expanded since the mid-2000s. To date, the market offers a wide range of freezing and warming kits for both medical and veterinary applications, featuring different formulations, protocols, and devices (e.g., HSV, Cryotip, Rapid-i, and Cryolock).

Vitrification is an ultra-rapid, manually controlled cooling process that leads to a glass-like solidification of cells and their surrounding medium (Chang et al., 2022). The technique requires high concentrations of CPAs in combination with small sample volumes and extremely fast cooling and warming rates it is indeed a highly complex process characterized by a reciprocal relationship between the cooling rate necessary to achieve vitrification and the concentration of cryoprotectants and solutes (Karlsson, 2001).

During the process, cells undergo osmotic dehydration upon exposure to highly concentrated vitrification solutions. Intracellular water exits the cell and is replaced by permeating CPAs. As a result, the cell becomes highly dehydrated and is suspended in a concentrated CPA medium. The sample is then directly immersed in liquid nitrogen (LN_2), causing rapid cooling. Due to the substantial reduction in water content, the system transitions from a liquid to a solid amorphous state without the formation of ice crystals. This ice-free, glass-like matrix preserves cellular structures by avoiding ice-induced mechanical damage (Da Luz et al., 2022).

In the warming process, the cells rehydrate while the cryoprotectants are diluted (Cobo et al., 2017). The faster the warming the higher the survival. At the highest warming rate of 2950°C/min, survivals are above 80% at all cooling rates between 187 and 1827°C/min. They are almost 70% even with cooling rate of 37°C/min (Seki & Mazur, 2009).

The warming phase is a critical step in the vitrification process, as survival depends not only on cooling but also on how intracellular water and ice behave upon rewarming. As outlined by Seki et al. (Seki & Mazur, 2009), three scenarios may occur: when large intracellular ice crystals have formed, survival is virtually null regardless of the warming rate; when small crystals are present, rapid warming is essential to prevent recrystallization; and when water has vitrified, devitrification and subsequent crystallization occur, with the outcome strongly influenced by warming speed. Seki et al. prove that rapid to ultrarapid warming is the key determinant overriding conditions created at any cooling rate. These observations highlight the central role of warming as a determinant of vitrification success.

Vitrification has progressively established itself as the elective method for cryopreservation, Levi-Setti et al. (Levi-Setti et al., 2016), in a retrospective analysis of Italian human assisted reproduction centres, reported an increasing use of vitrification between 2009 and 2014. The study showed improved outcomes and a progressive shift from slow freezing to vitrification. Nevertheless, while vitrification outperformed slow freezing, its results remained inferior compared with fresh oocytes. Furthermore, vitrification exerts a milder impact on gamete metabolism: although cryoinjury remains present and significant, Gardner et al. (Gardner et al., 2007) demonstrated that vitrification has a lower effect on energetic metabolism and membrane integrity compared with slow freezing. From a practical perspective, the advantages in terms of time, cost, and efficiency are also evident, as the technique requires smaller amounts of consumables and solutions. On the other hand, its successful application demands highly trained personnel with substantial technical expertise.

The development and spread of vitrification have represented a milestone in the field of cryobiology and reproductive biotechnologies. However, the remarkable success achieved in human medicine has not been equally reflected in veterinary applications. Despite significant progress and numerous studies, the considerable variability among species—whether domesticated and used for production purposes, or wild and endangered—makes it difficult to establish a universal protocol suited to the specific requirements of each species (Kamoshita et al., 2024). This limitation is particularly evident in the vitrification of oocytes, whereas in the case of embryos and semen the technique is now widely applied and provides satisfactory outcomes, although still inferior to those obtained with non-cryopreserved gametes or embryos. The widespread adoption of this technique has, however, raised concerns about its potential long-term effects and, consequently, about the safety of its use. (Da Luz et al., 2022).

Several studies have investigated whether vitrification may have potential effects on offspring, both in animal models and in humans. In a rabbit model, a comparative study (Garcia-

Dominguez et al., 2020) reported differences between offspring derived from vitrified embryos and those conceived naturally, including altered growth rates and transcriptomic and proteomic profiles, with evidence of disturbed lipid metabolism and impaired mitochondrial function, possibly linked to placental development and function. In humans, Belva et al. (Belva et al., 2023) found that embryo vitrification was associated with higher birthweight and an increased risk of large-for-gestational-age (LGA) infants, but with a lower risk of small-for-gestational-age (SGA); however, anthropometry and growth parameters in early childhood did not differ from those of children born after fresh embryo transfer. Similarly, in human medicine, Van Reckem et al. (Van Reckem et al., 2021) compared two-year-old children born from vitrified and fresh donated oocytes and reported no significant differences in body length, weight, BMI, head circumference, arm circumference, or waist circumference. In livestock, Gómez et al. (E. Gómez et al., 2022) provided the first comparison of calves born from fresh, frozen, and vitrified in vitro–produced embryos, highlighting that embryo cryopreservation and/or culture can yield metabolically distinct calves, particularly regarding protein and acid–base metabolism.

Taken together, these studies suggest that offspring derived from vitrified gametes or embryos may present differences compared with those from fresh counterparts. Epigenetic modifications are known to occur during cryopreservation and in vitro production, yet it remains unclear to what extent these changes translate into clinically significant consequences.

Nevertheless, vitrification continues to be widely applied in human medicine due to its undeniable advantages, and ongoing research to adapt and refine protocols for animal gametes remains both necessary and highly valuable.

2.2. Factors affecting outcomes

Vitrification is a multifactorial technique whose success depends on the delicate balance and interplay of several variables. The efficiency of the process is not determined by a single element, but rather by the correct integration of multiple factors, each of which can significantly influence the outcome. Even the operator's skills and precision contribute to variability in results. In the following sections, only the main variables, and those most extensively documented in the veterinary literature, will be discussed.

2.2.1. Cryodevices

Cryodevices are specialized tools developed to optimize vitrification by enhancing cooling rates and heat transfer. They can be broadly divided into two main categories: tubing devices and surface devices (Hochi, 2022).

Tubing devices, such as the open pulled straw (OPS) (Vajta et al., 1998), allow very high cooling rates and were among the first tools successfully applied to minimum volume vitrification (MVC) of mammalian oocytes. Surface devices, including the Cryoloop (Lane, Bavister, et al., 1999; Lane, Schoolcraft, et al., 1999; Mukaida et al., 2003) and the Cryotop (Katayama et al., 2003; Kuwayama, 2007), as well as other supports such as electron microscope grids, nylon meshes, and multilayer silk fibroin (Liebermann et al., 2003; Vajta & Nagy, 2006) are designed to minimize the volume of surrounding vitrification solution. Their thin solution film, combined with direct contact with liquid nitrogen, enables ultrarapid cooling rates and has made them widely used in both human and veterinary medicine. These systems were specifically developed to maximize cooling efficiency, improve oocyte loading capacity, and facilitate the removal of excess solution.

Among surface devices, the Cryotop was introduced as a more efficient alternative to the OPS (Kuwayama, 2007). By loading oocytes into an extremely small droplet of solution (<1 µl), Cryotop achieve faster cooling and warming rates, resulting in higher proportions of oocytes with normal spindle configuration and improved cleavage and blastocyst rates in several species, including rabbit (Li et al., 2012), pig (Liu et al., 2008), and bovine (Morató et al., 2008). While OPS represented the starting point for early vitrification studies, technological advances have progressively shifted the field toward devices offering greater performance. Nonetheless, mastering the technique still requires extensive training and precision.

Despite their advantages, these devices also present limitations. Cooling rates may be less efficient in the interior of the sample, and open vitrification carriers inherently expose biological material to liquid nitrogen, with a theoretical risk of microbiological contamination (Amini & Benson, 2023). To address these concerns, closed systems have been developed, in which the sample remains physically separated from liquid nitrogen during cooling, storage, and warming (Porcu et al., 2021). This separation provides a more aseptic environment and reduces contamination risks, although it can also reduce thermal exchange and thereby compromise cooling efficiency. Indeed, a meta-analysis by Youm et al. (Youm et al., 2018) reported that closed vitrification might be associated with lower survival rates compared to open vitrification in human mature oocytes.

Vajta et al. (Vajta et al., 2015), in a review, highlighted that most vitrification devices, particularly the commonly used ones, do not ensure complete sterility for gametes during either the freezing or the warming phase. Concerns about pathogen transmission became particularly evident during and after the COVID-19 pandemic, although no cases have been attributed to the use of cryopreserved gametes in human medicine (Pomeroy & Schiewe, 2020). Nevertheless, no cases of disease transmission attributable to liquid nitrogen-mediated cross-contamination or other cryopreservation-related sources have been reported to date in human o veterinary medicine.

Overall, the choice between open and closed systems reflects a balance between efficiency and safety. Open devices remain more widely adopted due to their consistently higher success rates, whereas closed systems are increasingly considered in contexts where biosafety is a priority.

2.2.2. Cryoprotective agents (CPAs)

One of the most critical aspects of vitrification is the composition and concentration of CPAs, as these determine the likelihood of achieving high survival rates and maintaining oocyte viability after warming. CPAs are chemical agents that mitigate or prevent ice formation and reduce cryoinjury (Pegg, 2015). They can be broadly classified into two categories: penetrating CPAs and non-penetrating CPAs.

Penetrating CPAs cross the cell membrane and include compounds such as ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (DMSO), glycerol (GLY), formaldehyde, and methanol. Their main function is to reduce osmotic shrinkage of cells and to prevent excessive intracellular electrolyte concentrations, which would otherwise occur if only non-permeating solutes were used. Non-penetrating CPAs, such as trehalose, sucrose, polyethylene glycol, polyvinyl alcohol (PVA), and polyvinylpyrrolidone (PVP), remain extracellular and primarily act as osmotic buffers and membrane stabilizers.

The mechanism of CPA action is mainly based on preventing ice crystal formation. By increasing solute concentration, water mobility is reduced, the melting point decreases, and intracellular water solidifies into an amorphous glass-like structure instead of forming damaging crystals (Amini & Benson, 2023). The permeability of different CPAs varies, with the order being glycerol > EG > DMSO > PG (Lotz et al., 2021). Oocytes, however, are characterized by low water and cryoprotectant permeability, making them particularly vulnerable to osmotic damage (Canesin et al., 2017) and to temperature-induced injury (Pickering et al., 1990).

CPA toxicity depends on several factors, including concentration, exposure time, and temperature. The literature shows variability in which CPA is deemed most or least toxic, likely reflecting differences in experimental conditions, cell type, and species. Generally, higher CPA concentrations can cause significant damage to oocytes, even after short exposure times. Concentrations of around 1.5 M are typically used in slow-freezing protocols, while vitrification requires much higher concentrations, making precise timing and handling essential to avoid toxicity.

In slow-freezing, CPAs replace part of the intracellular water during gradual cooling, thereby reducing damage by harmonizing cooling rates with membrane permeability (Jang et al., 2017). Their protective mechanism is not yet fully understood but may be linked to their ability to lower the freezing point and prevent ice crystal formation by forming hydrogen bonds with water molecules (Towey et al., 2012). During warming, CPA removal must be gradual to avoid osmotic shock and cell lysis; stepwise dilution, often in the presence of extracellular CPAs such as sucrose, is commonly used to buffer osmotic stress (Pomeroy et al., 2022; Yong et al., 2020). In addition, so-called ice blockers, such as polyglycerol and polyvinyl alcohol, represent a class of non-permeating agents that help inhibit ice growth during warming (Pomeroy et al., 2022).

Several other factors also influence cell survival during cryopreservation, including:

- The osmotic response of the cell in permeating CPA solutions.
- Changes in extracellular solute concentration as temperature decreases and ice forms.
- Osmotic water loss caused by extracellular ice formation.
- Cooling rate, particularly between -5°C and -35°C.
- Storage temperature and handling during storage.
- Warming rate of the cryopreserved sample.
- The osmotic response of the cell during CPA removal

(Pomeroy et al., 2022).

Ultimately, the success of cryopreservation and warming depends not only on the solutions used but also on proper training and precise execution of the protocols (Pomeroy et al., 2022).

In veterinary medicine, the composition and concentration of cryoprotectants are among the most critical variables influencing the success of vitrification protocols. Their choice largely depends on the species, as both the permeability of the oocyte membrane and the susceptibility to osmotic and toxic effects vary.

On bovine oocytes, studies have highlighted how the type and concentration of CPAs strongly influence survival and developmental competence after vitrification. Martins et al. (Martins et

al., 2005) reported that different equilibration times (0.5, 5, or 15 min) had no significant impact on post-thaw survival, but the concentration of ethylene glycol played a crucial role. The highest concentration tested (40% EG) was detrimental, while an intermediate concentration (20% EG) supported the best outcomes, by contrast, very low concentrations (3% EG) yielded only limited maturation rates (18-19%). The low permeability of bovine oocytes, particularly at the MII stage, further complicates CPA movement across the plasma membrane (Mogas, 2018). Another important aspect of oocyte cryopreservation is the concentration of cryoprotectants. Magnusson et al. (Magnusson et al., 2008) showed that increasing EG concentration in vitrification media negatively affected the developmental competence of bovine oocytes, with higher concentrations leading to reduced embryo formation. Similar observations were reported by Papis et al. (Papis et al., 2000), who obtained the best outcomes using a combination of EG (5.5 M) and sucrose (1 M) after a short equilibration step in low EG concentration (3%), confirming that excessively high CPA concentrations compromise oocyte developmental potential.

Among penetrating CPAs, EG and DMSO remain the most widely employed. Other compounds such as propylene glycol, glycerol, and polymers like Ficoll 70 have also been tested (Abdel-Gawa et al., 2016; Chian et al., 2004; El-Shahat & Hammam, 2014). For example, Abdel-Gawad et al. compared different CPA combinations, thawed blastocyst recovery was markedly higher with Ficoll (79.3%) than with DMSO (45%). In contrast, Anchamparuthy et al. (Anchamparuthy et al., 2009) observed reduced blastocyst production when Ficoll was combined with EG and sucrose. Similarly, Martins et al. (Martins et al., 2005) reported that sucrose supplementation yielded better maturation rates than trehalose.

Overall, EG appears to be less damaging than DMSO for bovine oocytes, consistent with Cetin and Bastan (Cetin & Bastan, 2006). Nevertheless, DMSO continues to be widely applied, likely due to its smaller molecular size and higher membrane permeability compared with glycerol (Yamada et al., 2007). The same author further highlighted the importance of CPA choice by demonstrating that glycerol, when combined with EG, produced the lowest maturation rates compared with other CPA mixtures. Glycerol, one of the earliest cryoprotectants introduced in cryobiology, is commonly employed in the cryopreservation of spermatozoa due to its ability to penetrate the cell membrane and protect against intracellular ice formation. However, its relatively low permeability and tendency to induce pronounced osmotic shrinkage make it less suitable for oocytes compared with EG or DMSO.

As highlighted by Paynter (Paynter, 2005), glycerol is generally unsuitable for mature oocytes, particularly in mice, due to its low permeability and the severe osmotic shrinkage it induces

compared with other CPAs. Temperature was also identified as a key variable, as higher incubation temperatures reduce osmotic stress by minimizing cell shrinkage, but simultaneously increase the risk of CPA toxicity. For instance, at 30°C, EG induced greater oocyte shrinkage compared to DMSO, whereas at 10°C the differences were negligible.

In the horse, the most widely used permeating CPA is ethylene glycol, which has higher membrane permeability compared with dimethyl sulfoxide and glycerol. This property allows EG to cross the plasma membrane more efficiently, ensuring better intracellular protection during cryopreservation (Içli et al., 2021). EG is also considered relatively safe, showing no significant toxicity in mammalian oocytes, including in murine models (Szurek & Eroglu, 2011). Equine vitrification protocols often combine EG with DMSO and sucrose, as the use of multiple CPAs reduces the toxicity associated with high concentrations of individual agents (Vajta & Kuwayama, 2006). EG has been a standard cryoprotectant for decades (Bautista & Kanagawa, 1998), and its efficacy was further confirmed by more recent successes, including the production of live foals (Canesin et al., 2018). Propylene glycol has been less frequently employed in equine oocyte vitrification, but promising results have been reported when combined with DMSO or EG. Canesin et al. (Canesin et al., 2017), for example, obtained equine blastocyst development using EG-PG with sucrose, while Lotz et al. (Lotz et al., 2021) demonstrated higher membrane permeability for PG than EG. Angel-Velez et al. (Angel-Velez et al., 2021) reported that solutions containing EG-PG with galactose showed maturation and blastocyst rates comparable to EG-DMSO with sucrose, but with slightly higher blastocyst yields (9.4% vs. 5%).

In swine, oocyte cryopreservation has historically been more challenging due to their high lipid content, which increases susceptibility to chilling injury and it has not yet become clinically available (Somfai, 2024). The most commonly used permeating CPAs in porcine oocytes are EG and DMSO, often combined with trehalose or sucrose as non-permeating solutes. Somfai et al. (Somfai et al., 2015) demonstrated that the combination of PG+EG at 4% concentration was more efficient than EG+DMSO, particularly when exposure times were shorter than 15 minutes. However, oocyte survival and developmental competence after vitrification remain low compared with fresh control and, more broadly, with other mammalian models.

The toxicity of CPAs remains a crucial concern, but it has been shown that their adverse effects can be mitigated by combining two or more cryoprotectants (Kuwayama, 2007). Although PG can induce toxicity at high concentrations, this effect can be mitigated by combining lower doses of PG with DMSO (Szurek & Eroglu, 2011). Similarly, studies on DMSO in human oocytes have shown transcriptomic alterations associated with vitrification (Wiltshire et al.,

2023), but its specific impact on the oocyte genome remains unclear. Importantly, other studies reported that DMSO exposure at moderate concentrations did not impair survival, fertilization, or blastocyst development, nor did it increase chromosomal abnormalities (Szurek & Eroglu, 2011). In bovine oocytes, Angel-Velez et al. (Angel-Velez et al., 2021) demonstrated that both high and low CPA concentrations reduced blastocyst rates compared with fresh oocytes, highlighting the detrimental effect of vitrification on embryo development.

Non-permeating CPAs, such as sucrose and trehalose, and macromolecules like Ficoll, bovine serum albumin, or synthetic polymers (e.g., polyvinylpyrrolidone, polyvinyl alcohol, SuperCool X-1000), are commonly used to minimize the toxic effects of permeating CPAs. By increasing extracellular viscosity, these agents reduce the risk of intracellular ice formation and lower the concentration of permeating CPAs required (B. Jin et al., 2011). For example, Curcio et al., (Curcio et al., 2014) reported 36% maturation in immature equine oocytes vitrified with a solution enriched with 1% ice blockers. In horses, sucrose remains the most employed non-permeating CPA, and its combination with EG and DMSO has led to the production of live foals (Ortiz-Escribano et al., 2018). Nevertheless, trehalose has shown superior results compared to sucrose in some studies, with higher maturation, cleavage, and blastocyst development rates (Canesin et al., 2017).

Additional strategies have been tested to reduce cryodamage. In bovine oocytes, glutathione supplementation during post-thaw culture improved blastocyst rates, reduced apoptotic cells, and counteracted cytoskeletal damage (Olexiková et al., 2022). Similarly, EGTA (Gutierrez-Castillo et al., 2023) and methyl-β-cyclodextrin (CLC) have been explored to stabilize membranes and enhance competence. In the horse, other molecules such as ice blockers (Curcio et al., 2014), melatonin (Clérico et al., 2021), and naloxone (Gugole et al., 2025) have been tested. Antifreeze proteins (AFPs) have been investigated as potential additives, as they can interact with ice crystals and inhibit recrystallization. While positive results have been reported in mice (Jo et al., 2011), rabbits (Nishijima et al., 2014), and sheep embryos (Correia et al., 2024), Chaves et al. (Chaves et al., 2016) did not observe protective effects during bovine oocyte or embryo vitrification. These interventions produced encouraging outcomes but did not consistently improve maturation or blastocyst rates.

In conclusion, defining an optimal vitrification solution composition for oocytes remains highly challenging. The success of cryopreservation depends on a delicate balance between CPA concentration, exposure time, and handling conditions, but these variables interact differently across species due to diverse oocyte characteristics. This makes it difficult to design a universal protocol applicable to all mammalian oocytes. Moreover, CPA-related toxicity continues to

represent a major limitation: while combinations and additives can mitigate some of its effects, they cannot fully eliminate the risks of cellular damage or reduced developmental competence. These challenges underline the need for continued research aimed at tailoring species-specific protocols and improving the safety and efficiency of oocyte cryopreservation.

2.2.3. Meiotic state

The meiotic stage of the oocyte plays a critical role in determining the outcome of vitrification. Oocytes at different stages, germinal vesicle (GV) or metaphase II (MII), display distinct ultrastructural characteristics that influence their susceptibility to cryodamage. Vitrification can compromise not only the ultrastructural integrity of oocytes but also their ability to repair damage, which varies between developmental stages (López et al., 2021). For instance, exposure of MII oocytes to temperatures below room temperature can irreversibly disrupt meiosis, causing spindle damage and chromosome scattering (Pickering et al., 1990)(Pickering et al., 1990), while GV oocytes exposed to low temperature may suffer altered cortical granule distribution and premature zona pellucida hardening (Canesin et al., 2017).
GV oocytes offer certain practical advantages: they can be collected and cryopreserved before reaching full maturation, expanding the window for breeding and providing greater flexibility in scheduling. Moreover, in large animals like horses, a higher number of GV oocytes can usually be retrieved per ovum pick-up (OPU) session compared to MII oocytes (Hinrichs, 2018). However, GV oocytes are less tolerant to cryopreservation due to their higher water content and immature cytoskeletal organization, making them more vulnerable to cryoinjury. At the same time, their chromatin configuration, being decondensed and protected by a nuclear envelope, may shield them from chromosome aberrations, a risk more pronounced in MII oocytes (Hinrichs, 2020). Despite these features, vitrification of GV oocytes remains technically challenging and is generally associated with reduced blastocyst development across mammalian species (Somfai, 2024; Tharasanit et al., 2009; Zhou et al., 2010).

In human medicine, vitrification is primarily applied to MII oocytes obtained from hyperstimulated donors yet attempts to vitrify GV oocytes have been reported. These studies typically show lower maturation potential (Cao & Chian, 2009), although some describe normal morphology and zona pellucida birefringence (Nazari et al., 2011; Shahedi et al., 2013). Interestingly, Lee et al. (Lee et al., 2014) demonstrated superior mitochondrial, chromatin, and spindle maturation in vitrified GV oocytes compared with vitrified MII oocytes, suggesting that

immature oocytes could offer some resilience. GV oocytes are also considered a possible option for fertility preservation in cancer patients for whom ovarian stimulation is not feasible (Mohsenzadeh et al., 2019).
MII oocytes, by contrast, are fully mature and immediately competent for fertilization, eliminating the need for additional maturation steps. This stage is therefore the preferred choice in human medicine (S. U. Chen et al., 2003). However, their meiotic spindle is highly sensitive to temperature fluctuations and freezing, leading to reduced developmental competence after thawing (S. U. Chen et al., 2003; Saunders & Parks, 1999). In some studies, vitrification of GV oocytes has been suggested as a safer alternative (Hochi et al., 1994), though developmental efficiency remains suboptimal.

In veterinary species, outcomes vary widely. In horses, live foals have been obtained from vitrified oocytes, with better efficiency reported for MII oocytes matured in vivo (Maclellan et al., 2002). Comparative studies in cattle and horses show consistently higher survival and maturation rates in bovine oocytes at both stages, with MII oocytes generally outperforming GV oocytes in both species (Agnieszka et al., 2021; Hurtt et al., 2000). In contrast, several studies demonstrated that mammalian oocytes such as those of humans, cattle, and pigs are more resilient to certain cryopreservation protocols at the mature stage than at the immature stage (Goud et al., 2000; Otoi et al., 1995; Rojas et al., 2004).
Additional work in equine oocytes highlights the influence of cumulus cells, with some studies reporting better cleavage and blastocyst development in MII oocytes, while others suggest GV oocytes with intact cumulus structures may fare better (Angel et al., 2020; Tharasanit et al., 2006). Notably, the few successful pregnancies reported in horses from vitrified oocytes have been obtained from GV oocytes (Clérico et al., 2021; Ortiz-Escribano et al., 2018).
In summary, both GV and MII stages present advantages and limitations for vitrification. GV oocytes may offer greater flexibility for collection and reduced chromosomal risks, but they are more sensitive to osmotic and cytoskeletal damage. MII oocytes are developmentally competent and the preferred option in clinical settings, yet they remain highly vulnerable to spindle disruption. Evidence from both human and veterinary medicine suggests that no stage is universally superior: the optimal choice depends on the species, technical approach, and intended application.

2.2.4. Corona radiata or Cumulus Oocytes Complexes

The role of cumulus cells during oocyte vitrification has long been debated, as they can provide both protective and detrimental effects depending on the stage of maturation and the cryopreservation protocol used.

Cumulus cells are thought to protect the oocyte from low temperature damage, particularly at the GV stage, but at the same time they represent a physical barrier that slows the penetration of cryoprotectants. This is a critical limitation in ultrarapid vitrification protocols, where CPA permeation must occur within seconds.

At the MII stage, the role of cumulus cells appears less crucial, since the oocyte has already matured. In theory, removal of the cumulus should therefore have fewer consequences for developmental competence. Nevertheless, cumulus cells secrete factors and maintain gap-junctional contact with the oocyte, which can enhance sperm penetration and fertilization rates in conventional IVF (in vitro fertilization), as reported in cattle (Tanghe et al., 2003) and cats (Godard et al., 2009). While better blastocyst rate were reported in mature or immature cumulus-oocytes in ovine (Dos Santos-Neto et al., 2020) and in mice (Nikseresht, 2015). I

Interestingly, some authors have even proposed a protective effect of cumulus during cryopreservation of MII oocytes (Imoedemhe & Sigue, 1992). Evidence in the horse also supports this view: equine oocytes vitrified at the MII stage with cumulus cells intact were able to produce viable pregnancies (Maclellan et al., 2002), while denuded oocytes showed high spindle abnormalities and poor developmental outcomes following intracytoplasmic sperm injection (ICSI) (Tharasanit et al., 2006). By contrast, in human medicine, current protocols almost universally involve cumulus removal prior to vitrification, with studies showing no reduction in viability (Fabbri et al., 2000) and even improved competence in bovine oocytes vitrified with the Cryotop method (Chian et al., 2004).

In human medicine, oocytes are generally denuded to assess their meiotic stage prior to vitrification. Several studies have examined the potential influence of cumulus cells on the outcomes of oocyte vitrification. In one study (Tong et al., 2012), cryopreservation-related changes such as the number of cortical granules and zona hardening did not differ between oocytes vitrified with cumulus and those denuded. However, fertilization rates (after IVF) were higher in cumulus-enclosed oocytes, although cleavage and blastocyst rates were ultimately comparable. A second study reported that oocytes vitrified without cumulus recovered their meiotic spindle more rapidly than those with cumulus (Minasi et al., 2012) . Only one (H.-X.

Jin et al., 2012) report describes improved outcomes, such as post-thaw survival, cleavage, implantation, and pregnancy rates, in oocytes frozen with cumulus.

The state of cumulus expansion may also influence vitrification outcomes. Cumulus–oocyte complexes (COCs) can be classified as compact or expanded (Hinrichs et al., 1993). Expanded cumulus oocytes have already initiated cytoplasmic maturation, enabling faster progression to MII in vitro compared to compact COCs (Alm & Hinrichs, 1996). This developmental advantage may affect their resilience to cryopreservation.

Recent studies in the horse have further investigated this aspect. Angel et al. (2020) compared immature oocytes with corona radiata (IMM), matured oocytes with corona radiata (MAT CR+), and matured oocytes without cumulus (MAT CR–), vitrified using Cryotech®. They found the highest cleavage rates in MAT CR– oocytes, although the only blastocyst obtained derived from MAT CR+. These results support the idea that vitrification at the MII stage surrounded by corona radiata cells, offers higher developmental competence than vitrification at the GV stage, in partial contrast with Tharasanit et al. (Tharasanit et al., 2006), who reported better cleavage rates from GV with cumulus vitrification.

Taken together, the presence of cumulus cells during vitrification exerts both protective and restrictive influences. At the GV stage, cumulus cells may provide shielding but hinder CPA penetration; at the MII stage, their role appears less critical, though some studies highlight improved outcomes when cumulus is maintained. Results remain species-specific: in horses, intact cumulus seems advantageous, while in human medicine denudation has become standard practice.

Ultimately, the impact of cumulus cells must be evaluated in relation to the meiotic stage, vitrification protocol, and species, with ongoing research needed to clarify their exact contribution to post-warming competence.

2.2.5. Exposure time

The duration of exposure to cryoprotectants is a critical factor in the success of oocyte vitrification. Finding the right balance is essential: exposure must be long enough to ensure adequate dehydration and prevent intracellular ice formation, yet short enough to minimize the toxic effects of CPAs on oocyte structure and function.

During CPA exposure, osmotic volume changes occur due to the movement of water out of the cell and CPAs into the cytoplasm. In mature (MII) oocytes, these changes may disrupt the meiotic spindle, whereas in immature (GV) oocytes they can interfere with microfilament

organization (Heo et al., 2011). Prolonged exposure increases the risk of toxicity, leading to reduced survival, fertilization, and developmental competence, while excessively short exposures may leave the oocyte insufficiently protected. Toxicity is influenced not only by exposure time but also by CPA type, concentration, and temperature. Penetrating CPAs such as EG and DMSO become more harmful at higher concentrations, with longer exposure durations, or under warmer conditions. Proposed mechanisms for CPA toxicity include hydrophobic interactions with proteins, disruption of hydrogen bonding with water, intracellular pH changes, calcium release (Gardner et al., 2007), and even the formation of formaldehyde in preservation media (Karran & Legge, 1996). The meiotic stage also plays a role: GV oocytes, due to their higher water content and cytoskeletal immaturity, often require longer equilibration times for proper dehydration, while MII oocytes are more rapidly permeabilized but also more sensitive to structural damage. The presence of cumulus cells can further slow CPA influx, risking suboptimal intracellular concentrations, since water efflux occurs quickly (within 20 s), while CPA entry is slower (B. Jin et al., 2011).

Experimental studies highlight these dynamics. Ortiz-Escribano et al. (Ortiz-Escribano et al., 2018) compared short and long vitrification protocols in equine, and found that although cleavage rates were not significantly different, blastocyst formation occurred only in oocytes vitrified with the shorter protocol. Similarly, studies on murine oocytes suggested that prolonged exposure did not always reduce maturation rates but strongly impaired blastocyst development. In equine, Canesin et al. (Canesin et al., 2017) reported limited developmental success with long protocols, although differences in devices and warming solutions may partly explain variability between studies. Early investigations also showed that freezing and CPA exposure alone can induce cytoskeletal disorganization, spindle depolymerization, and abnormal chromosome segregation (Aman & Parks, 1994; Eroglu et al., 1998), contributing to increased chromosomal abnormalities (Glenister et al., 1987). These findings suggest that while species-specific variability exists (Szurek & Eroglu, 2011), minimizing exposure time is generally favourable to reduce the window of CPA toxicity.

Modern vitrification protocols therefore emphasize extremely short equilibration steps with relatively high CPA concentrations, followed by rapid cooling. Current approaches often reduce the final CPA exposure to less than one minute (Shaw et al., 1992), balancing adequate dehydration with minimal toxicity. However, Yamada (Yamada et al., 2007) showed that bovine oocytes exposed to vitrification solutions for 60 s, compared with 30 s, displayed signs of toxicity at both low and high CPA concentrations. Nevertheless, the highest post-vitrification maturation rates were obtained in the group exposed to the higher CPA concentration.

Additional strategies, such as stepwise equilibration and the use of non-permeating CPAs (e.g., sucrose) as osmotic buffers, further mitigate osmotic shock and protect oocyte integrity (Chang et al., 2022).

A recent study conducted on equine oocytes, by Du et al. (Du et al., 2024), further investigated the impact of CPA exposure by testing multiple combinations of equilibration and vitrification conditions. The authors compared equilibrium solutions with increasing concentrations of EG and DMSO with vitrification solutions of increasing CPA concentrations (30%, 35%, and 40%) and combined these with progressively longer exposure times (40, 80, and 120 s), generating a total of 27 experimental groups. The best outcome was achieved with the lowest concentration equilibration solution**,** the lowest concentration vitrification solution (30%)**,** and an intermediate exposure time (80 s)**,** which yielded a maturation rate of about 19%**.** Notably, the control group in that study reached only 26% maturation, a value generally lower than those reported in most other studies.

The timing of CPA exposure is a decisive variable in oocyte vitrification, influencing osmotic balance, cytoskeletal integrity, and developmental competence. While prolonged exposure increases toxicity, overly brief exposure risks insufficient protection. Evidence across species indicates that shorter, well-calibrated exposure protocols, combined with supportive additives, offer the best compromise, though optimal timings remain species- and stage-dependent.

2.3. Cryoinjuries

Cryoinjuries refer to the damages that occur during the process of cryopreservation, these damages can include cellular dehydration, intracellular ice formation, and toxicity effects caused by the CPAs, which all can ultimately lead to cell death. These damages can occur due to exposure to cryoprotective agents, cooling, or a combination of both.

Since the earliest development of cryopreservation techniques, it has been observed and demonstrated that oocytes are more sensitive to cryo-damage than embryos (Friedler et al., 1988).

Chang et al. (Chang et al., 2022) summarized several key risks:

- Oocyte spindles, in both mouse and human models, are extremely sensitive to low temperatures and depolymerize rapidly; however, CPAs can stabilize microtubules and mitigate this effect.

- CPAs like DMSO and EG can trigger transient calcium influx and zona hardening, though oocytes often recover these calcium stores after warming.
- CPAs may exert genotoxic effects, potentially leading to chromosomal abnormalities; their long-term safety is still under investigation.
- Epigenetic modifications, including transgenerational effects, have been reported in animal models after vitrification and warming, though little is known in humans.

It is important to consider that different stages of oocyte maturation exhibit distinct intracellular structures, such as the conformation of the genetic material in the GV stage compared with MI or MII, reflecting a different susceptibility to cryo-damage.

During vitrification, various intracellular structures can be injured by freezing stress. The nucleus, for example, is not composed solely of genetic material but also of the nuclear envelope, a bilaminar structure essential for numerous nuclear and cytoplasmic processes, and the nuclear lamina, which not only provides mechanical support but also participates in genetic regulation and DNA replication (Hutchison, 2002).

Although chromatin and other nuclear components may reorganize after freezing, alterations in the shape or function of the nuclear lamina could compromise oocyte developmental competence.

Cytoplasmic structures are also vulnerable, especially in relation to the oocyte's meiotic stage. Proper cytoskeletal organization is essential for correct chromosome segregation, spindle rotation, cytokinesis, and pronuclear or nuclear formation (Maro et al., 1986; Schatten et al., 1985).

Microtubules, the main components of the meiotic spindle, are particularly sensitive. Breakage or disorganization of microtubules can displace the spindle and alter the chromosomal complement of the cell, leading to aneuploidy and compromising embryonic or foetal development. Importantly, GV-stage oocytes contain only a few asters and lack prominent microtubular structures, suggesting that GV oocytes may in principle be more resilient to cryopreservation than MII oocytes (Boiso et al., 2002) . However, Boiso and colleagues observed that although GV-intact oocytes survived thawing at higher rates than MII oocytes, the spindle and chromosome configuration were severely compromised in both groups, noting also that such assessments are partly subjective and dependent on microscopic evaluation.

Another crucial cytoskeletal component is actin, which forms microfilaments. Actin microfilaments play fundamental roles in spindle rotation, polar body extrusion, pronuclear migration, intracellular trafficking of molecules and organelles, and cytokinesis.
Although cooling alone does not seem to markedly affect actin polymerization (Bernard, 1996), it may indirectly influence microfilaments by promoting microtubule depolymerization (Webb et al., 1986). Furthermore, cryoprotectants—particularly dimethyl sulfoxide (DMSO) and 1,2-propanediol—can disrupt polymerized cortical actin (Saragusty & Arav, 2011; Vincent et al., 1990; Vincent & Johnson, 1992). If actin polymerization and microfilament function are compromised, intracellular organelle migration may be altered, causing, for example, premature release of cortical granules or abnormal clustering of mitochondria around the perinuclear region (Van Blerkom et al., 2000). Disruption of normal microfilament function during cytokinesis can also interfere with polar body extrusion, the first embryonic cleavage, or subsequent blastomere divisions.

Another structure vulnerable to cryo-damage is the zona pellucida, the glycoprotein envelope surrounding the oocyte that plays an essential role in sperm binding and the block to polyspermy (Wassarman, 1990). Cortical granule exocytosis normally induces the cortical reaction, which modifies the zona pellucida and/or the oolemma to prevent polyspermy (Wolf, 1981).
However, premature release of cortical granules during vitrification can compromise sperm penetration and fertilization. In addition, dehydration and cell-shape deformation during freezing can cause zona pellucida hardening or fracturing (Pickering et al., 1990) and rupture of the oolemma (Arav et al., 2000; Men et al., 2002); with the oocyte collapsing into a concave shape. Such physical damage has been linked to an increased incidence of polyspermic fertilization following oocyte cryopreservation (Agca et al., 2000; Asada & Fukui, 2000).

Unlike what is observed in veterinary medicine, where oocyte vitrification has so far yielded poor and inconsistent results, in the human model the outcomes are markedly better in terms of survival, fertilization, and implantation, despite the potential damage that may occur (Casciani et al., 2023). Meta-analyses, such as those summarized by Rienzi et al.(Rienzi et al., 2016), reported no significant differences in clinical outcomes between fresh and vitrified sibling oocytes, and a recent large cohort study confirmed that when the number of warmed oocytes equalled that of fresh ones, all reproductive outcomes were comparable. Moreover, Martinez-Burgos et al. (Martínez-Burgos et al., 2011) demonstrated that, following ultrarapid cooling, human oocytes recover their cellular volume more efficiently and show better spindle

preservation compared with slow-freezing protocols, with no increase in DNA fragmentation. These findings suggest that vitrified human oocytes not only survive at higher rates but also preserve critical subcellular structures more effectively.

3. The Role of Cryopreservation in Veterinary Medicine

Veterinary medicine applies cryopreservation techniques across various contexts, taking advantage of their versatility to preserve gametes for different purposes. The field of reproduction spans a wide range of applications, from commercial use to the conservation of endangered species, highlighting the multifaceted role of these techniques.

3.1. Use in assisted reproductive technologies (ART)

Vitrification is intrinsically part of the broad landscape of reproductive biotechnologies. As previously mentioned, it allows for the preservation of gametes, embryos, or even portions of gonadal tissue at extremely low temperatures for a virtually unlimited period of time. Alongside in vitro maturation, fertilization, and embryo culture, cryopreservation, most commonly through vitrification, has emerged as the most versatile, effective, and straightforward technique. Cryopreserved oocytes could be included in a genome resource bank (GRB) and could be used later to increase the gene bank of endangered breed populations, save species from extinction and potentially re-establish extinct species (Smits et al., 2012), avoid hereditary disease due to intensive selection cause by highly inbreeding, raising the probability of developing genetic diseases, propensity to develop muscular skeletal injuries and low fertility (Dini et al., 2020; Hill et al., 2023). The ability to preserve gametes and embryos enables their commercialization and supports genetic improvement in livestock breeds. It allows breeders to obtain animals that would otherwise be inaccessible due to logistical or economic constraints. Cryopreservation has therefore unlocked a range of possibilities previously unimaginable, facilitating the dissemination of valuable genetic resources even to geographically distant regions. Cloning is used commercially to increase or restore the reproductive potential of animals with a high genetic value, but nuclear transfer efficiency remains low (Gambini & Maserati, 2017; Olivera et al., 2016). Specialized laboratories could potentially use cryobanked sport-performing animals' oocytes, like equines, for oocyte-consuming procedures, like cloning. The production of blastocysts and the birth of healthy clones from cryopreserved oocytes in mouse (Hirata et al., 2011; Sung et al., 2010), cattle (Hou et al., 2005; M. J. Park et al., 2015), sheep (Moawad et al., 2011) and buffalo (Saini et al., 2018) have been reported, but no reports on cloned equine embryos using cryopreserved oocytes have been published so far.

An alternative approach is to freeze ovarian tissue, which, after thawing, can be transplanted or cultured with the objective of producing mature fertilizable oocytes (Aguiar et al., 2020; Souza et al., 2021) has been successfully applied in mice (Motohashi et al., 2011; O'Brien et al., 2003) but not in other species with the same results so far. Only lately, in humans, some progresses have been achieved (Zolfaghar et al., 2020). Beyond its commercial applications, vitrification plays a central role in research projects focused on in vitro embryo production, facilitating the storage of embryos. Moreover, it is the subject of dedicated studies aimed at improving survival rates and the ability of the cryopreserved cells to develop into viable and transferable blastocysts. While the results obtained so far are promising, they are not yet fully satisfactory. Ongoing research, including the present work, seeks to deepen our understanding of the cellular effects of vitrification and to continuously improve protocols, with the goal of developing a sufficiently safe and reliable method for preserving gamete viability and function.

3.1.1. Genetic resource preservation

One of the main purposes of cryopreservation is the conservation of genetic diversity in threatened populations, often as a last resort. For example, one of the Sustainable Development Goals indicators established by the FAO (Food and Agriculture Organization of the United States) considers the number of animal breeds with sufficient stored genetic material to allow reconstitution in case of extinction (FAO, 2022). The preservation of genetic material from animals with high genetic value has contributed to overcoming the limitations of traditional genetic selection and breed development programs, which are often time-consuming, inefficient, and result in the culling of individuals deemed economically, culturally, or environmentally unsuitable (Blackburn et al., 2024). The reintroduction of genetic material through cryopreserved samples can restore lost diversity and help limit inbreeding in livestock populations. Long-term cryopreservation of reproductive materials, such as semen, oocytes, or embryos, makes it possible to recover genetic traits from past individuals and reintroduce them into current populations to enhance variability. In livestock species, intensive genetic selection has led to a marked reduction in genetic diversity, often relying on a narrow pool of sires whose semen is widely used in breeding programs, thereby increasing inbreeding levels (Prentice & Anzar, 2010). Currently, there are more than 100 biobanks worldwide that store genetic material such as semen, oocytes, embryos, ovaries, testes, primordial germ cells, fibroblasts, and other tissues for DNA extraction. Countries like the United Kingdom and Canada collect samples from both common and rare breeds, while others, such as Brazil, have expanded their

collections to include material from local wild species undergoing domestication, aiming to enhance food security strategies through greater genetic diversity. Samples collected as early as the 1960s have been used in recent years to address present-day challenges, highlighting the enduring value of these resources. This demonstrates that biobanking is not only a tool for present-day breeding but also a long-term strategic investment. Inbreeding poses significant threats to animal populations, with consequences on productivity, fertility, and athletic performance. These effects have been widely studied in various species, such as Nelore cattle (Mota et al., 2024), the Italian Holstein breed (Ablondi et al., 2023), Asturiana de las Valles cattle (Cortes et al., 2024), rabbits (Piles et al., 2023), and sheep (Justinski et al., 2023). Horses are not exempt from this trend, as seen in local breeds like the Pura Raza Española (Laseca et al., 2024). and North American Thoroughbred populations (Hill et al., 2023). Despite its advantages, the use of cryopreserved material has some limitations. While it can "refresh" the gene pool and preserve breed-specific traits, it may also reduce or dilute selected characteristics achieved through modern breeding programs. Nevertheless, a targeted and balanced use of cryopreserved samples can be beneficial, a study on a small population of the French native Abondance cattle demonstrated the effectiveness of carefully planned use of stored genetic material (Jacques et al., 2023).

3.1.2. Conservation of endangered breeds

The rapid expansion of human activity has endangered numerous animal species that have failed to evolve quickly enough to adapt to anthropogenic environmental changes. This has led to the extinction or endangerment of many species, including amphibians, insects, and mammals. While conservation strategies such as zoological parks and protected areas have supported individual survival, they have often proven ineffective for captive breeding. Artificial environments disrupted social structures, and limited availability of mating partners have posed significant barriers to natural reproduction. In this context, assisted reproductive technologies (ART) have emerged as valuable tools to obtain offspring from captive animals, enabling the continuation of conservation programs. More recently, advanced ART (aART) approaches, technologies that employ genetic material from somatic cells to generate offspring, have been highlighted as pivotal for restoring biodiversity. Notable examples include somatic cell nuclear transfer (SCNT) and induced pluripotent stem cells (iPSC) (M. C. Gómez et al., 2008; Hikabe et al., 2016). The opportunity offered by ART lies in the ability to store genetic material from multiple individuals, thereby expanding genetic diversity and preventing its loss. Moreover,

cryopreserved samples can benefit from future technological advancements. Cryobanks therefore represent, as Hainau (Hainaut, 2017) states, "a crucial unfilled gap – offering a backup storage of the extant genomes of living species that are already under threat or are likely to be soon". Cryopreservation is included in the "One Plan Approach" framework, developed by the IUCN (International Union for Conservation of Nature) Conservation Planning Specialist Group (Byers O., 2013; Lees C., 2011; Traylor-Holzer K., 2018). However, it is important to acknowledge that the application of ART to wildlife species does not yield the same results as those observed in domestic animals. Given these limitations, the development of species-specific protocols tailored to the unique requirements of each species is essential, given the numerous differences observed in cryopreservation outcomes between female and male gametes across various mammalian species (Mastromonaco G.F., 2020). It is also crucial to consider not only the factors limiting the efficiency of ART, but also the ethical implications of their use. While ART and aART may reduce the need for direct manipulation of live animals, often using tissues obtained from deceased individuals, they still require surrogate animals, either for gestation or for the implantation of recovered tissues.

Although such procedures are performed under the close supervision of highly qualified professionals, the potential risks and complications for surrogate animals cannot be overlooked. As emphasized by de Mori et al. (de Mori et al., 2021), ethical and welfare risk assessments should be mandatory prior to their implementation, especially as the welfare of an individual animal risks becoming a secondary concern in pursuit of the broader goal of species conservation. The sample collection efforts carried out in previous decades have enabled the use of modern technologies to repopulate species now at critical risk of extinction. A notable example is the Przewalski's horse (*Equus przewalskii*), which was successfully cloned in 2020 using genetic material cryopreserved in 1980 at the San Diego Zoo Institute for Conservation Research's Frozen Zoo (Novak et al., 2025). These advancements pave the way for more innovative conservation strategies, promoting the integration of traditional approaches with biotechnology-based safety nets for endangered species.

3.1.3. Reproduction veterinary models for human reproduction medicine

Animal models have always been essential for research in human medicine, including the field of gamete cryopreservation. Unsurprisingly, the earliest advancements in this area were achieved using animal models before being translated into human applications. Animal models have proven particularly useful for refining protocols and deepening our understanding of both

the techniques themselves and their effects on cells, and to investigate the effectiveness of freezing protocols, which later can be used efficiently with other reproductive technologies. Among the various models, the mouse has traditionally been the most widely used in reproductive and cryopreservation research followed by the rabbit and the hamster. However, large animal models such as cattle have also proven invaluable, both for identifying species-specific characteristics and for exploiting physiological similarities with humans to enhance our knowledge in this field. For instance, research on bovine gametes has provided important insights into membrane permeability and the mechanisms of action of cryoprotectants, through both experimental approaches and mathematical modelling applicable to human oocytes (García-Martínez et al., 2021). The bovine model is frequently proposed and employed since its oocyte physiology closely parallels that of humans, particularly in terms of size, lipid content, and meiotic timing. Unlike non-human primates, the use of cattle as a model offers logistical advantages, including easier management and fewer ethical constraints, making it more accessible for routine laboratory research. Nevertheless, studies involving non-human primates have also been conducted, especially when driven by the need to preserve the valuable genetic material of endangered species. These studies have also provided opportunities to investigate reproductive biology using models that are phylogenetically closer to humans (Sadeghi et al., 2025). In the context of male gamete cryopreservation, the ram, bull, stallion and many other species have also been explored as a useful model for human sperm freezing studies (reviewed by (Aponte et al., 2023). Moreover, species such as sheep have been investigated for oocyte vitrification, further expanding the repertoire of animal models in this field (Moawad et al., 2018). The mare has also been proposed as a promising model for studying human reproductive aging (Carnevale, 2008). As one of the few domesticated animals with a relatively long lifespan and natural reproductive senescence, the mare shares several endocrine and cyclic similarities with women, making it a compelling model for age-related fertility decline. Not only oocytes, semen, and embryos, but also ovarian tissue from various animal species can serve as models for human applications. For instance, Gandolfi et al. (Gandolfi et al., 2006) investigated bovine and porcine ovarian tissue as potential models, but the results were not satisfactory. This highlights an important challenge: interspecies differences in ovarian structure and physiology can significantly limit the development of universally applicable protocols.

While human medicine has achieved significant advancements in ART and cryopreservation, the identification of appropriate animal models remains complex and is not always straightforward (Tharasanit & Thuwanut, 2021). Common laboratory models benefit from well-

established protocols and a deep understanding of their physiological variability. In contrast, the integration of new animal models requires considerable time for in-depth characterization and for protocol standardization.

Therefore, animal experimental models must remain at the forefront of research aimed at validating new cryopreservation technologies or treatments, particularly given the strict ethical and legal constraints surrounding the use of human embryos and oocytes. On the basis of these experimental models, several strategies have been developed to improve the efficiency of oocyte cryopreservation (Chang et al., 2011).

The continued development and refinement of animal models in this area will ultimately support progress in both human and veterinary reproductive medicine.

3.2. Species of interest

Cryopreservation can be applied to a wide range of species across different taxa, including amphibians, birds, and mammals. This technique has been utilized in both domesticated animals and wildlife. However, the focus of this work will be primarily on livestock species, particularly those most relevant to the present research and that have been extensively studied in the scientific literature.

3.2.1. Cattle

The success of female gamete cryopreservation in domestic animal species has historically been lower compared to that achieved with other reproductive cells, such as spermatozoa. Only a few studies report successful pregnancies resulting from cryopreserved bovine oocytes. One of the main challenges lies in the high cytoplasmic lipid droplet content of oocytes from large domestic species, which makes them extremely sensitive to chilling injury. This feature results in low survival rates when oocytes are subjected to slow freezing protocols. In 1992, Hamano et al (Hamano et al., 1992). reported the first pregnancy resulting from the transfer of in vitro matured (IVM) bovine oocytes that had been vitrified at the MII stage. Despite this promising clinical outcome, the blastocyst development rate was only 10%. A few years later, in 1996, Martino (Martino et al., 1996) et al. described a 15% blastocyst rate using an ultrarapid freezing technique based on electron microscope grids as a cryodevice. Subsequently, Vajta (Vajta et al., 1998) introduced the Open Pulled Straw (OPS) system, representing a major improvement in vitrification efficiency and standardization, in subsequent studies reported a 25% blastocyst rate from immature vitrified oocytes, suggesting that satisfactory results may also be achieved

without prior maturation (Vajta et al., 1998). However, despite advancements in technique and cryodevice design, blastocyst rates remained modest. In another study, Abe et al (Abe et al., 2005). reported that 8% of bovine oocytes developed to the blastocyst stage after stepwise exposure to a solution containing ethylene glycol, Ficoll, and sucrose, and vitrification on a nylon mesh holder. In that case, the birth of a live calf following embryo transfer was also achieved. In 2013, Dutta et al. (Dutta et al., 2013) reported a 21% blastocyst rate following the vitrification of in vitro matured oocytes using French straws and three-step protocol with increasing concentrations of sucrose, DMSO, and ethylene glycol. Similarly, Punyawai (Punyawai et al., 2015) achieved developmental rates of 22.9% and 25.5% using the Cryotop and a microvolume air cooling device, respectively, on in vitro matured bovine oocytes. Subsequent studies have rarely exceeded 20%, generally reporting values between 10% and 15%. (Dujíčková et al., 2021). To date, no oocyte vitrification protocol, regardless of the combination of cryodevices or meiotic stages, has yielded the desired outcomes. Research has yet to establish an optimal vitrification protocol for the bovine species, even though the embryo market is already expanding significantly (IETS, 2021) and there is likely potential for the future commercialization of oocytes as well.

3.2.2. Horses

At present, equine oocyte cryopreservation is still very difficult; only two foals were obtained after vitrification of mature horse oocytes (Maclellan et al., 2002). Vitrification in horse reproduction enables the conservation of oocyte in several cases: (1) when ICSI is performed time after the oocyte collection; (2) when is required preserve oocytes collected from deceased animals in places where IVEP is not available; (3) when oocytes are collected and stored outside the reproductive season; and (4) for the preservation of high-quality gametes of young mares that are competing in races. Then, the storage of oocytes from abattoir-derived ovaries could provide source of material for research and cloning for countries where oocyte availability is limited (Hinrichs, 2018).The efficiency of equine oocyte vitrification remains limited, while survival and maturation rates can be acceptable, blastocyst formation remains low, and only a few studies report live foal births from vitrified oocytes (De Coster et al., 2020). The best outcomes have been observed with in vivo-matured oocytes vitrified using Cryotop and commercial CPA media. For example, in the study by MacLellan et al. (Maclellan et al., 2023), a blastocyst rate of 33% and a pregnancy rate of 66% were reported, resulting in the birth of four foals. However, collecting in vivo-matured oocytes is challenging due to the lack of

effective superovulation protocols in mares. For this reason, most research has focused on immature oocytes, which are more readily obtained via transvaginal aspiration. Although the results are less impressive, they remain encouraging. A study by Ortiz-Escribano et al. (Ortiz-Escribano et al., 2018) reported the first live foal born from an immature vitrified oocyte that was matured in vitro, fertilized via ICSI, and transferred after embryo culture. In that study, the blastocyst rate reached 7%. Additional work by Canesin et al. (Canesin et al., 2017, 2018) achieved blastocyst rates of up to 11% using immature oocytes surrounded only by the corona radiata, together with optimized CPA combinations and high-efficiency devices such as stainless-steel mesh. Overall, the data suggest that vitrification of in vivo-matured oocytes yields the best embryonic and clinical outcomes, but the practical limitations associated with their collection have shifted research interest toward immature oocytes. These are more accessible and potentially applicable on a broader scale, especially if in vitro maturation protocols are optimized. Nevertheless, the variability among studies and protocols highlights the need for further research aimed at refining current techniques and improving overall clinical efficiency.

3.2.3. Swine

In vitro embryo production technologies and embryo transfer are not widely adopted in the swine industry, unlike what has been observed in cattle. This limited implementation has significantly constrained the development and application of these techniques within swine reproductive science (Somfai, 2024). Moreover, porcine oocytes are particularly sensitive to cryopreservation techniques, even more so than those of other livestock species (Mullen & Fahy, 2012). One of the main reasons for this heightened sensitivity is the high content of lipid droplets in the oocyte cytoplasm (Nagashima et al., 1995) that lead also to a greater hypothermic sensitivity. The intrinsic fragility of porcine gametes, combined with technical and logistical challenges, has hindered the advancement of cryopreservation protocols in pigs, leaving them lagging those developed for other species. As early as the 1990s, it was already evident that porcine gametes poorly tolerated conventional cryopreservation methods such as slow freezing (Didion et al., 1990). Nonetheless, they appear to survive vitrification procedures more effectively (reviewed by Zhou & Li, 2009), with immature oocytes showing post-warming survival rates of up to 24.5% (Rubinsky et al., 1992). In 1998, Isachenko (Isachenko et al., 1998) vitrified GV oocytes and obtained the in vitro development although no blastocyst was obtained. Blastocyst from vitrified immature oocytes was first reported by Fujihira et al.

(Fujihira et al., 2004), although fertilization was achieved using intracytoplasmic sperm injection and by parthenogenically activation (Somfai et al., 2006). It was not until 2014 that Somfai et al. reported the first live piglets born from the transfer of in vitro-produced blastocysts derived from vitrified immature cumulus-oocyte complexes (COCs), fertilized by conventional IVF. Currently, the survival rate of oocytes after warming is reported to exceed 90%, and more than 10% of these oocytes can develop into blastocysts after IVF. Despite these promising figures, vitrification of immature porcine oocytes still yields significantly lower results compared to non-vitrified controls (Somfai, 2024; Somfai et al., 2014, 2015). Nevertheless, the blastocysts obtained from vitrified oocytes appear morphologically normal, both in terms of blastomere count and full-term developmental competence (Somfai et al., 2014).Porcine oocytes seemed to respond to vitrification differently from different meiotic stage. MII porcine oocytes had better resistance to cryodamage compared to GV stage (Rojas et al., 2004) probably because the immature oocytes present a higher resistance, they don't present the meiotic spindle. One strategy developed to address the challenge posed by the high intracellular lipid content is mechanical delipidation, first described by Nagashima et al. (Nagashima et al., 1995). When applied to immature oocytes before vitrification, this technique has yielded moderate success (Park et al., 2005). Another lipid removal by suspension and the centrifugation of oocytes in a hypertonic solution, this technique improved the cryotolerance of GV porcine oocytes (Hara et al., 2005) but delipated immature oocytes reached a lower maturation rate after vitrification (Hara et al., 2005; Park et al., 2005). However, its practical use is limited by several factors: the procedure involves partial removal of cumulus cells, which may play a role in subsequent embryonic development; it can compromise the integrity of the zona pellucida, potentially violating international sanitary standards for embryo handling (Stringfellow et al., 1998), and it is technically complex, making it difficult to apply uniformly across all gametes. Cryopreservation of porcine oocytes is much more problematic than other mammals, once a suitable method will be developed, it would enhance pig cloning technologies, gene banking for transgenic pigs and the potential use of pig model for testing the safety of related technologies in humans.

3.2.4. Small ruminants

The results obtained in small ruminants largely mirror those observed in cattle. Research on oocyte vitrification in sheep is relatively recent and has produced modest developmental outcomes. One of the earliest studies by (Succu et al., 2007a) evaluated the vitrification of in

vitro matured oocytes using three different cryodevices: Open Pulled Straw (OPS), Cryoloop, and Cryotop. Oocytes were exposed to a vitrification solution containing ethylene glycol (EG) dimethyl sulfoxide (DMSO), sucrose, TCM199 (Tissue Culture Media 199), and FCS (Foetal Calf Serum). The resulting blastocyst rates did not exceed 17%, compared to 50% in the fresh control group. Among the cryodevices tested, Cryotop yielded the lowest rate of membrane damage (23%).

In further experiments (Succu et al., 2007b),attempted vitrification of oocytes from prepubertal ewes, yet the results were comparable to those obtained with adult oocytes. In a follow-up study using Cryotop and more concentrated vitrification solutions, a blastocyst rate of 17% was again achieved, though a delay in blastocyst development was observed in comparison to the untreated control.

The meiotic stage of the oocyte has also been investigated as a critical factor in vitrification outcomes (Shirazi et al., 2012) found that matured oocytes (MII) had better resistance to vitrification than immature oocytes (GV), with cleavage rates of 53% and 37%, respectively. These findings were supported by Mo et al. (Mo et al., 2014), who demonstrated that MII oocytes exhibited superior survival and developmental competence compared to GV oocytes after warming.

Other studies focused on the presence of cumulus cells, Zhang and Mo et al. (Mo et al., 2014) found no significant differences in survival, cleavage, or blastocyst rates between cumulus-enclosed and denuded oocytes. In contrast, (Shirazi et al., 2012) reported higher cleavage rates in cumulus–oocyte complexes (COCs) (53%) than in denuded oocytes (41%). While data remain inconsistent, it is widely accepted that viable cumulus cells and intact gap junctions are essential for proper meiotic resumption and cytoplasmic maturation (Shirazi et al., 2012) but that cumulus cell cannot mitigate the effect of the cryoinjury on the embryonic development (Dos Santos-Neto et al., 2017).

Despite improvements in vitrification protocols and devices, the vitrification of mature oocytes remains particularly challenging in sheep, as in other species. Barrera et al (Barrera et al., 2018) observed a significant decrease in the developmental rate to morulae and blastocysts in Cryotop-vitrified oocytes (15.5%) compared to non-vitrified controls (53.8%). Similarly, Succu et al (Succu et al., 2007b) reported a lower blastocyst rate (12.5%) in mature oocytes vitrified with Cryoloop compared to fresh controls (50%).

Overall, while progress has been made, current vitrification protocols for ovine oocytes—regardless of meiotic stage, cryodevice, or cryoprotectant composition—have yet to achieve developmental outcomes comparable to fresh oocytes.

In goats, the cryopreservation of gametes remains a significant challenge as happen for cattle and ovine species, although the embryo transfer (ET) market is slowly expanding worldwide. While embryo cryopreservation has shown promising results and increasing commercial interest, the vitrification of immature and mature oocytes continues to yield limited developmental outcomes due to their high sensitivity to cryoinjury and variability in response to protocols.

Following vitrification and warming, morphological damage is commonly observed in goat oocytes. According to Kharche et al (Kharche et al., 2005), 19% of immature oocytes displayed reduced cumulus mass, cytoplasmic clarity, and abnormalities or rupture of the zona pellucida. Similarly, Sharma et al. (Sharma et al., 2006) reported that 16% of oocytes presented with zona nicking, dissolution, cytoplasmic shrinkage, and abnormal shape. These alterations are likely to contribute to the significantly reduced fertilization rate observed in vitrified oocytes (8%) compared to non-exposed controls (17%).

Several cryoprotective agent (CPA) combinations have been tested in goats, yielding varied results. For instance, the use of propylene glycol (PG), trehalose resulted in 94% post-warming survival, yet only 8% of oocytes extruded a polar body after IVF (Sharma et al., 2006). Similarly, propanediol led to nuclear maturation rates that were markedly lower than those of control groups (Kharche et al., 2005). Interestingly, oocytes exposed only to CPAs (without vitrification) performed better than those subjected to the full procedure, suggesting that both the CPA formulation and the vitrification itself contribute to reduced developmental competence.

The presence of cumulus cells appears to play a protective role. Purohit et al. (Purohit et al., 2012) showed that immature cumulus-oocyte complexes (COCs) yielded higher IVF rates (32%) than denuded oocytes vitrified either before or after IVM (25% and 17%, respectively). Their results also confirmed that immature compact COCs were more resistant to cryoinjury, suggesting the structural and biochemical support of cumulus cells during vitrification.

As well as presence of cumulus, also meiotic stage is still a controversial point, Purohit et al. (Purohit et al., 2012) proposed that germinal vesicle (GV) stage oocytes may be more cryotolerant, conversely, Quan et al. (Quan et al., 2014) reported better morphology and cleavage rates in MII-stage oocytes than in GV-stage oocytes.

From a practical and commercial perspective, embryo transfer in goats is still limited in scale compared to cattle, yet it is attracting growing global interest, according to the Internation Embryo Transfer Society 2023 report (IETS, 2003), a total of 11,826 in vivo-derived (IVD) goat embryos were transferred worldwide, with North America (11,457 embryos) and Europe

(369 embryos) representing the most active markets. Australia also showed growing engagement, reporting the export of 872 IVD embryos. Although these numbers are modest compared to bovine embryo transfer (~1.3 million transfers per year), they point to a rising demand for genetic improvement, breed conservation, and transgenic model development in caprine species.

These trends suggest that the commercial potential of goat embryo technologies is growing, even as the optimization of oocyte vitrification protocols remains a critical bottleneck for broader reproductive biotechnology applications in this species.

PART II
Experimental Work

4. Effects of holding and the addition of naloxone on vitrification of equine immature oocytes

Penelope Maria Gugole[a],
Augusta Zannoni[ab],
Monica Forni[bc],
Iacono Eleonora[ab],
Zambelli Filippo[d],
Merlo Barbara[ab]

[a] Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy.
[b] Health Science and Technologies Interdepartmental Center for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy
[c] Department of Medical and Surgical Sciences, University of Bologna, via Massarenti 9, 40138 Bologna, Italy
[d] Eugin, Barcelona, Spain

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4.1. Abstract

This study investigates the effects of overnight holding and naloxone (Nx) supplementation on the vitrification outcomes of equine immature oocytes. Oocytes were divided into six experimental groups based on treatment combinations: fresh (F) and held (H) control oocytes, oocytes vitrified with or without Nx (10^{-8} M) (VIT and VIT-Nx), oocytes vitrified after overnight holding with or without Nx (10^{-8} M) (H-VIT and H-VIT-Nx). They were assessed for survival, meiotic competence, intracellular oxidative stress, mitochondrial activity and distribution, apoptosis, and apoptotic gene expression. At survival rate determination, the degeneration rate was higher in VIT and VIT-Nx compared to F ($P<0.05$). The highest maturation rate was observed in VIT-Nx. A significant reduction in ROS levels was observed in H compared to F ($P<0.05$). ROS levels were similar between F and VIT, while the Nx

supplementation tended to increase them (VIT-Nx vs F: P=0.053; VIT-Nx vs VIT: P=0.069). Conversely, in oocytes vitrified after overnight holding, vitrification induced an increase in ROS levels (H vs VIT: P<0.05), which was not observed in H-VIT-Nx. GSH intracellular levels showed significant differences only in held oocytes, with higher GH levels in H compared to H-VIT and H-VIT-Nx (P<0.05). All treatments induced an increase in HMMP levels compared to F (P<0.05). In H oocytes, mitochondria were distributed throughout the entire oolemma (TOMM20) and active mitochondria (D-LAT) were detected in the outermost region. Incontrast, in H-VIT-Nx, potentially active mitochondria were spread throughout the cytoplasm. AnnexinV/PI staining revealed that the percentage of viable oocytes was higher (P<0.05) in F and H than in all vitrified/warmed oocytes, and H-VIT-Nx had the highest degeneration rate (P<0.05). RT-PCR analysis confirmed the detection for both reference genes, and target genes *BCL2* and *Survivin* in all samples. In contrast, *BAX* and *p53* transcripts were consistently undetectable. No significant differences were observed in the expression of *BCL2* and *Survivin* between groups. In conclusion, overnight holding at uncontrolled room temperature can alter oocyte characteristics and lead to variable results after vitrification. Nx demonstrated contrasting antioxidant effects depending on the vitrification timing, but it appeared to improve IVM outcomes in oocytes vitrified immediately after collection.

Keywords: horse, oocyte, vitrification, holding, naloxone

4.1.1. Introduction

In horses, cryopreservation of sperm can be considered successful and is routinely used for both commercial and research purposes. On the contrary, preservation of genetic material from mares is still a challenge.

Despite oocyte cryopreservation is routinely practiced in humans and laboratory animals, the efficiency is low in domestic animals [1]. The methods available for oocyte cryopreservation are slow-freezing, based on relatively low concentrations of cryoprotectants (CPAs) and long equilibration periods, and vitrification, based on high concentrations of CPAs and direct plunging into liquid nitrogen [2]. The large size of the oocyte, its high water content, and the peculiar intracellular structure, make this specialized cell very susceptible to cryoinjury [3]. Nonetheless, the advent of vitrification represented a milestone in human IVF. Vitrification was demonstrated to be superior over slow-freezing protocols and its efficiency in terms of embryo development and pregnancy outcomes was not different compared to fresh counterparts [4].

In horses, oocyte vitrification is still considered experimental, and to date, only three foals have been born from cryopreserved oocytes. The first foal born from vitrified oocytes was obtained from *in vivo* matured oocytes transferred into the oviduct of inseminated mares (*in vivo* fertilized) [5]. Almost two decades later, the birth of two foals from vitrified/warmed in vitro matured oocytes fertilized by ICSI was reported [6,7]. Many factors have been investigated trying to optimize the cryopreservation protocols for horse oocytes vitrification, including nuclear maturation status and cumulus morphology [8,9], CPAs combinations [10–12], the effect of vitrification on the DNA fragmentation of cumulus cells [13], the effect of melatonin as antioxidant [7], and the maternal age [14]. However, the reason why developmental competence of equine vitrified oocytes decreases after vitrification has not been fully elucidated.

Endogenous opioid peptides are neuromodulators also playing a regulatory role in the reproductive system, influencing processes such as hormone release, ovulation, and reproductive behaviour [15]. Naloxone (Nx) is an opioid receptor antagonist which have opposite effects depending on concentration, indicating it may act as a partial agonist at higher concentrations [16] and counteract the modulatory action of opioid agonists on the μ-opioid receptor (MOR), influencing ion channels and second messenger effectors [17]. The dual effect of Nx on *in vitro* maturation (IVM) was initially demonstrated on bovine oocytes [18]. The presence of μ-opioid receptor (MOR) was then demonstrated in the mare oviduct [19,20], and in the cumulus-oocyte complex (COC) [21], with a different seasonal expression and a role in regulating meiotic competence. At a high concentration (10^{-3} M), Nx acted as an agonist at MOR, similar to β-endorphins, reducing the rate of MII oocytes and increasing the incidence of oocytes with incomplete or incorrect chromosome migration [21]. In contrast, at a low concentration (10^{-8} M), Nx exhibited antagonist activity, improving maturation rates and decreasing the occurrence of abnormal chromatin patterns [21]. Naloxone's action might be related to mitogen-activated protein kinase (MAPK) signalling pathway, as endogeneous opiods are known to affect MAPK in oocytes [18]. The expression of MOR has been studied also in canine, human, and porcine oocytes [22–24]. Similarly to what observed in bovine and equine oocytes, the addition of Nx during IVM at high concentrations reduced the rate of MII oocytes in canine [22] and porcine [24] oocytes. Conversely, at low concentration, it had a beneficial effect on maturation rate and ratio of inner cell mass to total cells in blastocysts in pig [24]. Indeed, in porcine oocyte IVM, Nx at a low concentration synergistically increased the MII rate with cyclic adenosine monophosphate (cAMP), likely due to Nx's antagonistic action at MOR, which mediates the process of the opioid inhibition of cAMP production [24].

Moreover, a recent study [25] testing the hypothesis that Nx could prevent oxidative stress in PC12 cells (derived from a pheochromocytoma cell line) treated with H_2O_2, revealed that it protects cells from reactive oxygen species (ROS) production by acting as an antioxidant agent. It counteracted intracellular ROS production, reduced H_2O_2-induced apoptosis levels, and prevented the oxidative damage-dependent increases of the percentage of cells in G2/M phase [25]. In addition, a preliminary study investigating embryo development after ICSI of vitrified equine oocytes pre- and post-IVM, with or without the addition of Nx, found that blastocysts were obtained only in groups treated with Nx [26]. However, due to the low number of oocytes used, it was not possible to draw definitive conclusions about the effect of Nx.

Even though oocyte vitrification can be easily learned by relatively inexperienced technicians [27], it might be more practical to transport immature oocytes intended for cryopreservation to ICSI laboratories at room temperature, as for commercial equine OPU/ICSI programs, and vitrify them at the lab, rather than transporting them in liquid nitrogen after field vitrification. Overnight holding of equine immature oocytes may induce a pre-selection of the most competent oocytes [28]. Foals have been obtained after vitrification of oocytes matured immediately [6] and those matured after overnight holding [7], but there is only a brief preliminary study about the effect of overnight holding on the maturation rate of vitrified immature horse oocytes [10].

The aims of this study were to evaluate the effect of holding on vitrification of equine immature oocytes and the possible beneficial action of Nx when added to vitrification solutions.

4.2. Materials and methods

All chemicals were purchased from Sigma–Aldrich (Merck, Italy) unless otherwise stated. Plasticware was purchased from Thermo Fisher Scientific (Monza, Italy).

4.2.1. Oocyte collection and study design

Ovaries were collected from slaughtered mares and transported to the laboratory within 2-3 hours at 25°C in an insulated container. Oocytes were collected as previously described (Merlo et al., 2018). Briefly, ovaries were rinsed with demineralized water, and oocytes were recovered by aspirating the content of 10-30 mm follicles using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg). The aspirated follicular fluid was collected into 250 mL glass flasks and filtered through a 65 μm mesh nylon filter (EmSafe, Minitube, Germany). Cumulus-oocyte-complexes (COCs) were then searched at a stereomicroscope and

classified as previously reported [29]. Briefly, COCs with at least 3 to 5 layers of cumulus cells attached were classified as compact (having a tight, complete compact cumulus with a distinct, smooth hillock), expanded (having a granular or expanded cumulus), and corona radiata (having only corona radiata present). The COCs were evenly distributed into six groups: 1) fresh control (F) (n=118); 2) overnight holding (H) (n=119); 3) vitrified (VIT) (n=137); 4) vitrified with the addition of Nx 10^{-8} M (VIT-Nx) (n=137); 5) vitrified after overnight holding (H-VIT) (n=121); 6) vitrified with the addition of Nx 10^{-8} M after overnight holding (H-VIT-Nx) (n=139). The overnight holding groups were kept in HSOF (Hepes Synthetic Oviductal Fluid) at room temperature (range 21-26°C) in the dark for 20-22 hours. The study design is illustrated in Figure 1.

4.2.2. Oocyte vitrification/warming

COCs were vitrified after a 3 steps exposure to cryoprotectants on a Cryotop (Cryotop, Kitazato Supply, Japan) and immediately immerse in liquid nitrogen. Briefly, 4-5 COCs were exposed for 30 s to the first vitrification solution (V1) (HSOF containing 5% ethylene glycol (EG) and 5% dimethyl sulfoxide (DMSO), with or without 10^{-8} M Nx [18]), 30 s in V2 (HSOF containing 10% EG and 10% DMSO, with or without 10^{-8} M Nx), and finally 30 s in V3 (HSOF containing 20% EG, 20% DMSO, sucrose 0,65 M, and Ficoll 10 mg/ml , with or without 10^{-8} M Nx). Oocytes were stored in liquid nitrogen for at least 3 days before warming. For warming, COCs were exposed to decreasing sucrose-containing solutions (0.250 M, 0.188M, and 0.125 M in HSOF) for 30 s each. Subsequently, COCs intended for various analyses, except for IVM, were incubated at 38.5°C in humidified air with 5 % CO_2 for two h in HSOF before being denuded in a 0.25% trypsin solution for 60 s, washed once in HSOF supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific, Italy) to inactivate trypsin, and finally washed twice in HSOF. Control COCs were similarly denuded after collection (F) or overnight holding (H). All oocytes were evaluated under a stereomicroscope for survival. Those exhibiting a disrupted plasma membrane were classified as degenerate (see Tab. 2) and excluded from further analyses.

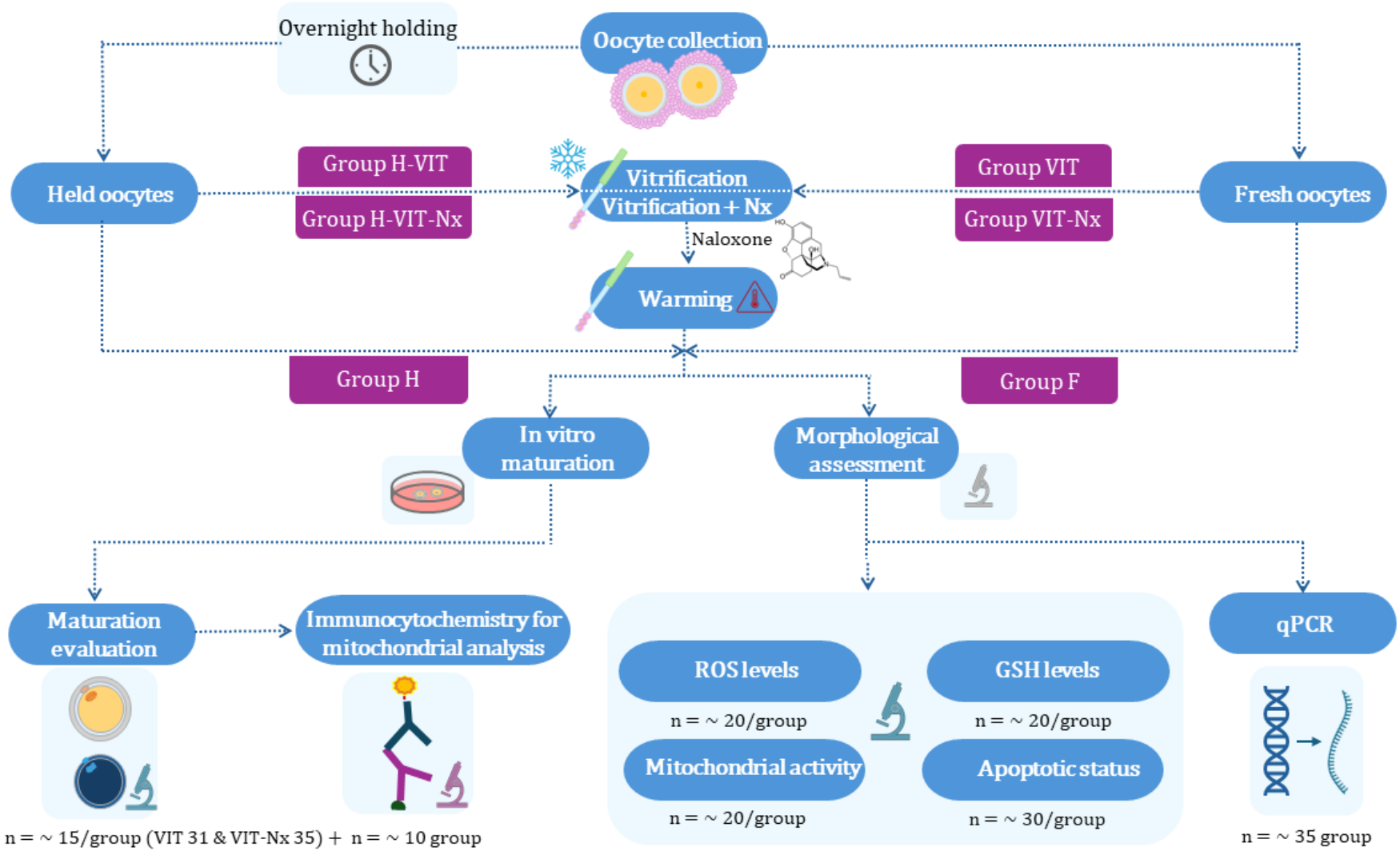

Figure 1. Experimental Design. Schematic illustration of the distribution of equine immature COCs into six experimental groups based on immediate or delayed (after overnight holding) vitrification, with or without naloxone (Nx), compared to fresh and held control groups. The following parameters were assessed: meiotic competence, reactive oxygen species (ROS) and glutathione (GSH) levels, mitochondrial activity, mitochondrial analysis post-maturation, apoptosis, and the expression of apoptosis-related genes. Experimental groups: F = fresh control; VIT = immediately vitrified; VIT-Nx = immediately vitrified with Nx (10^{-8} M); H = held control; H-VIT = vitrified after overnight holding; H-VIT-Nx = vitrified with Nx (10^{-8} M) after overnight holding.

4.2.3. Evaluation of oocyte meiotic competence

Fresh and held control COCs, and vitrified/warmed COCs were *in vitro* matured for 30 h in Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% (v/v) FBS, 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 0.1 IU/mL porcine FSH-LH (Pluset, Calier, Italy) at 38.5 °C, in humidified air at 5% CO_2.
At the end of the maturation period, oocytes were denuded as previously described and stained with Hoechst 33342 (bisbenzimide, 10 μg/ml in phosphate buffered saline (PBS) supplemented with 0.1% polyvinyl alcohol (PVA) for 15 min in the dark at room temperature. They were then washed once in PBS + PVA, mounted on glass slides, and evaluated under an epifluorescence microscope (Nikon Europe BV, The Netherlands) equipped with UV-2A (330-380 nm) excitation filter. Only oocytes displaying an extruded polar body and a visible metaphase plate (MII) were considered mature, those presenting nuclear configurations ranging from germinal vesicle to the metaphase I stage were categorized as immature, while those with disrupted membranes or an undefined nuclear configuration were classified as degenerate. The experiment was conducted in 3 replicates with 5-6 oocytes per group. An additional replicate was performed for the VIT (n = 17) and VIT-Nx (n = 20) groups to confirm the results.

4.2.4. Detection of reactive oxygen species (ROS) and glutathione (GSH) levels

Intracellular ROS and GSH levels were determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen™, Thermo Fisher Scientific, Italy) and 4-chloromethyl-6.8-difluoro- 7-hydroxycoumarin (CellTracker Blue, CMF2HC, Invitrogen™, Thermo Fisher Scientific, Italy) respectively. H2DCFDA is used as an indirect measure of ROS activity in cells, since when ROS levels increase, the H2DCFDA is oxidized to its fluorescent form (2',7'-dichlorofluorescein - DCF). CMF2HC dye is a cell-permeable, non-fluorescent compound that becomes fluorescent upon reaction with intracellular thiols. Four replicates were performed for each staining with 4-5 immature oocytes per group. Oocytes were incubated 30 min in the dark at room temperature in PBS + PVA and 10 μM H2DCFDA or 10 μM CellTracker Blue. After staining, oocytes were washed once in PBS+PVA, mounted on glass slides with vaseline anticompression layer, sealed with cover slips and examined under a Nikon Eclipse E400 epifluorescence microscope equipped with UV-2A (330-380 nm) and FITC (465-495 nm) excitation filters. Each group of oocytes was mounted and immediately imaged (Digital Sight camera, DS-U3, Nikon Europe BV, The Netherlands), using the software NI-

Elements D3.2 Laboratory Imaging, Nikon Europe BV, The Netherlands), keeping the same acquisition parameters for all groups. Images of fluorescent oocytes were analysed with a widely used open source software (FIJI ImageJ) that allows users to visualize, inspect, quantify, and validate scientific image data. The area of interest was selected using the selection tool. From the Analyze menu "set measurements" was selected and we made sure to have area integrated intensity and mean grey value selected. Then, we selected "Measure" from the analyze menu. It was important to select a region next to the cell that has no fluorescence, the background. Then, area, mean, minimum and maximum, integrated density, raw integrated density and length were calculated. These parameters are calculated from the pixel values along the line. CTCF (corrected total cell fluorescence) formula (CTCF (pixel) = Integrated Density – [Area of selected cell x Mean fluorescence of background readings]) was used to calculate cell fluorescence [30]. Final values are expressed as arbitrary units.

4.2.5. Detection of mitochondrial activity

Mitochondrial activity of equine immature oocytes was assessed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen, Italy), which stains mitochondria depending on mitochondrial membrane potential (MMP). Four replicates were performed with 4-5 oocytes per group. JC-1 is a dye, naturally exhibit green fluorescence, which is able to enter and accumulate into the mitochondria, and form reversible complexes called J aggregates (yellow-orange fluorescence) when the inner membranes are hyperpolarized [31]. Denuded oocytes were incubated in 25 µl of HSOF supplemented with 2 µl of a 150 µM JC-1 solution [32] for 30 min in the dark at room temperature, then they were washed once in PBS+PVA, mounted on glass slides with vaseline anticompression layer, sealed with cover slips, and examined under an epifluorescence microscope equipped with TRITC (540/25 nm) and FITC (465-495 nm) excitation filters. Each group of oocytes was mounted and immediately imaged using the same acquisition parameters for all groups. Only one fluorescent image (red channel) was acquired for each oocyte to detect mitochondria with high membrane potential (HMMP). Green fluorescence was not clearly detectable and attempts to enhance it up to the autofluorescence limit of negative controls were considered neither worthwhile nor reliable. Such adjustment could be misleading in assessing the red/green signal ratio, especially given the weak and poorly distributed green fluorescence observed in confocal microscopy images of equine oocytes stained with JC-1 [33]. Images were analysed as previously described for ROS and GSH.

4.2.6. Immunocytochemistry for mitochondrial analysis

Mitochondrial localization and activity were analysed in equine oocytes after IVM. Oocytes were denuded, and those with a clearly visible extruded polar body were fixed in 4% paraformaldehyde for 15 min and stored in PBS at 4°C (~ 10 oocytes per group). Before staining, the zona pellucida was removed with an acidic Tyrode's solution, then oocytes were permeabilized in 4% Triton-X for 1h, with a subsequent blocking with goat serum for 3 hours. The oocytes were then stained overnight at 4°C with primary antibodies for a subunit of the pyruvate dehydrogenase (dihydrolipoamide S-acetyltransferase, D-LAT, 1:100, mouse monoclonal, Thermo Fisher Scientific, Spain) and a mitochondrial membrane protein (translocase of outer mitochondrial membrane, TOMM20, 1:100, recombinant rabbit monoclonal, Thermo Fisher Scientific, Spain), to assess potential mitochondrial activity and localization, respectively. The expression of DLAT is indicative of mitochondrial activity because it is an essential enzyme within the pyruvate dehydrogenase complex, located in the mitochondrial matrix. Oocytes were then incubated with secondary antibodies, goat anti-mouse Alexa Fluor 488 (1:500, Thermo Fisher Scientific, Spain) and goat anti-rabbit Alexa Fluor 568 (1:500, Thermo Fisher Scientific, Spain) for D-LAT and TOMM20, respectively. The stained oocytes were mounted on glass slides with cover slips using Rapid Clear 1.47 mounting medium (Thermo Fisher Scientific, Spain) and DAPI. Each oocyte was visualized under a microscope, morphology and staining conditions were evaluated, the stage of the oocytes was checked, to confirm the previous MII morphological assessment, and only MII oocytes were analysed. Sample visualization with 60X oil objective was facilitated using the Dragonfly High Speed Confocal Microscope System (Oxford Instruments, UK) equipped with Fusion program. Dragonfly is an advanced imaging platform with high contrast and multi-dimensional capabilities encompassing four key imaging modalities. It operates as a multi-point confocal system, enabling high-speed and high-sensitivity imaging. This microscope is characterized by a capture speed of at least 10 times faster than conventional confocal technology. Using a confocal microscope, it was possible to visualize inside the oocytes in order to visualize all its "layers". With the Fusion program, it was possible to acquire images of each sample and each stack. A specific light source was chosen for each antibody: 488 GFP Sona1 for DLAT (color: green), 561 mCHERRY Sona1 for TOMM20 (color: red), and 405 Dapi Sona1: for DAPI (color: blue). Afterwards, the image processing program FIJI ImageJ was used. Images were analysed as previously described. An area of 30 μm inward from the oocyte cortex, to exclude the area without active mitochondria, was quantified.

4.2.7. Evaluation of the apoptotic status

Annexin V/propidium iodide (PI) staining was performed according to the manufacturer's instructions (Dead Cell Apoptosis Kit, Invitrogen™, Thermo Fisher Scientific, Italy). The experiment was done in 7 replicates with 4-5 immature oocytes per group. Samples were incubated for 30 min at 4°C in the dark for staining with Annexin V, a phospholipid-binding protein that detects the translocation of phospholipid phosphatidylserine from the inner to the outer cytoplasmic membrane, which is known to occur during the early stages of apoptosis, and PI to distinguish live cells from dead cells. Then oocytes were washed twice in buffer solution and observed at an epifluorescence microscope. Oocytes were classified into three groups: viable oocytes without annexin staining in the membrane; early apoptotic oocytes with a homogeneous positive annexin signal in the membrane; and dead oocytes with PI-positive red nuclei, which is indicative of membrane damage [32].

Table 1. Primer sequences for RT-PCR, values of PCR Efficiency (%), range Cycle quantity (Cq). ND= Not Detectable

Gene	Accession number	Primer sequence	% PCR Efficiency	Range Cq	Reference
BCL2	XM_001499714.1	F 5'-GCGTGGAAAGCGTAGACAAGGAGATG-3' R 5'-AGGCTCTAGGTGGTCATTCAGGTAAGTG-3'	94.7	26.79-33.89	[Present paper]
BAX	XM_001489207.1	F 5'-ATCGGAGATGAGCTGGACAGTAAC-3' R 5'-GGCAAAGTAGAAAAGGGCAACAAC-3'	94.5	ND	[Present paper]
p53	XM_001918153.1	F 5'-CTCACTATCATCACCCTGGAAGAC-3' R 5'-GTGTTACTGGACAATACTCGCTTAG-3'	89.2	ND	[Present paper]
Survivin	XM_001915400.1	F 5'-TTCATCCACTGTCCCACTGA-3' R 5'-GTTCCTCTATGGGGTCGTCA-3'	92.3	27.24-33.84	[35]
GAPDH	NM_001163856	F 5'- TGGTGAAGGTCGGAGTAAAC -3' R 5'- TGTAGTTGAGGTCAATGAAGGG -3'	84.1	22.8-32.9	[36]
ACTB	AF035774.1	F 5'- ATCGTGCGTGACATCAAGGA -3' R 5'- AGGAAGGAGGGCTGGAAGAG -3'	92.1	25-33.5	[36]

4.2.8. Quantitative real-time PCR (RT-PCR) gene expression analysis for BCL2, BAX, p53 and Survivin

The experiment was done in 3 replicates with 10-13 immature oocytes per group. Oocytes were denuded by digestion of zona pellucida in pronase solution (0.5% w/v in PBS), washed twice in PBS, and snap-frozen in molecular grade water (20 µl) before storage at -80°C. The lysis of the oocytes was performed by using SideStep lysis and Stabilization buffer (Agilent Technologies, Santa Clara, CA, USA), as described according to Galeati et al. 2016 [34].

Briefly, pool of oocytes were added with 2 µl of SideStep lysis and Stabilization buffer and mixed very well by pipetting. To avoid DNA genomic contamination, the lysed samples (14 µl) were added with 2 µl of the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). After DNase reaction protocol, all the volume (16 µl) were retrotranscribed with cDNA using 5X RT Supermix (BioRad) following the manufacturer's instructions, in a 20 µl final volume to obtain cDNA. The kit used for retrotranscription was primed both oligo d(T) and random examers. Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. Primers sequence for *BCL2*, *BAX*, *p53*, and Survivin were designed by using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA).

Regarding to the reference genes, *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), and *ACTB* (Actin B), and *HPRT* (Hypoxanthine Phosphoribosyltransferase 1) were selected. The *HPRT* transcripts resulted not detectable in any sample. Then, the stability of the two reference genes (*GPDH* and *ACTB*) was evaluated through M and CV mean values (0.8035 and 0.322 respectively) by the CFX software (BioRad). All the primers used (*BCL2*; *BAX*; *p53*; *Survivin*; *GAPDH* and *ACTB*) were located on different exons and they were reported in Table 1.

A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 10 µl of iTaq Universal SYBR Green Supermix (Bio-RAD), 0.8 µl of the forward and reverse primers (5 mM each) of each target gene, 2 µl cDNA, and 7.2 µl of water. The qPCR protocol used for the transcriptional characterization was: 10 min at 95°C, 40 cycles at 95°C for 15 s and at 60°C for 30 s, followed by a melting step from 55°C to 95°C (80 cycles of 0.5°C increase/cycle). The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. Real-time efficiency was evaluated by amplification of a standardized amount of cDNA, derived from cDNA of equine corpus luteum, starting from 150 ng with subsequent 5-fold dilutions (75, 15, 3, 0.6, and 0.12 ng). RT-PCR efficiency showed a values between 94.7% and 84.1% (*BCL2*- 94.7%; *BAX*-94.5%; *p53*-89.2%; *Surivin*-92.3%; *GAPDH*-84.1%; *ACTB*-92.1%) (Table 1). The relative

mRNA expressions of tested genes were normalized by using the ΔCt method (ΔCt= Ct geometric mean reference genes–Ct interest gene) [37] and then the relative expression was calculated as fold of change ($2^{-\Delta\Delta Ct}$ method) [38] in respect to the oocyte control group obtained under different condition (F, H, or VIT).

4.2.9. 2.9 Statistical analysis

Data from survival evaluation, Annexin V/PI staining, and maturation ability are expressed as percentages and were compared using the Chi Square test. Data on the intensity of different stainings are expressed as mean ± standard deviation, as well as for maturation rates in the additional experiment comparing only VIT and VIT-Nx (including all replicates for these groups). Data were checked for normality using the Shapiro-Wilk test. Then a Generalized Linear Model (GLM) for a gamma distribution and log link function was used, with Wald pairwise comparisons. When analysing the overall effect of vitrification, the comparison was made between non-vitrified (F and H) and vitrified/warmed (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) oocytes. Data were analysed using IBM SPSS Statistics 29.0 (IBM Corporation, Milan, Italy), with significance assessed at $P<0.05$. Only data obtained from at least five oocytes per group were included in the analysis. Consequently, the immunocytochemistry data for mitochondrial analysis from the F, VIT, and VIT-Nx groups were excluded.
For gene expression data (ΔCt values), normal distributions were evaluated by means of Shapiro–Wilk and Kolmogorov-Smirnov tests, and, according to the results, a statistic parametric test was performed (one-way ANOVA with significance level of $P < 0.05$; GraphPad Prism software, version 9.1, La Jolla, CA).

4.3. Results

A total of 771 equine immature oocytes were used for IVM and various staining procedures, while 214 oocytes were stored for qPCR. The survival rate, as determined by evaluation at the stereomicroscope after cumulus removal, was similar among vitrification groups ($P>0.05$). However, the degeneration rate was higher in oocytes vitrified immediately after collection compared to fresh control ($P<0.05$), while no difference was observed between held control (H) and oocytes vitrified after overnight holding (H-VIT, and H-VIT-Nx) ($P>0.05$) (Table 2).

Table 2. Survival rate of equine immature oocytes vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by morphological evaluation 2 h after warming.

Groups	N oocytes	Survived (%)	Degenerate (%)
F	118	107 (90.7) [a]	11 (9.3) [a]
VIT	137	110 (80.3) [b]	27 (19.7) [b]
VIT-Nx	137	106 (77.4) [b]	31 (22.6) [b]
H	119	100 (84.0) [a,b]	19 (16.0) [a,b]
H-VIT	121	104 (86.0) [a,b]	17 (14.0) [a,b]
H-VIT-Nx	139	111 (79.9) [b]	28 (20.1) [b]

Different superscript letters in columns indicate statistical significance (P<0.05). F = fresh; H=holding; VIT=vitrified; Nx=naloxone.

Analysing the ability of vitrified oocytes to resume meiosis and reach the MII stage (Tab. 3), no significant differences were observed among the vitrification groups (P>0.05), likely due to the low number of oocytes. However, only VIT-Nx achieved a maturation rate not significantly different from F and H (P>0.05). To confirm the positive effect of Nx on oocytes vitrified immediately after collection, additional oocytes were vitrified in the VIT and VIT-Nx groups, with a total of 31 and 35 oocytes, respectively. The presence of Nx significantly improved the maturation rate (P < 0.05; VIT: 37.8 ± 13.1% vs. VIT-Nx: 59.2 ± 8.2), primarily due to a lower degeneration rate (P < 0.05; VIT: 58.6 ± 8.0% vs. VIT-Nx: 38.3 ± 11.8), as the percentage of non-matured oocytes was similar between groups (P > 0.05; VIT: 3.6 ± 5.1% vs. VIT-Nx: 2.5 ± 3.5).

Table 3. Maturation rate of equine immature oocytes vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by Hoechst 33342 staining.

Groups	N oocytes	Mature (%)	Immature (%)	Degenerate (%)
F	18	12 (77.8) [a]	1 (5.6) [a]	3 (16.7) [a]
VIT	14	4 (28.6) [b]	1 (7.1) [a]	9 (64.3) [b]
VIT-Nx	15	8 (53.3) [a,b]	0 (0.0) [a]	7 (46.7) [a,b]
H	16	12 (75.0) [a]	1 (6.3) [a]	3 (18.8) [a]
H-VIT	16	5 (31.3) [b]	3 (18.8) [a,b]	8 (50.0) [b]
H-VIT-Nx	16	5 (31.3) [b]	6 (37.5) [b]	5 (31.3) [a,b]

Different superscript letters in columns indicate statistical significance (P<0.05). F = fresh; H=holding; VIT=vitrified; Nx=naloxone.

When comparing all groups for ROS intracellular levels, significant differences were observed (Fig 2A) (P<0.05). Specifically, a significant reduction in ROS levels was observed in held oocytes (H) compared to fresh (F) controls (P<0.05). In oocytes vitrified immediately after collection, vitrification (VIT vs F) did not affect ROS levels (P>0.05), while the addition of Nx (VIT-Nx) tended to increase ROS levels (VIT-Nx vs F: P=0.053; VIT-Nx vs VIT: P=0.069). Conversely, in oocytes vitrified after a holding period, vitrification (H-VIT vs H) induced a significant increase in ROS levels (P<0.05), whereas the presence of Nx during vitrification (H-VIT-Nx) did not lead to a significant increase compared to the held control group (H-VIT-Nx vs H: P>0.05). No differences in ROS levels were observed among the vitrification groups

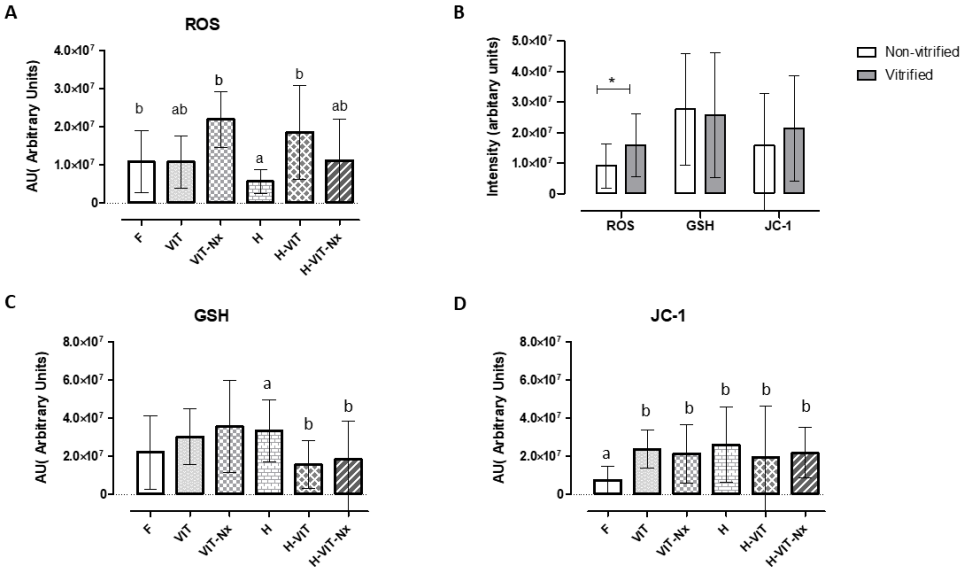

Figure 2. Evaluation of equine immature oocytes vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed 2 h after warming by A) H2DCFDA for reactive oxygen species (ROS) levels determination in various experimental groups. Different letters indicate statistically significant differences among groups (P < 0.05) CellTracker Blue for glutathione (GSH) levels, JC-1 for high mitochondrial membrane potential (HMMP) in vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) and non-vitrified (F and H) equine immature oocytes. The symbol * indicates statistically significant differences among groups (P < 0.05); C) CellTracker Blue for GSH levels determination in various experimental groups. Different letters indicate statistically significant differences among held groups (P < 0.05); D) JC-1 for HMMP determination in various experimental groups. Different letters indicate statistically significant differences among groups (P < 0.05)

(P>0.05). Additionally, intracellular ROS levels were higher in vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) oocytes compared to non-vitrified (F and H) ones (P<0.05) (Fig 2B).

GSH intracellular levels were not significantly different when comparing all groups, vitrification groups, or fresh control group and immediately vitrified oocytes (P>0.05) (Fig 2C). On the other hand, considering held oocytes, GSH levels were higher in the control group

compared to vitrified oocytes (P<0.05) (Fig. 2C). Overall, intracellular GSH levels were similar in vitrified oocytes compared to non-vitrified ones (P>0.05) (Fig 2B).

High mitochondrial membrane potential, assessed by JC-1 (orange fluorescence), showed significant differences (P<0.05) between groups (Fig. 2D). All treatments (holding and vitrification with or without naloxone) induced an increase in HMMP levels compared to fresh control oocytes. However, no significant difference in HMMP levels were observed between vitrified and non-vitrified oocytes (P<0.05) (Fig 2B).

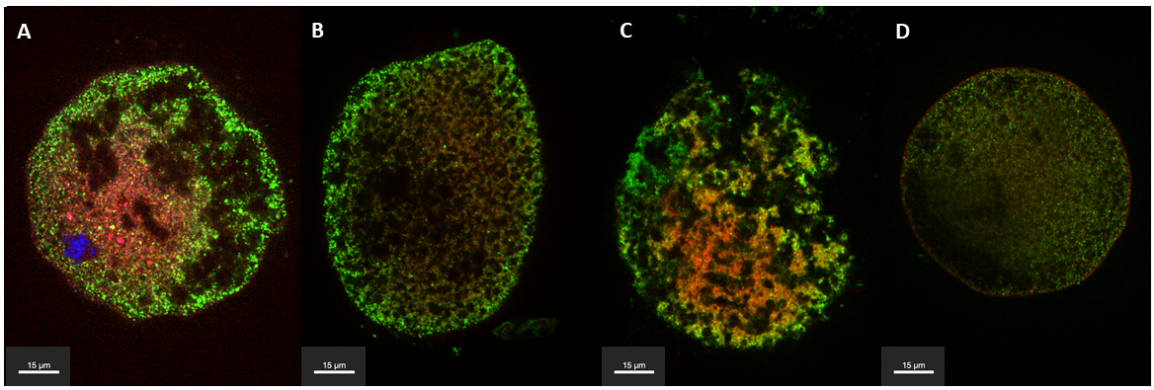

Figure 3. Confocal microscope images of equine oocyte mitochondrial activity (stained in green by D-LAT) and localization (stained red by TOMM20) after IVM. A) Control oocyte from the overnight holding group (H) at maturation stage confirmation. Note the MII plate (stained in blu by DAPI), and the absence of the first polar body, which was lost during zona pellucida removal; B) Another control oocyte of the overnight holding group (H); C) Oocyte vitrified after overnight holding (H-VIT); D) Oocyte vitrified after overnight holding in presence of naloxone (H-VIT-Nx).

In held matured oocytes, mitochondria were distributed throughout the entire oolemma, as shown by TOMM20 staining (Fig. 3A). The presence of DLAT, used as a marker for active mitochondria, was detected in the outermost region of the oocytes, with no signal in the centre (Fig.3A). This same pattern was predominantly observed in both control (4/5) (Fig. 3B) and vitrified (5/7) oocytes (Fig. 3C), but not in oocytes vitrified in presence of Nx (3/7) (Fig. 3D). In these oocytes, the signal of potentially active mitochondria was spread throughout the cytoplasm (4/7). Moreover, when comparing the amount of DLAT (active mitochondria), no significant differences were observed between groups, nor between vitrified and non-vitrified oocytes (P>0.05) (Fig. 5).

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Figure 4. Evaluation of apoptotic status of equine non-vitrified (F and H) and vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) immature oocytes as assessed by Annexin V/PI staining 2 h after warming. Different letters indicate statistically significant differences among groups (P < 0.05).

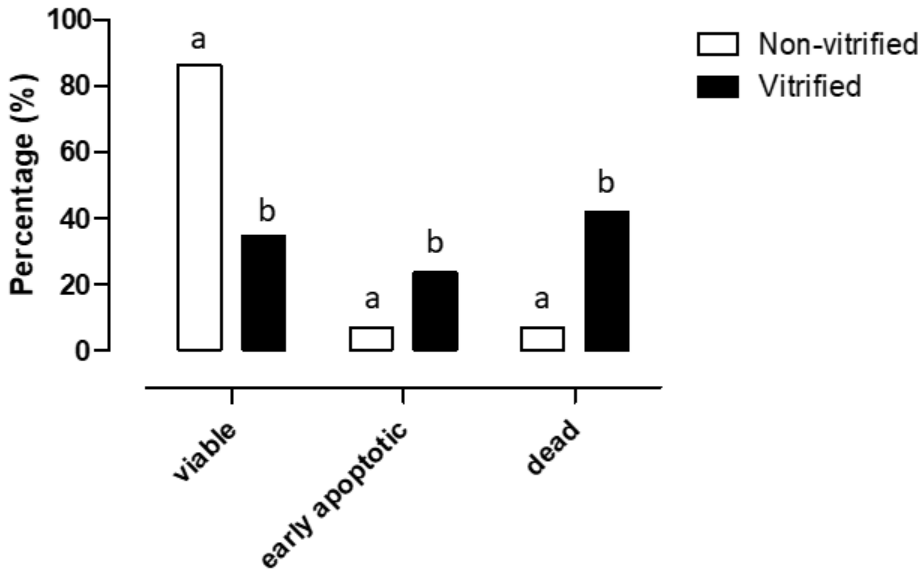

Table 4. Viable, early apoptotic, and dead rates of equine immature oocytes, vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by AnnexinV/PI staining. Different superscript letters in columns indicate statistical significance (P<0.05). F = fresh; H=holding; VIT=vitrified; Nx=naloxone.

Groups	N oocytes	Viable (%)	Early apoptotic (%)	Dead (%)
F	30	24 (80.0) [a]	2 (6.7) [a]	4 (13.3) [b]
VIT	29	15 (51.7) [b]	6 (20.7) [a,b]	8 (27.6) [b,c]
VIT-Nx	26	9 (34.6) [b]	5 (19.2) [a,b]	12 (46.2) [c,d]
H	29	27 (93.1) [a]	2 (6.9) [a]	0 (0.0) [a]
H-VIT	28	9 (32.1) [b]	9 (32.1) [b]	10 (35.7) [c,d]
H-VIT-Nx	32	7 (21.9) [c]	7 (21.9) [a,b]	18 (56.3) [d]

Results from AnnexinV/PI staining are showed in Table 4. The percentage of viable oocytes was higher (P<0.05) in non-vitrified than in all vitrified/warmed oocytes, and H-VIT-Nx protocol was the less efficient protocol (P<0.05). Early apoptotic oocytes were more present (P<0.05) in H-VIT compared to non-vitrified groups (F and H), while the other vitrification protocols showed intermediate results. No dead oocytes were found in H group, so the rate was significantly lower than in all groups (P<0.05). The percentage of dead oocytes was lower in VIT compared to H-VIT-Nx (P<0.05), and intermediate for VIT-Nx and H-VIT. Considering oocytes treated immediately after collection, the rate of dead oocytes was significantly increased compared to control only in presence of Nx (P<0.05).

Comparing total non-vitrified and vitrified oocytes (Fig. 4), the vitrification process significantly reduced the rate of viable oocytes, while simultaneously increasing the rates of early apoptotic and dead oocytes (P<0.05).

RT-PCR analysis confirmed the detection for both reference genes, and target genes *BCL2* and *Survivin* in all samples. In contrast, *BAX* and *p53* transcripts were consistently undetectable. When comparing fresh and held groups separately, no significant differences were observed in the expression of *BCL2* and *Survivin* (Fig. 6A, 6B). However, the expression of both genes was significantly increased in vitrified oocytes compared to non-vitrified ones (P<0.05) (Fig. 6C).

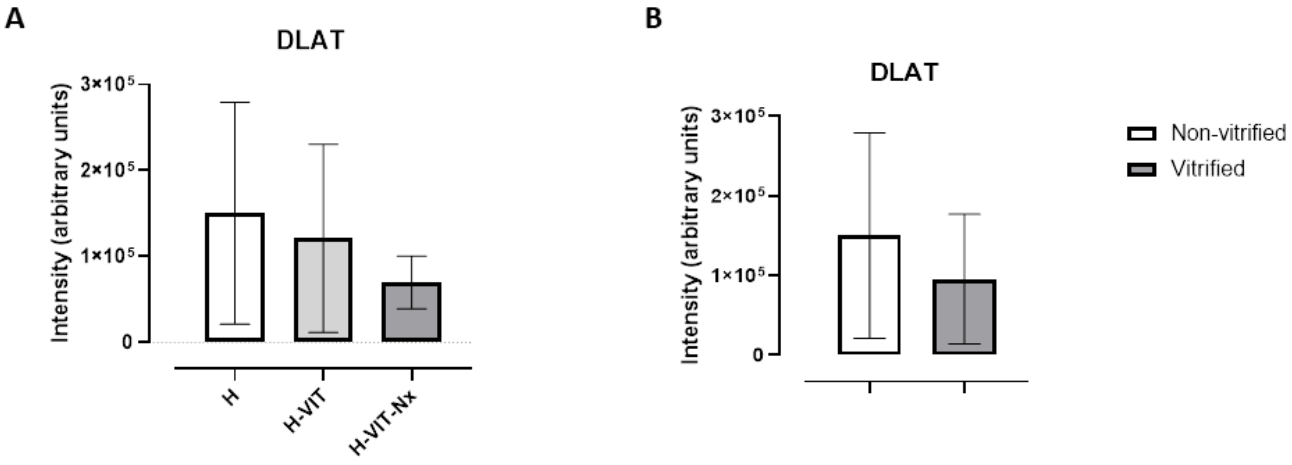

Figure 5. Relative expression levels of Bcl-2 and Survivin genes evaluated by qPCR in equine immature oocytes vitrified under different experimental conditions: A) expression was normalized to fresh control oocytes (F); B) expression was normalized to held control oocytes (H); C) expression was normalized to non-vitrified oocytes (F and H). The symbol * indicates statistically significant differences among groups (P < 0.05).

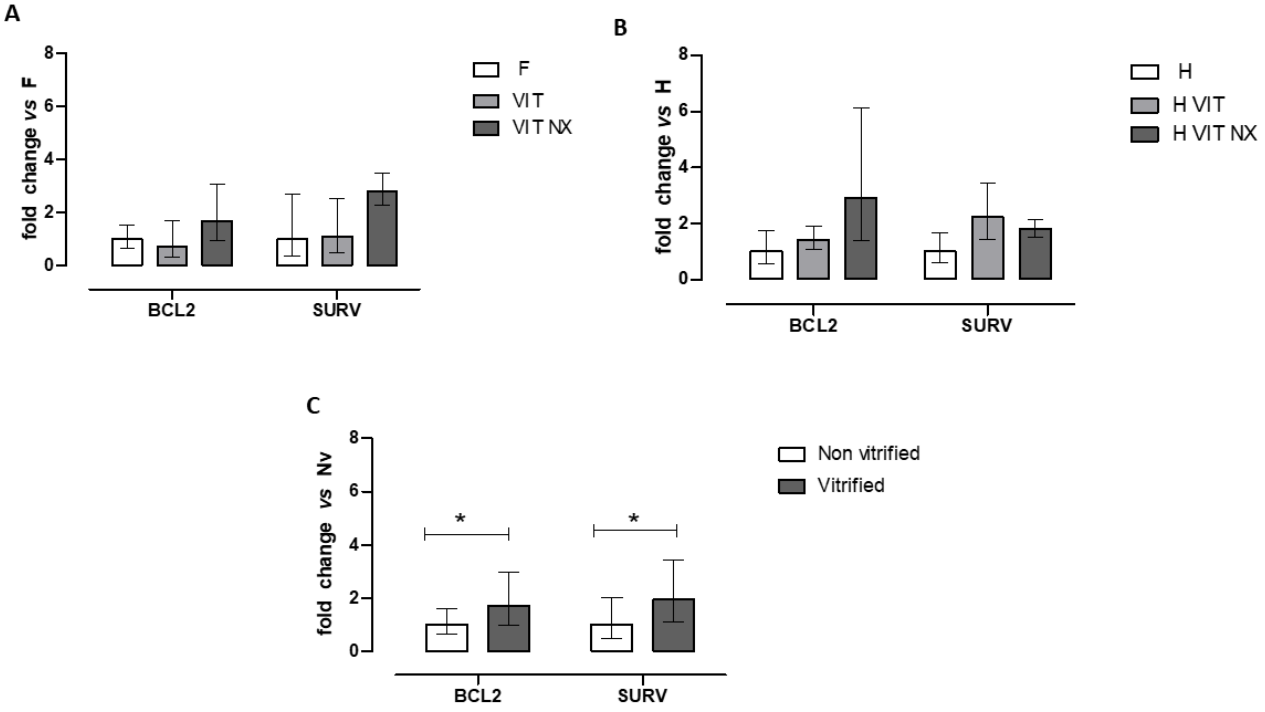

Figure 6. Amount of DLAT (active mitochondria) in equine in vitro matured held oocytes before and after vitrification with or without naloxone (10^{-8} M): A) comparison between held control oocytes (H: n=5) and oocytes vitrified after overnight holding without (H-VIT: n=7) or with naloxone (H-VIT-Nx: n=7); B) comparison between non-vitrified (H) and vitrified (H-VIT, and H-VIT-Nx) oocytes.

4.4. Discussion

In the present study, equine immature oocytes were vitrified using Cryotop® following a previously described protocol [5,26]. The research aimed to investigate the effects of adding naloxone to the vitrification solutions and to explore the impact of overnight holding on vitrification outcomes.

Survival rates, assessed morphologically two hours after warming, were similar across various treatments, ranging from 77.4% to 86.0%. However, the degeneration rate appeared higher for oocytes vitrified immediately after collection compared to non-vitrified control, while it was similar among groups of oocytes held overnight. Previous studies [28,39] have observed that oocytes already partially compromised may be less tolerant to overnight holding, resulting in an increased degeneration rate after maturation. This may explain why the holding period appeared to minimize the differences in outcomes between vitrified and control held oocytes. During the holding period, any partially compromised oocytes likely degenerated, while in the fresh control group such compromised oocytes were not detectable at morphological evaluation.

Furthermore, as previously observed in canine oocytes [40], morphological evaluation under the stereomicroscope is a reliable method for detecting oocytes with ruptured plasma membranes, but it does not allow for the identification of oocytes with damaged membrane. Indeed, in our study, dead oocytes were detected using Annexin V/PI staining, even though only oocytes that appeared to have intact plasma membranes during morphological evaluation were stained. Moreover, although the relatively low number of oocytes analysed may limit the robustness of the result interpretation, no dead oocytes were found in the held control group, confirming that partially compromised oocytes may degenerate during overnight holding.

After IVM of vitrified/warmed immature oocytes, approximatively one-third successfully reached the MII stage, with 55.3% achieving MII when oocytes were vitrified immediately after collection in the presence of naloxone (compared to 77.8% MII for fresh control oocytes). Similar maturation rates have been reported [9,14]. However, direct comparison between studies is challenging because the success of vitrification depends on both oocyte quality and the specific techniques employed. Significant variability arises when working with oocytes recovered from ovaries sourced at slaughterhouses, where critical factors such as time from slaughter to oocyte recovery, tissue handling, and mare reproductive status and age are unknown. Despite these differences and considering that the low number of oocytes may limit result interpretation, naloxone appeared to have a positive effect on meiotic competence of oocytes vitrified immediately after collection but not on those held prior to vitrification. Holding at room temperature maintains meiotic arrest in horse oocytes [41], which may have interfered with Nx's action.

A recent study revealed that Nx has protective effects on oxidative stress [25]. In the present study, Nx exhibited opposite effects depending on the timing of vitrification. It tended to increase ROS levels in oocytes vitrified immediately after collection, while it prevented the rise in ROS levels in held oocytes following vitrification. Additionally, overnight holding reduced ROS levels in non-vitrified oocytes. This suggests that the holding period influences the activity of Nx on equine immature oocytes. Oocytes physiologically utilize oxygen for energy production through mitochondrial oxidative phosphorylation, and ROS production increases during IVM [42]. It can be hypothesized that overnight holding not only maintains meiotic arrest, but also reduces the metabolic activity of the oocyte, thereby decreasing the physiological ROS levels. The effect of holding on ROS production was investigated in one study that assessed mitochondrial energy/redox potential in both immature and matured equine oocytes [43]. ROS levels were measured in relation to nuclear chromatin configuration, and although the mean intracellular ROS levels were numerically lower in held oocytes than in

those immediately stained across all configurations, the difference was not statistically significant [43]. Additionally, different holding media were used (Earle's/Hank's' M199-based medium [43] vs HSOF in the present study), which may have played an important role in influencing the observed differences. ROS levels were evaluated in another study involving immature vitrified/warmed equine oocyte matured in presence of melatonin [7], where oocytes were held overnight prior to vitrification. In that study, the vitrification and warming process increased intracellular oxidative stress, as evidenced by higher ROS levels [7]. Further studies are needed to better understand the modifications induced by overnight holding in equine immature oocytes, particularly to explain the different responses to Nx, which may be linked to changes in MOR expression.

Glutathione plays a crucial role in protecting cells from the detrimental effects of ROS [44], modulates protein and DNA synthesis by affecting redox status, and is involved in the assembly of microtubules [45]. However, an inverse correlation between GSH and ROS levels was not observed in this study. This phenomenon has already been reported for some antioxidants in cattle [46,47]. In horses, there are no reports of GSH determination in oocytes using CellTracker Blue staining. Nevertheless, it has been demonstrated that GSH concentrations are lower in germinal vesicle oocytes compared to post-maturation oocytes, both *in vivo* and *in vitro* [48]. The synthesis of GSH during oocyte maturation is crucial for sperm chromatin decondensation and male pronuclear formation [49], playing a pivotal role in subsequent steps of in vitro embryo production [50]. Our overall results could suggest that vitrification does not affect GSH content in equine immature oocytes, which contrast with findings in immature cat [51] and silver fox [52] oocytes, where vitrification has been shown to impact GSH levels adversely. However, a decrease in GSH levels, regardless the presence of Nx, was observed in oocytes vitrified after overnight holding. It would be valuable to investigate whether these oocytes can attain optimal GSH levels following maturation, or if GSH supplementation during post-warming recovery culture [53] could alleviate the detrimental effects of vitrification on GSH levels in held equine immature oocytes.

Regarding active mitochondria, both holding and vitrification increased HMMP levels, whereas the addition of Nx did not influence this rise. The higher MMP observed in held oocytes compared to freshly collected ones in this study contrasts with findings from another study [43]. In that study, holding oocytes at un-controlled room temperature (22-27°C), similar to our conditions, affected chromatin configuration, increasing the proportion of meiotically competent oocytes (condensed chromatin (CC) and prometaphase I/metaphase I configurations), but did not alter mitochondrial activity as detected by MitoTracker Orange

CMTM Ros [43]. Nonetheless, oocytes in the CC configuration exhibited a more mature mitochondrial distribution and higher mitochondrial activity compared to less advanced stages [43]. Since CC configuration was most frequent in held oocytes [43], it is plausible that our holding conditions primarily selected CC oocytes, enabling JC-1 staining to detect a significant increase in HMMP. Moreover, the rise in HMMP observed in immediately vitrified oocytes is likely attributable to a different mechanism. ROS tipically induce mitochondrial permeability, which reduces MMP [54]. Vitrification has been shown to decrease MMP in human [55], porcine [56], bovine [57], and sheep [58] oocytes. However, it was observed that this reduction in MMP in human MII oocytes is temporary; within 4 h of warming, MMP levels spontaneously recover to those observed in fresh oocytes [55]. In the present study, vitrified-warmed oocytes were incubated for 2 hours at 38.5°C prior to staining, thus it is not possible to determine if MMP decreased after vitrification. In our experimental conditions, as reported for human MII oocytes, the MMP of immediately vitrified equine oocytes increased after warming followed by incubation, potentially in preparation for meiosis resumption stimulated by the increased temperature [43].

As observed in human [59] and bovine [60] species, equine mature oocytes are characterized by numerous mitochondria distributed throughout the cytoplasm [61]. The abundance of inactive mitochondria is thought to support the oocyte's prolonged quiescence, protecting it from damage caused by ROS generated by active mitochondria [62]. A distinctive feature of these cells is the presence of a layer of active mitochondria in the subcortical area, with no mitochondrial activity detectable in the central region. The accumulation of active mitochondria in the peripheral cytoplasm and/or around the nucleus has been identified as a marker of full cytoplasmic maturation in equine oocytes [63]. Notably, considering the low number of analysed oocytes, it appears that vitrification did not alter this pattern in held oocytes, whereas the addition of Nx resulted in an even distribution of active mitochondria throughout the cytoplasm. This alteration could indicate an immature cytoplasmic condition [63].

In general, ROS are associated with induction of death in apoptotic process [64]. The increase of ROS after oocyte vitrification has been associated with an higher rate of early apoptotic oocytes (assessed using Annexin V/PI staining) in cattle [65]. In porcine oocytes, vitrification induced a rise in the percentage of early apoptotic oocytes (Annexin V + and PI-) and a contemporaneous increase of dead oocytes (PI+) [32]. On the other hand, in prepubertal goat oocytes, even if vitrified oocytes showed higher ROS levels, no differences were found in the number of early apoptotic oocytes (Annexin V + and PI-), while a decrease in live oocytes and an increase in dead oocytes was observed [66]. In the present study, the vitrification process

reduced the rate of viable oocytes and, similarly to what observed in the pig [32], increased early apoptotic and dead oocytes. Although the number of analysed oocytes was relatively limited, the poorest outcome was observed for held oocytes vitrified in presence of naloxone, and the rate of dead oocytes was increased by the addition of Nx in oocytes vitrified immediately after collection. While early apoptosis, characterized by mitochondrial depolarization and phosphatidylserine externalization, is typically a precursor to cell death, it is not always irreversible. When the stressor is removed and favorable conditions are provided, early apoptotic processes can sometimes be halted or reversed [67]. For instance, oocytes recovering from changes induced by cryopreservation have demonstrated restored functionality when subjected to antioxidant supplementation and culture techniques that mitigate oxidative stress [7,53,65,68,69]. In this context, the potential antioxidant effect of Nx was not evident, particularly in freshly vitrified oocytes, as it actually tended to increased ROS levels post-warming.

The increased expression of anti-apoptotic genes such as *BCL2* and *Survivin* observed in vitrified-warmed oocytes, alongside the absence of consistent or significant levels of the pro-apoptotic gene *BAX* and the tumor suppressor gene *p53*, suggests a possible protective mechanism against mitochondrial-mediated apoptosis during cryopreservation. Given that fully grown oocytes at the GV stage are transcriptionally dormant, these changes likely result from post-transcriptional regulation, where previously stored mRNAs are stabilized or degraded in response to vitrification-induced stress, rather than new mRNA synthesis. The *BCL2* family plays a pivotal role in outer mitochondrial membrane permeabilization, with *BAX* promoting cytochrome c release and apoptosis, while *BCL2* inhibits this release, thus preventing apoptotic progression [65]. *Survivin*, recognized as a marker for developmental potential, further contributes to anti-apoptotic mechanisms by binding to and inhibiting caspases, particularly caspase-3, thereby preventing apoptosome formation [70]. The lack of *p53* expression, typically associated with stress-induced apoptosis, suggests that vitrification-induced apoptosis in oocytes may proceed via alternative, *p53*-independent pathways. This aligns with previous findings indicating that *p53* expression does not correlate with the morphological quality of bovine embryos, suggesting its limited involvement in oocyte cryopreservation responses [71]. These observations, combined with the modulation of *BCL2* and *BAX* under various oocyte maturation and stress conditions [72,73], emphasize the critical role of oxidative stress and mitochondrial integrity in regulating apoptotic gene expression during cryopreservation. The post-transcriptional activation of stored mRNAs encoding anti-apoptotic proteins, likely triggered by vitrification and warming stress, could serve as a protective response to maintain

oocyte viability. However, these findings should be interpreted with caution, as they may reflect differential stabilization or degradation of mRNA transcripts rather than active transcriptional changes. Future studies focusing on the expression of these proteins should be conducted. Additionally, in this study, oocyte for qPCR were stored after enzymatic removal of the zona pellucida, a procedure that likely selected for higher-quality oocytes, excluding from the analysis necrotic ones and those with compromised plasma membranes.

Finally, there are some limitations to consider when interpreting the qPCR results regarding the selection of reference genes, a critical factor for accurate and consistent quantification. Unfortunately, the most reliable reference genes in equine oocytes have not been studied previously. Therefore, we select three potential reference genes (*HPRT*, *ACTB*, and *GAPDH*) from different functional classes to reduce the change of co-regulation. Before performing the normalization, we evaluated the gene stability parameter (M value) for the detectable transcripts of the reference genes (GAPDH and ACTB), as lower values indicate higher expression stability. The observed M value (M=0.8035) was higher than the accepted cut-off value (M=0.5). However, we consider a range between 0.6 and 0.9 to reflect relatively good stability according to Smits et al. [74], based on a study conducted on equine *in vivo*, fresh and frozen *in vitro* blastocysts.

4.5. Conclusion

Vitrification increased oxidative stress, apoptosis, and the proportion of mitochondria with high MMP in equine immature oocytes. Overnight holding at uncontrolled room temperature reduced oxidative stress. However, this approach also reduced GSH levels after vitrification, potentially compromising oocyte developmental competence, and modified the oocyte's response to Nx. In fact, while naloxone supplementation positively influenced meiotic competence in oocytes vitrified immediately after collection, its effects were less consistent when combined with overnight holding. Conversely, Nx exhibited antioxidant activity in oocytes vitrified after holding but had the opposite effect in those vitrified immediately. This consideration is essential when evaluating the effects of antioxidants or other molecules to improve vitrification outcomes for immature equine oocytes, as overnight holding at uncontrolled room temperature may alter oocyte characteristics and lead to variable results. Overall, Nx displayed contrasting antioxidant effects depending on the vitrification timing, but it appeared to improve IVM outcomes in oocytes vitrified immediately after collection. The relatively low number of oocytes used in each analysis might limit the interpretation of the

study results. Nevertheless, these findings emphasize the importance of tailoring vitrification protocols to the condition of the oocytes and suggest the need for further investigation into Nx's properties. Its potential application during the post-warming period warrants exploration to enhance cryopreservation outcomes for equine immature oocytes.

4.5.1. Acknowledgements

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5. Vitrification of equine mature oocytes: the role of naloxone in a comparative analysis with the bovine model

Penelope Maria Gugole [a],
Augusta Zannoni [ab],
Iacono Eleonora [ab],
Merlo Barbara [ab]

[a] Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy.
[b] Health Science and Technologies Interdepartmental Centre for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy

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5.1. Abstract

The cryopreservation of equine oocytes remains a major challenge in assisted reproduction. This study aimed to evaluate whether naloxone (NX), an opioid receptor antagonist with reported antioxidant properties, could improve vitrification outcomes and whether bovine oocytes provide a suitable preliminary model. Two experiments were conducted. In experiment 1, immature (bGV) and mature (bMII) bovine oocytes were vitrified without (VIT) or with NX (VIT-NX) to assess meiotic competence, viability, and protocol efficiency. In experiment 2, equine mature oocytes (eMII) were vitrified with or without NX, and analyzed for viability, reactive oxygen species (ROS), glutathione (GSH), high mitochondrial membrane potential (HMMP), and developmental competence via intracytoplasmic sperm injection (ICSI). Apoptotic gene expression (BCL2, BAX, p53, *survivin*) was assessed by qRT-PCR. In bovine oocytes, NX tended to improve vitrification efficiency of GV oocytes but did not affect maturation rates (bGV-VIT 24.6 ± 4.4 vs. bGV-VIT-NX 18.5 ± 3.6), while MII vitrification achieved higher overall efficiency (overall viable MII oocytes from bGV 13.5%, vs. bMII

46.1%) (P<0.05). In equine oocytes, NX did not affect post-warming viability (eMII-VIT 74.9 ± 25.0 vs. eMII-VIT-NX 64.7 ± 17.3), GSH, HMMP, or developmental competence, and tended to reduce ROS intracellular levels. Vitrification significantly reduced (P<0.05) cleavage rates and increased degeneration, with no significant differences between NX-supplemented and control protocols. qRT-PCR revealed stable BCL2 expression and low, inconsistent detection of pro-apoptotic genes, indicating limited transcriptional activation of apoptosis. In conclusion, naloxone supplementation did not improve equine MII oocyte survival or developmental competence, although a modest reduction in ROS suggests limited antioxidant activity. Bovine oocytes confirmed the value of a preliminary model but could not reliably predict equine responses. Future work should dissect species- and stage-specific mechanisms to guide more effective strategies for equine oocyte cryopreservation.

Keywords: horse**,** equine, bovine, oocyte, vitrification, cryopreservation, naloxone

5.2. Introduction

In human medicine, oocyte cryopreservation is widely applied, whereas in veterinary medicine the outcomes remain poor [1], limiting the widespread adoption of this technique. To date, only three foals have been produced from vitrified oocytes. In the first case, oocytes were matured in vivo, vitrified, and subsequently transferred into the oviducts of inseminated mares [2]. More recently, two studies reported the births of foals from oocytes matured in vitro and fertilized by ICSI [3,4]. Despite numerous attempts to improve outcomes, no vitrification protocol has yet proven sufficiently reliable or efficient for the commercial application of in vitro embryo production from cryopreserved equine oocytes. Most recently, satisfactory outcomes were achieved only with in vivo-matured oocytes, reaching a blastocyst rate of 40% and a pregnancy rate of 67% [5].

Cryopreservation at the immature stage is theoretically less harmful to the spindle, which is not yet fully formed. Although spindle repolarization has been observed after warming in some human oocytes vitrified at the mature stage [6], a similar recovery does not appear to occur in vitrified equine oocytes [7]. Vitrification affects the cytoskeleton, spindle formation, calcium ion transport, and homeostasis [8]. At the mitochondrial level, swelling and reduced matrix density have been reported in equine oocytes following vitrification [9,10].

Like the oocytes of many other species, equine oocytes are rich in lipids, which can exacerbate oxidative stress during freezing, particularly during the lipid phase. Promising results in other

species have been achieved by culturing oocytes in media that reduce lipid accumulation and supplementing both culture and vitrification media with antioxidants [11,12]. In equine mature oocytes, melatonin supplementation improved cleavage rates, although blastocyst production remained limited [4].

Naloxone, an antagonist on the μ-opioid receptor (MOR), exhibits concentration-dependent effects and can act as a partial agonist at higher concentrations [13]. MOR expression has been detected in the mare oviduct [14,15] and in equine cumulus-oocyte complexes (COCs) [16]. At the COC level, a high concentration naloxone acted as an agonist, imparing meiosis and increaing chromosomal abnormalities, whereas at a low concentration it functioned as an antagonist, enhancing maturation and reducing chromatin defects [16]. Similarly, supplementation of IVM media with high-dose naloxone reduced the maturation rates in both canine [17] and porcine [18] oocytes, while a low concentration improved oocyte maturation and increased the inner cell mass to total cell ratio in porcine blastocysts [18]. Beyond its receptor-mediated activity, naloxone has also been investigated for potential antioxidant properties, as demonstrated in studies using PC12 cells [19]. In a preliminary study on equine oocytes, low-dose naloxone supplementation during vitrification improved blastocyst rates for both immature and mature COCs; however, the limited sample size precluded definitive conclusions [20]. More recently, a deeper investigation into its role during vitrification of equine immature COCs revealed a dual antioxidant effect depending on vitrification timing and the presence of a holding phase [21]. Specifically, naloxone enhanced meiotic competence in oocytes vitrified immediately after collection, with less consistent effects when combined with overnight holding, whereas it exhibited antioxidant activity in oocytes vitrified after holding but an opposing effect in those vitrified immediately [21].

A major limitation in equine oocyte research is their limited availability. To overcome this, bovine embryos have been used as model for testing new freezing protocols for equine embryos [22], owing to the greater availability and ease of collection of bovine oocytes. Although both bovine and equine oocytes are susceptible to cryodamage, bovine oocytes demonstrated greater robustness in vitrification, making them an ideal model for preliminary studies [23]. Additionally, cattle exibit a more regular reproductive cycle, which facilitates sample collection and the standardization of experimental conditions. These advantages, combined with the greater availability of in vitro culture and fertilization techniques for bovines [24], highlight cattle as a valuable model for advancing reproductive practices in equines.

The objective of this study was to investigate the effect of naloxone supplementation during the vitrification of equine mature oocytes, using bovine oocytes as a model system.

5.3. Materials and methods

All chemicals were purchased from Sigma–Aldrich (Merck, Milan, Italy) unless otherwise stated. Plasticware was purchased from Thermo Fisher Scientific (Monza, Italy).

5.3.1. Study experimental design

The study was structured into two experiments, as illustrated Fig. 7. The first experiment aimed to validate the use of bovine oocytes as a model for equine oocytes, given the limited availability of the latter. In this phase, both immature (bGV-VIT, bGV-VIT-NX) and mature (bMII-VIT, bMII-VIT-NX) bovine oocytes were vitrified using a protocol originally developed for equine oocytes, with or without naloxone supplementation. GV oocytes were evaluated for their ability to resume meiosis and reach the MII stage, while post-warming viability was assessed for mature oocytes. In the second experiment, the same vitrification protocol was applied to equine mature oocytes (eMII-VIT, eMII-VIT-NX) to assess the effect of naloxone, with non-vitrified oocytes (eMII) serving as controls in subsequent analyses. Viability, reactive oxygen species (ROS) levels, intracellular glutathione (GSH) content, and high mitochondrial membrane potential (HMMP) levels were evaluated in warmed equine oocytes using fluorescent staining. To further assess the developmental competence, intracytoplasmic sperm injection (ICSI) was performed post-warming, and embryo development was evaluated 48 h after fertilization.

5.3.2. Bovine oocyte collection and maturation

Bovine ovaries were collected at a local slaughterhouse (Inalca S.p.A, Modena) and transported to the laboratory within 2 to 3 h at 25 °C in a thermos case. In the lab, the ovaries were washed with demineralized water, and the cumulus-oocyte complexes (COCs) were recovered by aspirating follicular fluid using a 21-gauge butterfly infusion set connected to a vacuum pump. Recovered oocytes were classified using the IETS (International Embryo Technologies Society) grade scale, and oocytes with compact cumulus oophorous with at least 4 layers of cells and homogeneous cytoplasm were selected. Part of the collected oocytes was vitrified immediately after collection at the GV stage, while the remaining oocytes underwent IVM before vitrification.

For IVM, COCs were washed with HEPES Synthetic Oviductal Fluid (H-SOF) and cultured for 20 h in 2 ml maturation medium at 38.5 °C, in a humidified atmosphere of 5% CO_2 in air.

ng/ml epidermal growth factor (EGF), 100 ng/ml insulin-like growth factor (IGF-I), 0.1 IU/ml porcine FSH-LH (Pluset, Calier, Italy), 1.2 mM L-cysteine, 1 mM Na-pyruvate, 75 µg/ml kanamycin, and 10% of foetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Italy). At the end of the maturation period, oocytes were denuded using a fine pipette. Denuded oocytes with a normal appearance, and a visible extruded polar body (PB), were considered suitable for vitrification.

5.3.3. Equine oocyte collection and maturation

Equine ovaries were collected from slaughtered mares (Zerbini e Ragazzi S.n.c, Reggio Emilia) and transported to the laboratory within 2-3 hours at 25°C in an insulated container. Oocytes were collected as previously described [25]. Briefly, ovaries were rinsed with demineralized water, and oocytes were recovered by aspirating the content of 10-30 mm follicles using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg). The aspirated follicular fluid was collected into 250 mL glass flasks and filtered through a 65 µm mesh nylon filter (EmSafe, Minitube, Germany). COCs were then searched at a stereomicroscope. The oocytes were subjected to overnight holding, kept in HSOF (Hepes Synthetic Oviductal Fluid) at room temperature in the dark for 20-22 hours.

For IVM, oocytes were cultured in 500 µl maturation medium in four-well plates at 38.5 ◦C, in a humidified atmosphere of 5 % CO_2 in air. Maturation medium consisted of Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10 % (v/v) FBS, 50 ng/ml EGF, 100 ng/ml IGF-1, and 0.1 IU/ml porcine FSH-LH.

At the end of the maturation period, oocytes were denuded in a 0.25% trypsin solution for 60 s, washed once in HSOF plus 10% FBS to inactivate trypsin, and washed twice in HSOF. Denuded oocytes with a normal appearance, including a visible extruded PB, were considered suitable for vitrification

5.3.4. Vitrification and warming

Immature COCs or matured denuded oocytes were vitrified after a 3 steps exposure to cryoprotectants on a cryotop (Cryotop, Kitazato Supply, Japan) and immediately immerse in liquid nitrogen. Briefly, 4-5 COCs were exposed for 30 s to the first vitrification solution (V1) (HSOF containing 5% ethylene glycol (EG) and 5% dimethyl sulfoxide (DMSO), with or without 10^{-8} M Nx), 30 s in V2 (HSOF containing 10% EG and 10% DMSO, with or without

10^{-8} M Nx), and finally 30 s in V3 (HSOF containing 20% EG, 20% DMSO, sucrose 0,65 M, and Ficoll 10 mg/ml, with or without 10^{-8} M Nx). Oocytes were stored in liquid nitrogen for at least 3 days before warming. For warming, they were exposed to decreasing sucrose-containing solutions (0.250 M, 0.188M, and 0.125 M in HSOF) for 30 s each.

5.3.5. Evaluation of oocyte meiotic competence

A thin glass pipette was used to remove cumulus cells from bovine vitrified/warmed COCs after IVM. Oocytes were then morphologically evaluated under a stereomicroscope, and those exhibiting a disrupted plasma membrane were classified as degenerate and excluded from further analyses. For staining, bisbenzimide fluorescent dye (Hoechst 33342) 10 μg/ml in phosphate-buffered saline (PBS) supplemented with 0.1% w/v polyvinyl alcohol (PVA) was used. Oocytes were incubated for 15 min in the dark at room temperature and finally evaluated under a Nikon Eclipse E400 epifluorescence microscope (Nikon Europe BV, The Netherlands) to assess nuclear configuration. Oocytes were classified as mature (metaphase II, MII), immature (from germinal vesicle, GV, to metaphase I, MI), or degenerate (disrupted membranes or undefined nuclear configuration). The analysis was done in 3 replicates.

5.3.6. Evaluation of oocyte viability

Warmed mature oocytes from both species (6 replicates for bovine and 9 replicates for equine) were incubated for 2 h in HSOF at 38.5°C in humidified air with 5 % CO_2. Viability was then evaluated using Hoechst 33342/propidium iodide (PI, 10 μg/ml in PBS+PVA) staining. Only oocytes with a visibly intact plasma membrane were selected for staining. Oocytes were incubated for 30 min at room temperature in the dark and then evaluated under an epifluorescence microscope. Those with a damaged cell membrane fluoresced red and were classified as non-viable.

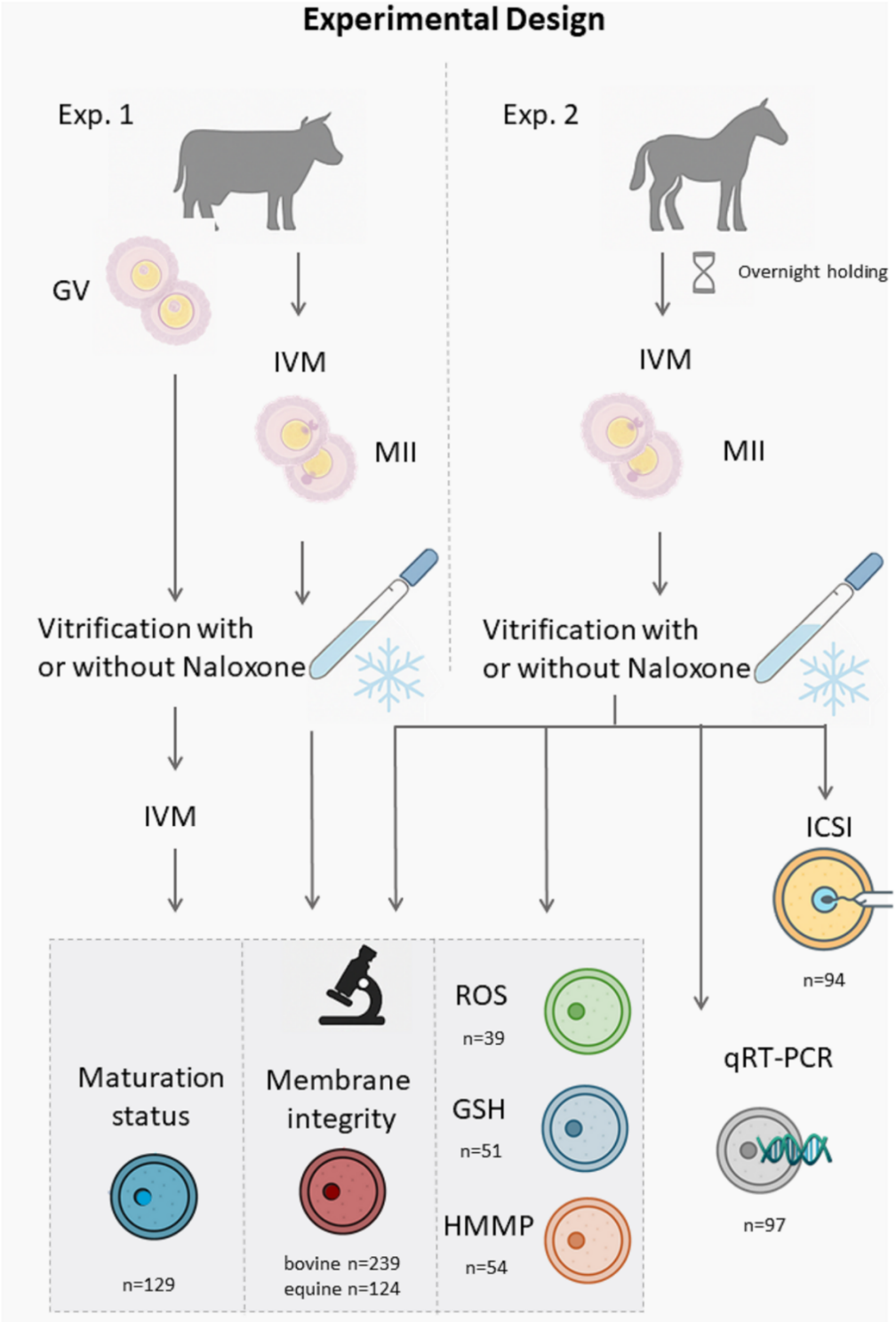

Figure 7. Schematic illustration of the distribution of bovine and equine oocytes into experimental groups based on meiotic status (immature: GV or mature: MII) and vitrification protocol, with or without naloxone supplementation. Maturation status was assessed in bovine GV after warming and in vitro maturation (IVM), while membrane integrity was evaluated in both warmed bovine and equine MII oocytes. In equine MII oocytes, levels of reactive oxygen species (ROS), intracellular glutathione (GSH), and high mitochondrial membrane potential (HMMP) were measured, along with the expression of apoptosis-related genes by quantitative real-time PCR (qRT-PCR). Oocyte developmental competence was further assessed in equine MII oocytes by intracytoplasmic sperm injection (ICSI).

5.3.7. Detection of reactive oxygen species (ROS) and glutathione (GSH) levels

Intracellular ROS and GSH levels were determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen™, Thermo Fisher Scientific, Italy) and 4-chloromethyl-6.8-difluoro- 7-hydroxycoumarin (CellTracker Blue, CMF2HC, Invitrogen™, Thermo Fisher Scientific, Italy) respectively. Oocytes were incubated 20 min in the dark at room temperature in PBS+PVA and 10 µM H2DCFDA or 10 µM CellTracker Blue. After staining, oocytes were washed once in PBS+PVA and examined under an epifluorescence microscope. Images of fluorescent oocytes were acquired, keeping the same acquisition parameters for all groups, and subsequently analysed with an open-source software (ImageJ). For each image, oocyte fluorescence intensity was measured and normalized to the background, as previously described [21]. Final values are expressed as arbitrary units.

5.3.8. Mitochondrial membrane potential detection

Mitochondrial membrane potential of oocytes was assessed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen, Italy). Denuded oocytes were incubated at 38.5°C for 30 min in the dark in 30 µl microdrops of HSOF supplemented with 0,2 µg of JC-1. Then, the oocytes were immediately examined under an epifluorescence microscope. Only one fluorescent image (red channel) was acquired for each oocyte, to detect the mitochondria with high membrane potential (HMMP, orange stained). Images were analysed as previously reported for ROS and GSH.

5.3.9. Intracytoplasmic sperm injection (ICSI)

Control and vitrified/warmed equine oocytes displaying an intact plasma membrane and an extruded PB were injected with frozen/thawed spermatozoa from a stallion of proven fertility after simple washing. ICSI was first performed on control oocytes, which had to be injected immediately. For each warming of vitrified oocytes, a batch of fresh controls was included, resulting in 7 replicates for controls and 5 for vitrified oocytes.

Conventional ICSI was performed at 37 °C using a micromanipulator (Narishige Co. Ltd, Tokyo, Japan) mounted on an inverted microscope (Nikon TE 300: Nikon, Kawasaki, Japan). Following ICSI, oocytes were cultured in 20 µl droplets of SOF-IVC (SOF supplemented with MEM amino acids and 16 mg/ml fatty acid free-bovine serum albumin, FAF-BSA) under mineral oil at 38.5 °C in a modified atmosphere (5% CO_2, 7% O_2, and 88% N_2) for 48 h, before

cleavage assessment. After 48 h, cleaved embryos were stained with Hoechst 33342 (10 µg/ml bisbenzimide in PBS+PVA) for 30 min at room temperature, washed in PBS and observed using an epifluorescence microscope to assess the number of nuclei. ICSI was performed in 7 replicates (5 for vitrified oocytes).

5.3.10. Quantitative real-time PCR (qRT-PCR) gene expression analysis for Bcl2, Bax, p53 and *survivin*

The experiment was done in 3 replicates with 9-13 in vitro matured oocyte per group (eMII; eMII-VIT; eMII-VIT-NX). Oocytes were denuded by digestion of zona pellucida in pronase solution (0.5% w/v in PBS), washed twice in PBS, and snap-frozen in molecular grade water (20 µl) before storage at -80°C. The RNA extraction, retrotranscription and RT-PCR were performed according to Gugole et al., 2025 [21]. Briefly, pool of MII oocytes were added with 2 µl of SideStep lysis and Stabilization buffer (Agilent Technologies, Santa Clara, CA, USA), mixed very well by pipetting and then 16 µl were retrotranscribed with cDNA using 5X iScript RT Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), following the manufacturer's instructions, in a 20 µl final volume to obtain cDNA. Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. All the primers used for interest genes (Bcl2, Bax, p53 and *survivin*) and reference genes (GAPDH, ACTB) was reported, and the RT-PCR experiments were reported in our previous paper [21]. The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. The relative mRNA expressions of tested genes were normalized by using the ΔCt method (ΔCt= Ct mean reference genes–Ct interest gene) and then the relative expression was calculated as fold of change ($2^{-\Delta\Delta Ct}$ method) in respect to the MII oocyte control group obtained under different condition (VIT or VIT-NX).

5.3.11. Statistical analysis

Data are expressed as mean ± standard deviation. Viability results in different treatments were compared using a binomial Generalized Linear Model (GLM) with logit function. Fluorescence intensity results were checked for normality using the Shapiro-Wilk test and then compared using a GLM for gamma log distribution and Wald pairwise comparison. Overall protocols' efficiency, comparing naloxone supplementation, immature versus mature oocytes, or different species, was assessed using a contingency table Chi square test. Data were analysed using IBM SPSS Statistics 29.0 (IBM Corporation, Milan, Italy), with significance assessed at $P<0.05$.

For gene expression data (ΔCt values), normal distributions were evaluated by means of Shapiro–Wilk and Kolmogorov-Smirnov tests, and, according to the results, statistic parametric test was performed (one-way ANOVA with significance level of $P \leq 0.05$; GraphPad Prism software, version 9.1, La Jolla, CA).

5.4. Results

5.4.1. Experiment 1

For bovine immature oocytes, no significant differences were observed between the two vitrification protocols, (P>0.05), although naloxone supplementation tended to increase the number of COCs suitable for IVM (P=0.064) and overall efficiency (P=0.078) (Table 5). Nonetheless, meiotic competence after IVM did not differ between groups (P>0.05) (Table 6). For bovine mature oocytes, no significant differences in overall efficiency or viability were observed between protocols (P>0.05) (Table 7).

Group	Vitrified oocytes	Recovered oocytes (%)	IVM oocytes (%)	Stained oocytes (%)	Overall stained oocytes (%)
bGV-VIT	100	90 (90.0)	78 (86.7)	58 (74.4)	58/90 (64.4)
bGV-VIT-NX	100	93 (90.3)	88 (94.6)	71 (80.7)	71/93 (76.3)

Table 5. Recovery, IVM selection, staining, and protocol efficiency (stained/recovered) of vitrified immature bovine oocytes using solutions without (bGV-VIT) or with (bGV-VIT-NX) naloxone supplementation.

Group	MII	IMM	DEG
bGV-VIT	24.6 ± 4.4	10.4 ± 11.9	64.8 ± 15.6
bGV-VIT-NX	18.5 ± 3.6	17.3 ± 15.1	64.2 ± 18.4

Table 6. Percentage (mean ± SD) of mature (MII), immature (IMM) and degenerate (DEG) bovine oocytes vitrified without (bGV-VIT) or with (bGV-VIT-NX) naloxone, as assessed by Hoechst 33342 staining after 22 h of IVM.

Group	Vitrified oocytes	Recovered oocytes (%)	H/PI stained oocytes (%)	Viable oocytes
bMII-VIT	128	123 (96.1)	118 (95.3)	54.6 ± 14.5

bMII-VIT-NX	135	127 (94.1)	121 (95.3)	61.2 ± 10.1

Table 7. Recovery, protocol efficiency (stained/recovered), and percentage of viable bovine mature oocytes (mean ± SD), assessed by Hoechst 33342/propidium iodide (H/PI) stain 2 h after warming, using vitrification solutions without (bMII-VIT) or with (bMII-VIT-NX) naloxone.

Given the absence of significant effects of naloxone supplementation on vitrification, data were pooled to compare the vitrification efficiency of immature versus mature bovine oocytes (Table 8). A lower proportion of oocytes was vitrified when using mature oocytes ($P<0.05$). The presence of cumulus cells in immature oocytes did not significantly affect the recovery rate ($P>0.05$) but reduced the number of oocytes with intact membranes at stereomicroscopic evaluation ($P<0.05$). Vitrified mature oocytes achieved higher overall efficiency in terms of viable MII oocytes compared to immature oocytes ($P<0.05$).

Group	Initial COCs	Vitrified oocytes (%)	Recovered oocytes (%)	Intact oocytes (%)	Overall viable MII oocytes (%)
bGV-V	200	200 (100.0) a	183 (91.5)	159 (86.9) a	27/200 (13.5) a
bMII-V	310	263 (84.8) b	250 (95.1)	239 (95.6) b	143/310 (46.1) b

Table 8. Comparison of vitrification efficiency in bovine immature (bGV-V) and mature (bMII-V) oocytes.

5.4.2. Experiment 2

A total of 536 equine mature oocytes were used, of which 392 were vitrified and 144 served as controls. Similar to bovine mature oocytes, 374 (95.4%) were recovered after warming, and 357 (90.8%) were included in the analyses ($P>0.05$).

No difference between protocols were observed in recovery rate and in the number of oocytes with intact membranes at stereomicroscopic evaluation ($P>0.05$) (Table 9). However, the viability of equine oocytes was significantly higher ($P<0.05$) in the control group compared with the vitrified group with naloxone, whereas the vitrified group without naloxone showed intermediate results ($P>0.05$) (Table 9).

Group	Vitrified oocytes	Recovered oocytes (%)	Overall intact oocytes (%)	H/PI stained oocytes (%)	Viable oocytes
eMII	/	/	/	32	91.1 ± 16.9 a
eMII-VIT	194	188 (96.9)	174 (92.5)	60	74.9 ± 25.0 ab
eMII-VIT-NX	198	186 (93.9)	173 (93.0)	64	64.7 ± 17.3 b

Table 9. Recovery, protocol efficiency (intact/recovered), and percentage (mean ± SD) of viable equine mature oocytes before (eMII) or after vitrification without (eMII-VIT) or with (eMII-VIT-NX) naloxone, assessed by Hoechst 33342/propidium iodide (H/PI) stain 2 h after warming.

The results of staining for ROS and GSH intracellular levels, as well as HMMP, are graphically summarised (Fig. 8). ROS intracellular levels tended to be different between groups (P=0.060), with a significant raise (P<0.05) in eMII-VIT compared to control, and only a tendence (P=0.070) for eMII-VIT-NX (Fig. 8A). On the contrary, no statistically significant differences (P>0.05) were observed for both GSH intracellular levels (Fig. 8B) and HMMP (Fig. 8C).

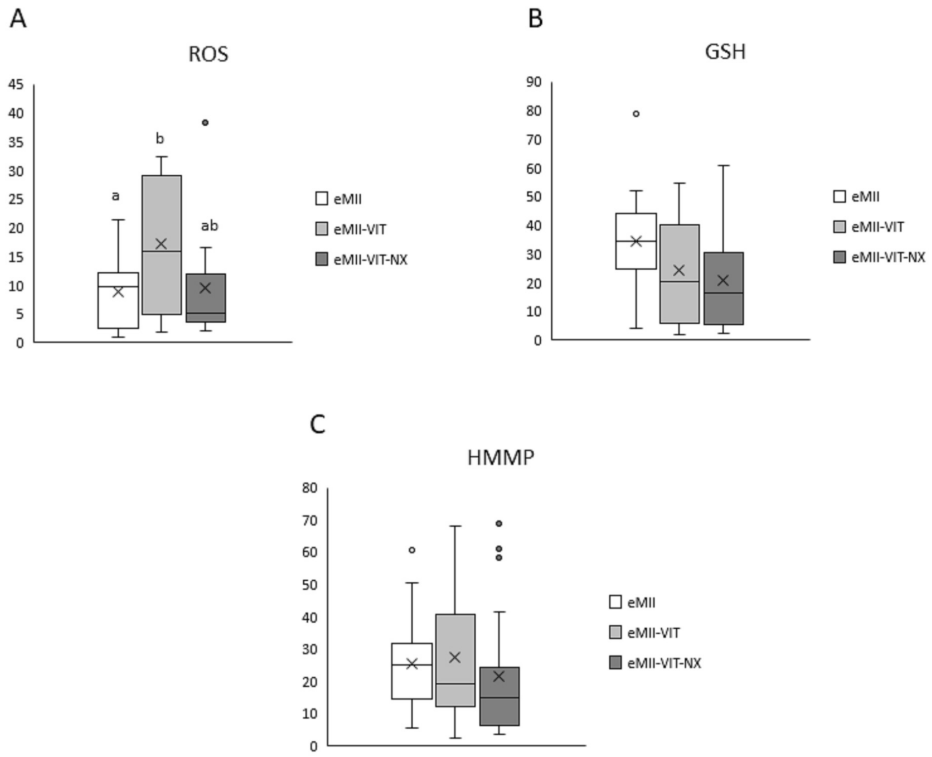

Figure 8. Quantification of oxidative stress, intracellular glutathione levels, and mitochondrial activity in equine MII oocytes after vitrification, through fluorescent staining 2 h after warming. (A) Reactive oxygen species (ROS) levels assessed by H2DCFDA; (B) Intracellular glutathione (GSH) levels assessed by CellTracker Blue; (C) Mitochondrial membrane potential measured by JC-1 staining assessed by JC-1. Three experimental groups are shown: control oocytes (eMII), vitrified oocytes without (eMII-VIT) and with (eMII-VIT-NX) naloxone. a vs b P<0.05

Oocyte developmental competence after ICSI was reduced by cryopreservation (P<0.05), which led to higher oocyte degeneration (P<0.05), with no significant differences observed between vitrification protocols (P>0.05) (Table 10). In the eMII group, a total of 9 embryos were obtained (3 at the 2-cell stage, 5 at 4-cell stage, and 1 at the 8 cell stage); in the eMII-VIT group, 2 embryos (1 at the 2-cell stage, and 1 at the 8 cell stage); and in the eMII-VIT-NX group, 1 embryo (at the 4 cell stage).

Group	ICSI (n)	Lysed	Cultured oocytes (n)	Cleaved	Degenerate
eMII	36	8	28	39,3±32,2 a	10,7±19,7 a
eMII-VIT	30	3	27	7,3±10,1 b	32,7±44,7 b
eMII-VIT-NX	28	2	26	2,9±6,4 b	37,1±51,1 b

Table 10. Percentage (mean ± SD) of cleaved and degenerate equine mature oocytes before(eMII) or after vitrification without (eMII-VIT) or with (eMII-VIT-NX), as assessed by Hoechst 33342 staining 48 h after ICSI.

qRT-PCR analysis on eMII, eMII-VIT and eMII-VIT-NX groups showed the detection for both reference genes (Table 11). Regarding interest genes, BCL2 was detectable in all replicates for each group (9/9), BAX in two replicates per group (6/9), and p53 only in two replicates of the control group and in one replicate for each vitrification group (4/9) (Table 11). Survivin was not detectable in any sample. To calculate fold changes reltive to control group, undetectable samples were assigned a Cq value of 40, corresponding to the maximum cycle of the PCR protocol. Samples exhibited high variability, particularly for BCL2 and BAX, and no statistically significant differences were observed (Fig. 9).

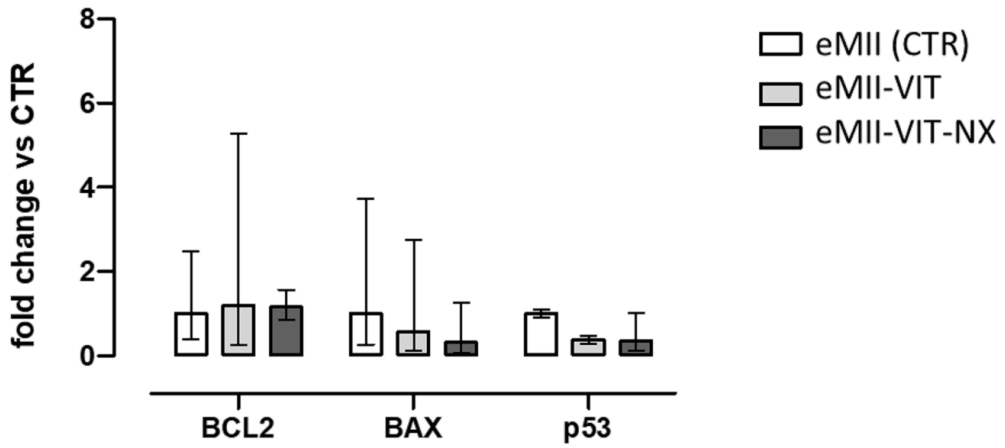

Figure 9. Relative expression levels of BCL2, BAX, and p53 evaluated by qRT-PCR in equine mature oocytes vitrified without (eMII-VIT) or with (eMII-VIT-NX) naloxone (VIT-NX) relative to the non-vitrified controls (eMII).

gene	eMII	eMII-VIT	eMII-VIT-NX
GAPDH	3/3 (range Cq 25.9-32.8)	3/3 (range Cq 26.8-31.6)	3/3 (range Cq 29.5-30.7)
BACT	3/3 (range Cq 25.4-35.7)	3/3 (range Cq 28.4-33.7)	3/3 (range Cq 30.3-34.5)

BCL2	3/3 (range Cq 24.8-33.8)	3/3 (range Cq 26.6-32.3)	3/3 (range Cq 28.9-2.8)
BAX	2/3 (range Cq 29.9-34.2)	2/3 (range Cq 31.6-36.4)	2/3 (range Cq 35.6)
P53	2/3 (range Cq 33.3-36)	1/3 (Cq 35.8)	1/3 (Cq 36.6)
Survivin	ND	ND	ND

Table 11. Number of samples in which each gene was detected, relative to the triplicates in each experimental group. The range of Cycle quantity (Cq) detected by RT-PCR is reported. ND= Not Detectable.

5.5. Discussion

The vitrification of oocytes in the veterinary field remains a significant challenge, and the effects of this technique on oocytes are still partially unknown. This study explored the potential of naloxone supplementation to improve equine mature oocyte vitrification, using the bovine as a model for the horse. This comparative approach allowed for a broader interpretation of naloxone's effects and highlighted the value of establishing a reliable model for equine oocytes, given the limited availability and intrinsic handling constraints of equine female gametes.

In the present study, NX tended to improve the vitrification efficiency of bovine immature oocytes, but without any effect on subsequent maturation rates. Conversely, equine oocytes displayed greater meiotic competence when vitrified under the same conditions (i.e., immediately vitrified in the presence of NX) [21]. This suggests a species-specific interaction between naloxone and COCs, independent of exposure to a holding period.

Nevertheless, in both bovine and equine mature oocytes, NX supplementation did not influence vitrification efficiency or oocyte viability 2 h after warming. However, in equine oocytes, when vitrified samples were compared with non-vitrified controls, NX negatively affected viability. In this context, bovine oocytes served as the immediately vitrified counterpart, whereas equine oocytes underwent overnight holding. Overnight holding at uncontrolled room temperature may have contributed to these differences, as already observed for equine immature oocytes [21].

It has been demonstrated that bovine oocytes can be held at room temperature for 6-10 hours without detrimental effects on maturation or embryo development [26], but overnight holding in the absence of maturation inhibitors has not been described. In horses, overnight holding is considered useful for oocyte shipment without impairing developmental competence [31]. On the other hand, although uncontrolled (22-27°C) overnight holding did not compromise viability or energy/redox status in equine oocytes, it did increased apparent cytoplasmic maturation before IVM, as reflected in a more mature mitochondrial distribution in condensed chromatin configuration oocytes [27]. It has also been suggested that overnight holding prior

to IVM may act as a pre-selection step, thereby enhancing the developmental competence of surviving oocytes [28].

Given the discrepancies observed between immature equine and bovine COCs vitrified immediately with or without NX, some concerns arise regarding the suitability of bovine non-held oocytes as a model for equine ones.

Our findings indicate that, in the bovine model, vitrification at the MII stage is more advantageous than at the GV stage. This observation is consistent with what observed in horses, considering the outcomes obtained with immature equine oocytes [21]. These results reinforce the notion that the maturation stage at the time of cryopreservation is a critical determinant of oocyte survival and subsequent competence, and that GV-stage vitrification still represents a major biological challenge. It has long been argued that GV oocytes represent the meiotic stage most resistant to cryodamage, particularly due to the absence of the meiotic spindle, a delicate structure present in mature oocytes [29]. However, bovine immature oocytes had a lower water permeability than mature oocytes and a higher permeability for ethylene glycol at low concentrations; this inverse relationship with CPA concentration was not observed in mature oocytes, likely reflecting differences in aquaporin expression between the two meiotic stages [30]. Nonetheless, recent findings suggest that damage to other delicate structures, such as the cumulus cell compartment or transzonal processes, may hinder the maturation, fertilization, and embryonic development of oocytes vitrified at the germinal vesicle stage [31]. Conversely, it has been reported that MII oocytes contain a higher amount of polyunsaturated fatty acids and a lower content of saturated fatty acids [32,33] which would allow their membranes to be more flexible and more resistant to low temperatures due to their lipid composition [34,35]. Moreover, the lipid content in oocytes matured in vitro is higher compared to oocytes matured in vivo, resembling the levels found in immature oocytes [36].

Equine mature oocytes subjected to vitrification showed lower survival rates compared to the non-vitrified group. The results obtained in this study agree with those reported in the literature for domestic animals, in which oocyte vitrification leads to a loss of gametes in terms of survival after warming [37]. Although vitrification is an effective preservation method that surpassed slow freezing decades ago [38], the outcomes achieved never fully match those of gametes that have not undergone cryopreservation.

Besides its role as an opioid antagonist, naloxone has also been reported to exert protective effects against oxidative stress [19]. Cryopreservation procedures are well known to provoke oxidative stress, via ROS production, lipid peroxidation, and related damage to membranes, DNA, and organelles. Other studies have highlighted the positive impact of antioxidant

molecules, such as melatonin, on cryopreserved equine oocytes [4]. In this study, NX attenuated ROS generation induced by vitrification and warming, confirming its antioxidant effect. However, the reduced ROS production was not associated to a higher survival rate, suggesting that it may influence the activation of degenerative and apoptotic processes. MOR receptors regulate calcium entry into oocytes and may influence their maturation in relation to reproductive seasonality [16]. Calcium is a critical intracellular messenger within oocytes, playing a central role in regulating metabolism, activation, fertilization, and embryonic development [39]. Any disruption in calcium homeostasis can compromise oocyte viability, fertilization ability, and developmental competence. Excessive intracellular Ca^{2+} levels are a key driver of apoptosis in mammalian oocytes [40]. The potential influence of MOR receptors modulated by naloxone on intracellular Ca^{2+} levels may have acted synergistically with vitrification to trigger apoptotic pathways.

Moreover, mitochondrial integrity is closely linked to calcium signalling, even minor structural damage to mitochondria, as induced by vitrification, leads to a complete loss of mitochondrial functionality in the pericortical region of cryopreserved oocytes [41]. Mitochondria are essential for maintaining basal Ca^{2+} levels in unfertilized oocytes and for restoring these levels after the oscillations induced by fertilization [42,43]. Cryoprotectants, such as DMSO and ethylene glycol, further complicate calcium homeostasis by increasing intracellular Ca^{2+} levels. Ethylene glycol facilitates calcium entry from the extracellular environment, while DMSO promotes calcium release from intracellular stores, such as the rough endoplasmic reticulum [44]. This dysregulation can result in calcium overloading, which alters membrane permeability, decreases mitochondrial membrane potential, and reduces ATP levels [45]. These factors could also explain the lower HMMP observed in oocytes vitrified with naloxone compared to those vitrified without it, although the difference was not significant, likely due to the variability among batches of ovaries.

Vitrification and naloxone supplementation did not appear to alter GSH content in equine MII oocytes, although their developmental competence was reduced. As for HMMP, the high variability between batches of oocytes may have masked the significance of the GSH reduction in vitrified oocytes. Glutathione, a thiol tripeptide (γ-glutamyl cysteinyl glycine), is the primary non-enzymatic defence against oxidative stress due to the reducing properties of its sulfhydryl group [46]. Its synthesis during oocyte maturation is crucial for sperm chromatin decondensation and the formation of the male pronucleus [47].

In fact, ICSI outcomes at 72 h were poorer in cryopreserved oocytes, consistent with previous findings indicating that ICSI on vitrified equine oocytes yields lower fertilization rates than in

fresh controls [20,7,48,49,5]. Vitrification not only increased the degeneration rate, but also directly reduced the developmental competence of non-degenerated oocytes, as evidenced by their failure to cleave after ICSI. The relatively low cleavage rate of non-vitrified oocytes observed in this study may be attributable to the use of a ICSI-technique without piezo assistance [50] and to the involvement of a newly trained operator. Furthermore, It must be noted that the use of oocytes obtained from slaughterhouse ovaries introduces inherent variability in gamete quality due to unknown animal histories, as well as factors related to tissue transport, handling, and organ preservation. This variability, while unavoidable, is a recognized challenge in studies of this nature. This variability, coupled with factors such as advanced age, known to alter meiotic spindle morphology and chromosome alignment, may further influence embryo viability and developmental competence, irrespective of vitrification [5].

Our RT-PCR results revealed stable detection of BCL2 and low or inconsistent detection of BAX and p53 across treatments, with no differences in relative expression. This pattern contrasts with findings in bovine oocytes, where vitrification has been reported to increase BAX mRNA levels and decrease BCL2 mRNA levels, contributing to mitochondrion-mediated apoptosis [51] Interestingly, our data more closely resemble those observed in vitrified canine oocytes, where BCL2 expression was increased whereas BAX was undetectable, suggesting a limited transcriptional activation of pro-apoptotic pathways [52]. Moreover, while an increased BAX/BCL2 ratio is a hallmark of metabolic stress in equine embryos exposed to high glucose concentrations [53], no such increase was observed here. Taken together, these interspecies comparisons suggest that equine MII oocytes may possess a species-specific resilience to vitrification-induced apoptotic signalling or alternatively activate these pathways at post-transcriptional or delayed stages, which warrants further temporal and protein-level investigations.

5.6. Conclusion

In conclusion, this study demonstrates that, despite the widespread use of vitrification, equine oocytes, particularly at the MII stage, remain highly sensitive to cryopreservation and do not benefit from naloxone supplementation. Naloxone failed to improve post-warming survival, mitochondrial membrane potential, or developmental competence, and it did not significantly alter the expression of key apoptotic genes, although a modest reduction in ROS suggests a limited antioxidant action. Comparative trials using bovine oocytes confirmed that this species can serve as a preliminary model but cannot reliably predict the equine response, especially

regarding the interaction of naloxone with the holding phase. Overall, our findings indicate that naloxone does not provide meaningful protection during equine oocyte vitrification and that the mechanisms underlying species- and stage-specific responses remain to be clarified. Future studies should focus on temporal and protein-level analyses of apoptosis and calcium homeostasis to better understand the interplay between cryoprotectants, naloxone, and oocyte physiology, and to develop more effective strategies for equine oocyte cryopreservation.

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6. Bovine oocytes cryopreservation using a human commercial modified vitrification protocol

Penelope Maria Gugole[a],
Eleonora Iacono[ab],
Costanza Casanti[a],
Simone Palini[c],
Barbara Merlo[ab]

[a] Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy.
[b] Health Science and Technologies Interdepartmental Center for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy
[c] Cervesi Hospital, Via Ludwig Van Beethoven 1, 47841 Cattolica (RN), Italy

6.1. Abstract

Although vitrification represents the most widely applied strategy for oocyte cryopreservation, the optimization of existing protocols remains an active area of research. Current efforts are focused on simplifying procedures, shortening exposure times, and reducing the cytotoxic impact of cryoprotective agents (CPAs). The aim of this study was to evaluate a shortened version of a commercial vitrification protocol originally designed for human oocytes, using bovine as an experimental model. The Rapid-i™ Vitrification System (Vitrolife Sweden AB, Västra Frölunda, Sweden), a DMSO-free, clinical-grade kit, was employed and tested a modified version, named Manhattan protocol, characterized by reduced oocyte exposure times to CPAs. In Experiment 1, matured bovine oocytes (MII stage) were vitrified using the Manhattan protocol and divided into two groups: a closed (C) and a semi-open (SO) system. No statistically significant differences were observed in post-warming oocyte recovery or viability (91.9 ± 9.4% C, 90.5 ± 11.8% SO), demonstrating that both systems ensure efficient vitrification and that shortened exposure times do not compromise oocyte survival (75.7 ± 10.3% C, 76.9 ± 19.3% SO). In Experiment 2, based on these results, matured bovine oocytes

were vitrified using the Manhattan protocol with the Cryotop, an open vitrification system commonly used in assisted reproduction laboratories. Intracellular reactive oxygen species, glutathione amount and mitochondrial activity were evaluated through fluorescent staining. No significant differences were detected in intracellular ROS and glutathione levels between the Manhattan and non-vitrified control group, suggesting that the protocol had minimal impact on oxidative stress. Conversely, vitrified oocytes exhibited a significantly higher mitochondrial membrane potential (MMP) compared with the control group ($p < 0.05$), which may indicate either a transient increase in energy demand during post-warming recovery or early apoptotic activity. In conclusion, the Manhattan protocol, applied to bovine oocytes using either closed, semi-open vitrification systems, ensures high post-warming viability and does not alter redox homeostasis. The increased mitochondrial activity observed warrants further investigation to determine its biological meaning. Future studies including fertilization and embryo development will help to better assess the functional competence of oocytes vitrified with Manhattan protocol.

Keywords: bovine, human, oocyte, vitrification.

6.2. Introduction

Fertility preservation allows women to protect their reproductive capacity in situations where fertility may be compromised, such as diagnosed oncologic disease, medical condition or delayed childbearing giving them greater control over when to become mothers and increased security in facing potential future medical challenges [1].

Ethical limitations and scarce availability of human gametes restrict direct research, but comparative studies using animal models offer valuable experimental opportunities to explore biological mechanisms and generate preliminary data under controlled conditions. The bovine species represents one of the most established models in reproductive physiology, owing to the extensive understanding of its ovarian function and the availability of numerous reproductive tools applicable to both production and experimental research [2]. In the dairy industry, techniques such as oestrus synchronization, artificial insemination (AI), sperm sexing, superovulation, in vitro fertilization, and embryo transfer (ET) have been successfully implemented and optimized to maximize the genetic improvement of cattle breeds [3].

Cattle, and livestock species in general, offer several advantages as research models: they are readily available, relatively inexpensive, easy to handle compared with nondomestic animals,

and large enough to enable detailed endocrinological and morphological investigations [4]. Moreover, the large global cattle population provides accessible biological material for experimentation, thereby reducing the need for traditional laboratory animals.

Bovine and human reproductive biology share numerous characteristics, both are monovular, exhibit continuous cyclicity when not pregnant, have a gestation period of approximately nine months, and possess ovaries of comparable size and morphology [5]. The cow has thus been proposed as a valuable model for studying human ovarian function [5–7] and has been successfully used to explore several aspects of follicular physiology [7–14]. Furthermore, key techniques such as ultrasound monitoring of individual follicles and ovulation induction were originally developed in cattle [5,15].

In the ART scene, vitrification has become the preferred method of cryopreservation in assisted reproduction, largely replacing slow-freezing due to its superior post-thaw survival and developmental outcomes [16]. One major area of interest is the reduction of equilibration and rehydration times during vitrification and warming. Shorter exposure times are desirable not only to simplify procedures and improve workflow consistency but also to limit the detrimental effects of prolonged exposure to cryoprotective agents (CPAs), which can induce osmotic, toxic, genetic, or epigenetic damage [17,18].

Traditional vitrification protocols, however, involve prolonged exposure (5–15 min) to equilibration media containing low CPA concentrations, followed by a short immersion in vitrification solution before liquid nitrogen storage. This approach allows gradual CPA permeation and dehydration but increases the risk of CPA toxicity with longer equilibration times. Consequently, several studies have focused on identifying the effect of the variation of exposure duration that ensures effective CPA loading while minimizing cellular stress on humans [19–21] and bovine [22–24].

In this context, the bovine oocyte has proven to be a valuable model for optimizing vitrification protocols applicable to both human and veterinary medicine. Due to its similarity in size, lipid content, and plasma membrane properties to the human oocyte, the bovine model allows controlled assessment of permeability kinetics and osmotic tolerance [24,25]. Recent work by García-Martínez et al. [24] proposed a shortened vitrification protocol for MII bovine oocytes, reducing the equilibration step from 2 min to 30 s at 38.5 °C without compromising survival or developmental competence. Their results demonstrated that maintaining CPA exposure within osmotic tolerance limits can effectively prevent toxic damage while ensuring adequate dehydration.

In addition to optimizing the duration of exposure to cryoprotectants, another key aspect of vitrification refinement concerns the type of carrier system employed. Two main approaches are currently used in oocyte vitrification: open systems, in which samples come into direct contact with liquid nitrogen, and closed systems, which provide a physical barrier between the biological material and the cryogenic medium. Both systems have been shown to support high post-warming survival and developmental rates [26].

Comparative studies show similar outcomes between open and closed systems, though some evidence points to slightly better results with closed vitrification [27,28]. However, when compared indirectly with fresh oocytes, closed systems appear to exert a less detrimental effect on oocyte competence, as reflected by higher blastocyst formation rates. Beyond developmental performance, closed devices also offer an additional biosafety advantage, preventing any potential contamination by liquid nitrogen or cross-contamination between samples during storage [29].

Although there is currently no evidence of pathogen transmission through cryogenic storage [30], the use of closed systems has been recommended as a precautionary measure, especially in clinical and germplasm banking contexts [31].

Within this framework, the present study was designed to evaluate not only the effect of shortened vitrification protocols, but also to compare the performance of two vitrification devices, one closed and one semi-open, in order to assess their impact on post-warming oocyte integrity and viability in the bovine model.

6.3. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Merck, Rome, Italy) unless otherwise stated. Plasticware was purchased from Thermo Fisher Scientific (Monza, Italy).

6.3.1. Experimental design

Two consecutive experiments were conducted to evaluate the effects of Manhattan vitrification protocol, two vitrification systems devices and co-culture conditions on vitrified bovine oocyte viability and developmental competence (see Figure 10).

- Experiment 1 – Comparison between closed and semi-open vitrification systems

Mature bovine oocytes were randomly allocated into two groups according to the vitrification support used: (1) Closed system (C)**,** in which oocytes did not come into direct contact with

liquid nitrogen; and (2) Semi-open system (SO**)**, in which oocytes came into direct contact with liquid nitrogen. All vitrification's were performed using the Manhattan protocol. Post-warming oocyte integrity and survival rates were assessed to compare the efficiency of the two systems. The experiment was done in 5 replicates.

- Experiment 2 – Assessment of oocyte quality after vitrification using Cryotop

Based on the results of Experiment 1, showing no significant differences between closed and semi-open systems, vitrification was subsequently carried out using the Cryotop device, an open support widely used in both human and veterinary ART. After warming, oocytes were evaluated for morphological integrity and viability and then subjected to fluorescent staining to assess reactive oxygen species (ROS) levels, intracellular glutathione (GSH) content, and mitochondrial membrane potential (HMMP) using JC-1 dye.

6.3.2. Collection and in vitro maturation of cumulus oocyte complexes

Bovine ovaries were collected at a local slaughterhouse (Inalca S.p.A, Modena) and transported to the Laboratory of Reproduction and Animal Biotechnologies, Department of Veterinary Medicine, University of Bologna, within 2 to 3 h at 25 °C in a thermos case. In the lab, the ovaries were washed with demineralized water and the cumulus-oocyte complexes (COCs) were recovered by aspirating follicular fluid using a 21-gauge butterfly infusion set connected to a vacuum pump. Recovered oocytes were classified using the IETS (International Embryo Technologies Society) grade scale and oocytes with a compact cumulus oophorus with at least 4 layers of cells and homogeneous cytoplasm were enrolled in the study.
For IVM, COCs were washed with HEPES Synthetic Oviductal Fluid (H-SOF) and cultured for 20 h in 2 ml of maturation medium at 38.5 °C, in a humidified atmosphere at 5% CO_2 in air. Maturation medium consisted in Tissue Culture Medium (TCM 199) supplemented with 10 ng/ml epidermal growth factor (EGF), 100 ng/ml insulin-like growth factor (IGF-I), 0.1 IU/ml porcine FSH-LH (Pluset, Calier, Como, Italy), 1.2 mM L-cystein, 1 mM Na-pyruvate, 75 µg/ml kanamycin, and 10% fetal bovine serum (FBS; Gibco). At the end of the maturation period, oocytes were denuded using a fine glass pipette. Denuded oocytes with a visible extruded polar body (PB), were considered suitable for vitrification.

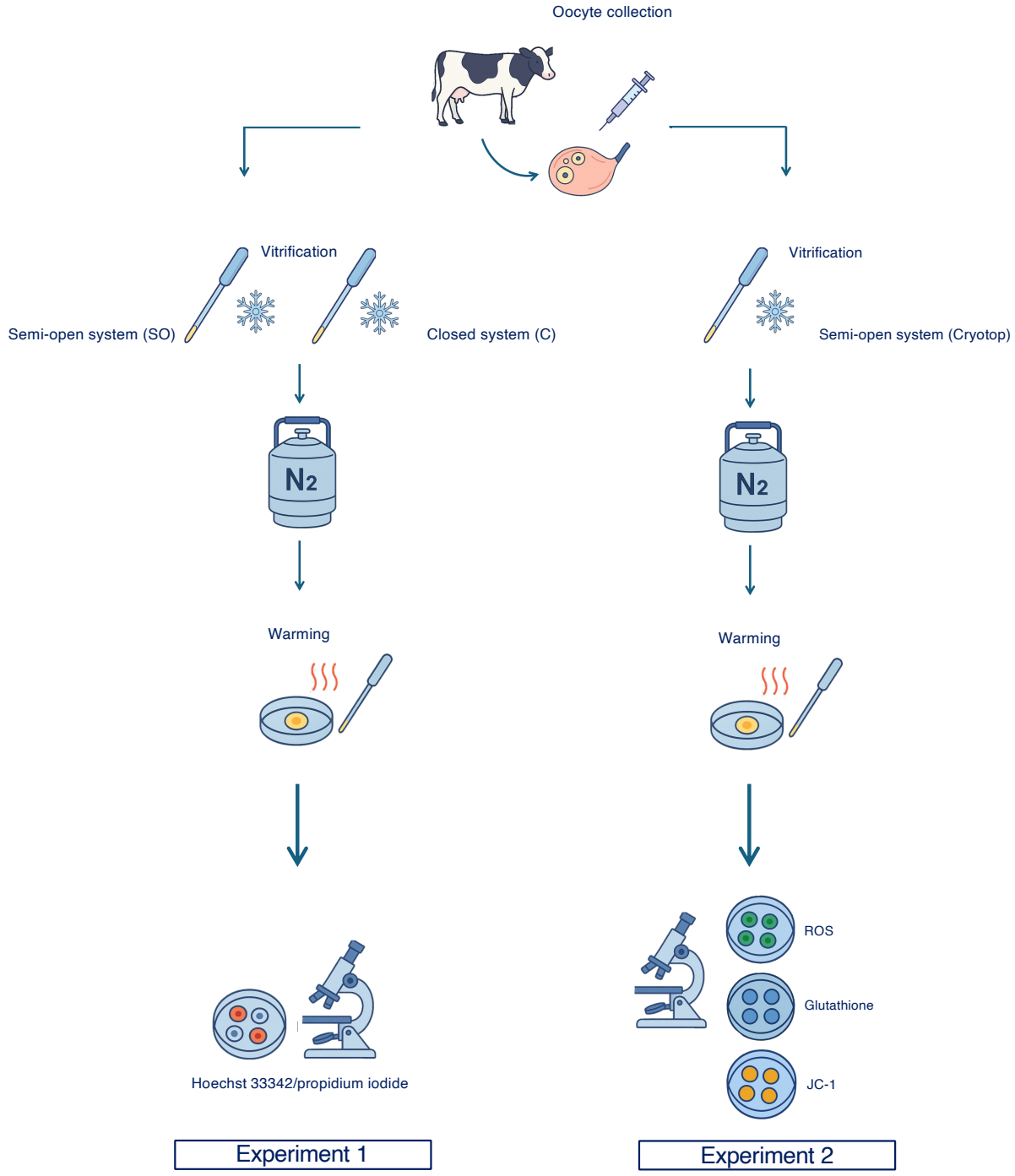

Figure 10 Schematic representation of the experimental design. Bovine oocytes were collected from slaughterhouse ovaries and divided into two experiments. In Experiment 1, matured oocytes (MII) were vitrified using the Manhattan protocol and compared between a semi-open (SO) and a closed (C) system. After warming, oocyte viability was evaluated using Hoechst 33342/propidium iodide staining. In Experiment 2, matured oocytes were vitrified using the same Manhattan protocol with the Cryotop, a semi-open vitrification system commonly used in assisted reproduction laboratories. Following warming and 2 h of incubation, intracellular reactive oxygen species (ROS), glutathione (GSH) levels, and mitochondrial membrane potential (MMP) were assessed by specific fluorescent staining (H_2DCFDA, CellTracker™ Blue, and JC-1, respectively).

6.3.3. Oocyte vitrification and warming

In Exp. 1, Oocyte vitrification was performed using the Rapid-i™ Vitrification System (Vitrolife Sweden AB, V. Frolunda, Sweden), that provides three different vitrification media (RapidVit™), four different warming media RapidWarm™, and the cryodevice Rapid-i™ straw. The RapidVit™ & RapidWarm™ Oocyte are MOPS (3-N-morpholino-propanesulfonic acid) buffered calcium-free media containing cryoprotectants and human serum albumin. After vitrification, the Rapid-i™ straw is ultrasonically sealed providing safe storage.

MII oocytes were washed with H-SOF and vitrified using the Manhattan protocol, which was designed to reduce the exposure time of oocytes to cryoprotectants, as follows:

- vitrification solution RapidVitri 2™ for 1 min,
- vitrification solution RapidVitri 3™ for 10 sec at 37°C
- loading onto a Rapid-i™ straw (4 oocytes).

For warming, the Rapid-i™ was taken out of liquid nitrogen and instantly placed in warming solution RapidWarm 1™ at 37 °C. After 1 min, oocytes were placed in RapidWarm 4™ for 10 sec, then transferred in H-SOF for two hours at 38.5 °C, in a humidified atmosphere of 5% CO2 in air, before survival assessment and staining.

In Exp. 2, in vitro matured bovine oocytes were vitrified using the Manhattan protocol. Based on the findings of Experiment 1, the Cryotop, a semi-open vitrification system widely used in assisted reproduction laboratories, was selected as cryodevice.

6.3.4. Viability assessment

Two hours after warming, oocytes were stained to confirm the maturation state and the membrane integrity. Oocytes were repeatedly washed with DPBS+PVA (Dulbecco's phosphate buffered saline + 0.1% w/v polyvinyl alcohol) before and after being transferred to a drop of 100 µl containing Hoechst 33342 (bisbenzimide, 10 µg/ml in DPBS+PVA) and propidium iodide (10 µg/ml in DPBS+PVA) for 15 min in the dark. The stained oocytes were then loaded onto a slide for visualization under a Nikon Eclipse E400 epifluorescence microscope (Nikon Europe BV, The Netherlands) using UV-2A (330–380 nm) and TRITC (540/25 nm) excitation filters. Only oocytes with a visible metaphase plate and an extruded polar body (MII oocytes) were classified as mature. Oocytes displaying a red cytoplasm were considered non-viable, whereas those with a red cytoplasm and no detectable nuclear material were classified as degenerate.

6.3.5. Detection of reactive oxygen species (ROS) and glutathione (GSH) levels

Intracellular ROS and GSH levels were determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen™, Thermo Fisher Scientific, Italy) and 4-chloromethyl-6.8-difluoro- 7-hydroxycoumarin (CellTracker Blue, CMF2HC, Invitrogen™, Thermo Fisher Scientific, Italy) respectively. Oocytes were incubated 20 min in the dark at room temperature in PBS+PVA and 10 µM H2DCFDA or 10 µM CellTracker Blue. After staining, oocytes were washed once in PBS+PVA and examined under an epifluorescence microscope. Images of fluorescent oocytes were analysed with an open-source software (ImageJ). For each image, the oocyte fluorescence intensity was measured and normalized with the background.

6.3.6. Mitochondrial membrane potential detection

Mitochondrial membrane potential of oocytes was assessed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen, Italy). Denuded oocytes were incubated at 38.5°C for 30 min in the dark in 30 µl microdrops of HSOF supplemented with 0,2 µg of JC-1. Then, the oocytes were immediately examined under an epifluorescence microscope. Only one fluorescent image (red channel) was acquired for each oocyte, to detect the mitochondria with high membrane potential (orange stained). Images were analysed as previously described for ROS and GSH.

6.4. Statistical analysis

Data are expressed as mean ± standard deviation and were analyzed using Statistics for data analysis v. 30 (SPS S.r.l., Italy). Data distribution was determined using Shapiro-Wilk test then analyzed by GLM (generalized linear model) for gamma distribution and log link function. Significance was assessed for $p< 0.05$.

6.5. Results

6.5.1. Experiment 1

In the present study a total of 590 bovine oocytes have been recovered from slaughtered ovaries and matured in vitro. As showed in Table 12, after in vitro maturation, 188 oocytes in MII phase have been vitrified using a closed system (C) and 188 have been vitrified using a semi open

system (SO). After warning, 174 oocytes were recovered in group C and 166 in group SO; no statistically significant differences were registered between groups in the number of recovered oocytes (p>0.05).

Table 12. Vitrified and recovered oocytes in semi-open and close systems.

Groups	Vitrified Oocytes (n)	Recovered Oocytes (%)
SO	188	90.5 ± 11.8
C	188	91.9 ± 9.4

After warming, recovered oocytes were stained to determine survival rate after vitrification and warming, using a semi-open and closed system. No statistically significant differences were registered between two research groups (p>0.05; Table 13).

Table 13. Survival rate after vitrification in semi-open and close systems

Groups	Oocytes (n)	Viable Oocytes (%)	Non-viable oocytes (%)	Degenerated oocytes (%)
SO	93	75.7 ± 10.3	19.8 ± 5.6	4.6 ± 6.3
C	84	76.9 ± 19.3	18.4 ± 17.5	4.7 ± 6.5

6.5.2. Experiment 2

The results of fluorescence staining for ROS, GSH, and mitochondrial activity (JC-1) are summarized in Figure 11. No significant differences ($P > 0.05$) were detected between groups for ROS (11A.) and GSH levels (11B.) indicating that vitrification with the Manhattan protocol did not significantly affect intracellular oxidative status or antioxidant content. Conversely, mitochondrial activity, assessed by JC-1 staining (11C.), was significantly higher in vitrified oocytes compared to controls ($P < 0.001$), indicating a significantly increased mitochondrial membrane potential following vitrification and warming.

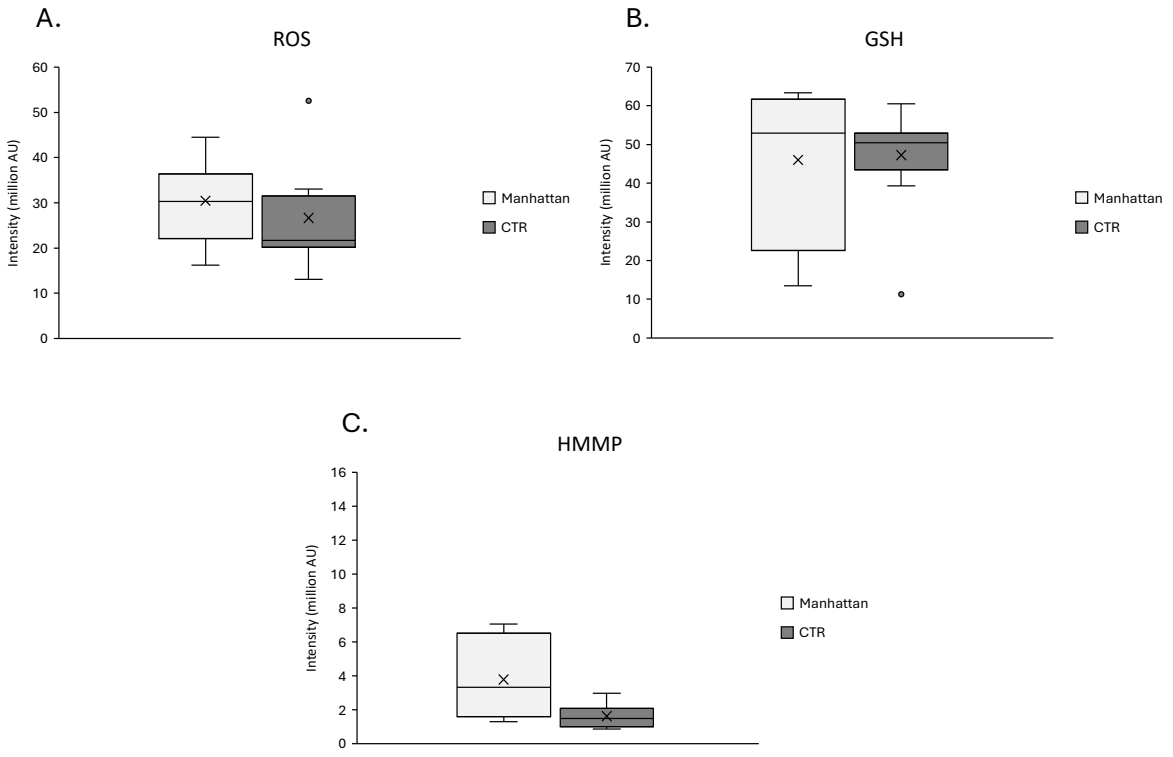

Figure 11 Quantification of oxidative stress, intracellular glutathione levels, and mitochondrial activity in bovine MII oocytes after vitrification, through fluorescent staining 2 h after warming. (A) Reactive oxygen species (ROS) levels assessed by H_2DCFDA; (B) Intracellular glutathione (GSH) levels assessed by CellTracker™ Blue; (C) High mitochondrial membrane potential (HMMP) measured by JC-1 staining. Two experimental groups are shown: control oocytes (CTR) and oocytes vitrified using the Manhattan protocol. No significant differences were observed in ROS and GSH levels between groups, whereas vitrified oocytes exhibited a significantly higher HMMP compared with controls ($p< 0.05$).

6.6. Discussion

The aim of this study was to evaluate a shortened version of a commercial vitrification protocol originally designed for human oocytes, using bovine oocytes as an experimental model. We employed the Rapid-i™ Vitrification System (Vitrolife Sweden AB, Västra Frölunda, Sweden), a human clinical-grade vitrification kit that uses DMSO-free solutions. The use of DMSO as a cryoprotectant has been questioned, as it may induce spindle and microfilament disorganization, asymmetric cytokinesis, untimely intracellular calcium oscillations, and premature cortical granule exocytosis [32]. Moreover, exposure to cryoprotective agents can affect cell function and survival, with concentration, temperature, and exposure time being critical factors. To minimize the potential cytotoxicity associated with prolonged CPA exposure, we tested a shortened version of the commercial protocol, referred to as the

Manhattan protocol, in which the duration of oocyte exposure to cryoprotectants was reduced. Several recent studies have already explored the possibility of shortening vitrification protocols, both in freezing [33] and warming [33–35] phase.

Additionally, the type of support used during vitrification is a crucial factor, particularly regarding whether the cryogen contacts the oocytes (open vitrification) or not (closed vitrification). The choice between these two approaches remains a topic of ongoing research, several studies have directly compared open vs closed vitrification systems in human oocytes and embryos [27,28]. While overall survival and clinical outcomes show no consistent superiority of one over the other, some evidence hints that closed systems may exert a less detrimental impact on developmental competence [36]. In this context, evaluating whether the type of system influences oocyte survival and post-warming recovery represents a relevant objective of the present study. For this reason, in the present study at the end of in vitro maturation period, bovine oocytes at MII stage have been divided in two research groups: vitrified using a closed system (C) and vitrified using a semi open system (SO).

After warming, no differences were registered between groups in the number of recovered oocytes. In the Rapid-i Kit oocytes are held by surface tension in a standardized volume; it is easy to see when the right volume of medium has been applied. This simplifies loading and reduces variability between operators. Results of post-warming recovered oocytes demonstrate the effectiveness of Rapid-i Kit not only in the closed system, for which it was designed, but also in the semi open system, by preventing oocytes from being lost in direct contact with liquid nitrogen.

In term of cell viability after vitrification, the results observed in the present study do not show differences between the two research groups, closed system (C) and semi open system (SO). Therefore, the timings used in the Manhattan protocol seem to allow high-quality cryopreservation of the oocytes, reducing exposure to CPAs, which might cause ultrastructural modification of the smooth endoplasmic reticulum complex and mitochondria, which are implicated in the physiological mechanism of calcium signalling triggered by the sperm fusion at the time of fertilization [37]. In addition to reduced exposure to cryoprotectants, the absence of DMSO seems to be an important factor for successful vitrification; indeed, Best et al. [38] has shown DMSO highly toxicity, especially at higher temperatures, leading to increased formaldehyde production. These effects make DMSO a cryoprotectant that must be used with caution. Pinasco et al. [39] conducted a comparative study on human oocytes using the Rapid-i™ Vitrification System as a closed device and the Kitazato vitrification media kit with Cryotop as a semi-open system. No significant differences were observed in oocyte viability rates

between the closed (C) and open (O) vitrification groups. Similarly, our results showed no significant differences in post-warming survival rates between the two vitrification systems tested. This suggests that both open and closed devices can ensure comparable efficiency in oocyte vitrification, allowing flexibility in system selection according to laboratory preferences or biosafety requirements.

Several reports have demonstrated that vitrification may disturb the reduction-oxidation (redox) status, reduce glutathione content and increase reactive oxygen species levels, resulting in damage to biomolecules such as DNA, proteins and membrane lipids and leading to mitochondrial dysfunction, which may induce apoptotic responses and reduce cleavage and embryo viability Castillo-Martin et al. [40]. For this reason, mitochondrial function, glutathione level and oxidative stress of vitrified bovine oocytes were determined using JC-1, GSH, and ROS staining, respectively. In the present study, vitrified bovine oocytes exhibited a higher mitochondrial membrane potential. Several studies have investigated mitochondrial activity immediately after warming, often reporting a transient decrease in mitochondrial membrane potential in 2PN embryos [41] or in MII oocytes, followed by recovery to control levels after a few hours of incubation. Similar results were described by Succu et al.[42] in ovine oocytes, where mitochondrial activity returned to baseline after post-warming incubation.

It is well established that increased mitochondrial activity can sometimes precede apoptotic events [43]. However, in bovine oocytes, Gutnisky et al [44]. observed a rise in mitochondrial activity three hours after warming, suggesting that this may reflect an increased energy demand associated with organelle reorganization during the post-warming recovery phase. Most recently, Gutierrez et al. [45] reported that oocytes cultured for 4 h post-warming exhibited mitochondrial activity levels comparable to those of the control group, suggesting that full mitochondrial recovery may take several hours after vitrification and warming. In our study, oocytes were incubated for 2 h in H-SOF medium after warming; thus, the higher mitochondrial activity detected at this time point could represent an ongoing recovery phase rather than a return to baseline levels.

Glutathione is a powerful antioxidant present in all cells, playing a fundamental role in protecting against oxidative damage, maintaining redox balance, and detoxifying cells from free radicals and toxic substances. In cell, glutathione occurs in reduced (GSH) and oxidized (GSSG) forms, maintains the redox balance in the cell, and serves to regenerate the reduced form of other antioxidants [46]. Alterations in GSH content during oocyte vitrification may affect embryo cleavage by preventing the formation of the male pronucleus, Sutovsky et al. [47] and García-Martínez et al [48] demonstrated that the addition of GSH to bovine oocytes

during IVM prior to vitrification may be beneficial for embryo development presumably as a source of additional antioxidant protection. For this reason, we investigated the concentration of glutathione in vitrified oocytes. From our data, it was observed that intracellular glutathione levels were comparable between the vitrified and control oocytes. However, a higher variability was noted in the treated group. It is important to consider that, in the present study, ovaries were collected from slaughtered animals. Although this approach allowed easy access to a large number of oocytes, it provided no information about the donors' physiological status or age. Therefore, it cannot be excluded that some oocytes had already experienced mitochondrial alterations prior to vitrification, potentially influencing post-warming mitochondrial responses. Regarding the oxidative stress, from our data, we could observe that intracellular ROS levels were comparable between the vitrified and control oocytes. Gutnisky et al. [44] reported higher ROS levels in vitrified bovine oocytes compared with the control group, but these levels decreased three hours after warming, suggesting a transient oxidative stress response. It is therefore possible that the vitrification protocol used in the present study had a milder impact, resulting in ROS levels that were already low and comparable to the control group after only two hours of post-warming incubation. Similarly, Cocoles et al. [49] also reported no significant differences in ROS levels between vitrified and control oocytes, although a non-significant trend was observed. Notably, the vitrification protocol used in that study contained DMSO, whereas the present protocol was DMSO-free. Although several studies have reported an increase in ROS following vitrification, it remains unclear whether this effect is attributable to specific cryoprotectants such as DMSO or to the vitrification process itself, which may act as the main trigger of oxidative stress.

6.7. Conclusion

In conclusion, we demonstrated that the Manhattan protocol and the human commercial Rapid-i™ Vitrification System (Vitrolife Sweden AB, Västra Frölunda, Sweden) ensure good post-warming viability of bovine oocytes. No differences in oocyte viability were observed between the semi-open (SO) and closed (C) systems, indicating that both represent efficient vitrification methods. The Manhattan protocol applied to an open system (Cryotop) does not increase intracellular ROS or glutathione levels, whereas a higher mitochondrial membrane potential (HMMP) was observed. Further studies are required to determine whether this increase in mitochondrial activity represents an early apoptotic event or, conversely, a physiological reorganization of organelles linked to post-warming recovery and cellular

health. Complementary experiments involving fertilization and embryo development would provide valuable insight into the functional competence of vitrified oocytes.

This study was commissioned by the Vitrolife Group (Vitrolife Sweden AB, Västra Frölunda, Sweden) to assess the effects of a modified human commercial vitrification protocol on bovine oocytes, used here as an animal model for human oocyte vitrification.

6.8. References

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