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Comparison of vitrification protocols in immature equine oocytes

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Ce mémoire intitulé

Comparison of vitrification protocols in immature equine oocytes

Présenté par

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A été évalué par un jury composé des personnes suivantes

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Résumé

La cryoconservation d'ovocytes est une méthode qui faciliterait la conservation du potentiel génétique chez la femelle et permettrait plus de flexibilité dans l'application des techniques de reproduction assistée chez les animaux domestiques et les espèces en voie de disparition. Chez le cheval, le taux de réussite de cette technique est faible comparée à celui obtenu chez d'autres espèces animales. Par conséquent, plus d'études seront nécessaires pour élucider les mécanismes spécifiques responsables du faible taux de succès après la cryopréservation. Le but de cette étude était d'évaluer l'effet de la vitrification d'ovocytes équins immatures sur leur taux de maturation, de clivage et le développement de blastocystes en utilisant un protocole de vitrification en trois étapes avec de l'ethylène glycol (EG) et du diméthylsulfoxyde (DMSO), ainsi que comparer l'effet des milieux hors congélation. Le protocole de vitrification utilisé dans la présente étude a été conçu en fonction des résultats obtenus au cours d'études préliminaires. Des ovocytes provenant de follicules immatures de juments ont été conservés pendant une nuit (14-18 heures) à température ambiante (~22ºC) dans un milieu de maintien. Le lendemain, les ovocytes ont été dénudés et placés dans une solution de base (BS) composée de 20% de sérum de veau fœtal (FBS) + M199/Hanks' salts. Les ovocytes ont ensuite été répartis au hasard dans différents groupes : contrôle, vitrification et exposés aux agents cryoprotecteurs (CPA)-. Les ovocytes du groupe contrôle ont été immédiatement mis en maturation *in vitro* (IVM). Trois ovocytes ont été exposés à un protocole de vitrification en trois étapes décomposées en (1) solution de pré-vitrification (PVS) 1 (5% EG / 5 DMSO) 40s. (2) PVS 2 (10% EG / 10% DMSO) 40s et enfin, (3) solution de vitrification (VIT) (17,5% EG / 17,5% DMSO / 3 M saccharose) 10s. Le groupe vitrification est plongé dans l'azote liquide alors que les groupes CPA-exposés ont été exposés aux cryoprotecteurs mais n'ont pas été congelés. Les ovocytes ont ensuite été transférés sur un maillage en acier inoxydable stérile puis réchauffés à 42 ° C dans un BS pendant 5 min. Les ovocytes ont ensuite été soumis à l'IVM, fécondés par injection intracytoplasmique d'un spermatozoïde puis mis en culture dans le but de produire des embryons. Les différences en termes de maturation, de clivage et de taux de blastocystes entre les groupes ont été analysées par le test exact de Fisher. Le taux de maturation des deux groupes vitrification et CPA-exposés ne différait pas significativement avec le groupe contrôle. Aucun blastocyste n'a cependant été obtenu des groupes vitrification et CPA-exposés. Ces résultats ont montré que les ovocytes

équins immatures peuvent maintenir une viabilité et une compétence méiotique après vitrification similaires à celles du groupe contrôle; de plus, l'exposition aux cryoprotecteurs n'a pas abouti à la formation de blastocystes en comparaison avec le groupe contrôle. Une étude plus approfondie sur la physiologie des ovocytes équins est nécessaire afin de pouvoir optimiser la production d'embryons.

Mots-clés : cryoconservation, vitrification, équidés, ovocytes immatures, cryoprotecteurs.

Abstract

Oocyte cryopreservation would facilitate the conservation of female genetic material and allow more flexibility in the application of assisted reproductive techniques in domestic animals and endangered species. The overall success rate of this technique in the horse is low compared with other species. Therefore, further research is required to elucidate the species-specific mechanisms responsible for poor survivability following vitrification. This study aimed to evaluate the effect on maturation rate, cleavage and blastocyst development of vitrified immature equine oocytes, using a three-step vitrification protocol with ethylene glycol (EG) and dimethyl sulfoxide (DMSO); and comparing the effect of media without freezing. The vitrification protocol was designed based on the results of preliminary experiments. Oocytes were recovered from immature follicles of live mares. Oocytes were held overnight at room temperature (14-24 hrs) in a holding medium. Oocytes were then denuded and placed in a base solution (BS) composed of 20% fetal bovine serum (FBS) + M199/Hanks' salts. Oocytes were randomly allotted to control, vitrification, and cryoprotectant agents (CPAs)-exposed groups. Control oocytes were cultured directly for *in-vitro* maturation (IVM). Three oocytes were exposed to a three-step vitrification protocol composed of a pre-vitrification solution (PVS) 1 (5% EG/ 5% DMSO); PVS 2 (10% EG/ 10% DMSO) during 40s each; and finally vitrification solution (VS) (17.5% EG/ 17.5% DMSO/ 3 M sucrose), during 10s. All media were diluted in M199/Hanks' salts + 20% FBS. Oocytes were then transferred to a 75-µm sterile stainless steel mesh. The oocytes were warmed at 42 °C in the BS for 5 minutes. Oocytes from the vitrified group were plunged into liquid nitrogen, while oocytes from CPA-exposed groups were only exposed to cryoprotectants. Oocytes were then subjected to IVM, fertilization and embryo culture. Fisher's Exact Test analyzed differences in maturation, cleavage and blastocyst rates between groups. The maturation rate of vitrified and CPA-exposed groups did not differ significantly from control oocytes. However, no blastocysts were obtained from CPA-exposed and vitrified groups. Vitrification and control groups showed that immature equine oocytes could maintain viability and meiotic competence**;** moreover, cryoprotectant exposure did not show any blastocyst formation as compared to control. Further investigation is necessary to understand the overall physiology of equine oocytes in order to optimize the developmental capacity of embryos.

Keywords: cryopreservation, vitrification, equine, immature oocytes, cryoprotectants.

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AI: artificial insemination
ART: assisted reproductive technology
ATP: adenosine triphosphate
BS: base solution
BSA: bovine serum albumin
C: carbon
Ca++: Intracellular calcium
CAT: catalase
COCs: cumulus oocyte complexes
CPAs: cryoprotectant agents
DMSO: dimethyl sulfoxide
DNA: Deoxyribonucleic acid
EG: ethylene glycol
FBS: fetal bovine serum
FSH: follicle-stimulating hormone
GSH: glutathione
GV: germinal vesicle
H: hydrogen
ICSI: intracytoplasmic sperm injection
IV: intravenous
IVF: *in vitro* fertilization
IVM: *in vitro* maturation
LH: luteinizing hormone
MAPK: mitogen activated protein
MI: metaphase I
MII: metaphase II
Mt: mitochondria
O: oxygen
OPS: open pulled straws
PG: propylene glycol
PV: pre-vitrification Solution
ROS: reactive oxygen species

SOD: superoxide dismutase

TVA: transvaginal ultrasound-guided follicular aspiration

TZPs: transzonal projections

VS: vitrification solution

Con todo cariño dedico este logro a mis padres, hermanos y Hemant

porque siempre han creído en mí y me han impulsado

para lograr mis metas.

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1. Introduction

The equine industry has experienced a slow but steady development of several assisted reproductive techniques (ART) to support the management of biological aspects of equine reproduction (1) ranging from simple methods such as artificial insemination (AI) (2) to more advanced techniques as nuclear transfer (3). The transfer of an embryo to a recipient mare to produce more than one foal per year or obtain foals from mares with fertility problems can be pursued by either oocyte recovery, intracytoplasmic sperm injection (ICSI) and embryo culture or direct embryo recovery (4). Although there is still a long process to follow for the improvement of ART in horses, the equine industry's increasing demand justifies the labor and expenses needed to obtain valuable horses (5, 6).

Equine oocyte cryopreservation is an important step in ART for preserving genetic material from domestic and endangered breeds by maximizing the availability of gametes and making the application of ART independent of time and geographic location (7, 8). For instance, in a clinical application, oocytes from valuable mares could be recovered and cryopreserved in areas where the availability of ART is limited and then shipped to an assisted reproduction laboratory for embryo production, making it possible to decide in the future the male parent. The research field can provide a reliable source of immature equine oocytes in countries without access to equine slaughterhouses, such as the United States (9). Finally, oocyte cryopreservation allows for preserving genetic material from valuable horses and endangered breeds (8).

Over the last few decades, cryopreservation has progressed rapidly in many fields. At present, this technique is routinely used for the preservation of oocytes (10, 11), sperm (12), and embryos (13) in both animals (14) and humans for ART (15). Although equine-assisted reproductive technologies, such as embryo (13) and semen cryopreservation (16), have evolved rapidly and relatively new techniques have further advanced the process, oocyte cryopreservation remains in its infancy (9, 17-20). Ever since the method of conventional “slow freezing” preservation proved to be less effective in the embryonic development of oocytes from humans (21) and animals (22), vitrification has been the most commonly used cryopreservation technique. Vitrification involves high concentrations of cryoprotectant agents (CPAs) and a fast cooling rate (23). Many conditions in the vitrification process can profoundly affect the survival rate of oocytes.

Among these, the type and concentration of the cryoprotectant (24) and the exposure to the cryoprotectant solution (25) are important factors for improving survival rates for further embryo development.

In humans, various approaches have been utilized to cryopreserve oocytes, such as slow freezing (21) and, more recently, the technique of vitrification (15, 26). Chen (27) reported for the first time the success of freezing human oocytes using a slow-freezing technique. In that study, only one of 12 human oocytes survived. Even though oocyte vitrification's clinical application started only a decade ago, thousands of children have been born with this technique (28). Moreover, in domestic animals, the most extensive research has been conducted in cattle. Calves have been born from vitrified oocytes following *in-vitro* fertilization (IVF) and embryo culture (14). On the other hand, a limited number of studies have been done on the vitrification of small ruminant oocytes, especially in sheep, for which poor developmental rates were obtained following oocyte vitrification (29). The application of vitrification techniques to mature porcine oocytes has resulted in limited embryo development rates after IVF (30) or ICSI (31). In recent years, a protocol for the vitrification of immature porcine oocytes has been developed and resulted in the production of 18 piglets (32). After certain modifications, the vitrification protocol has become more efficient (33). Also, in horses, immature oocytes undergo significant damage during controlled freezing. Only five studies have reported embryo development after the vitrification of immature oocytes (9, 17-20), and the efficiency of blastocyst production was low. From these reports, the highest rate of blastocyst development from equine oocytes is 15% (17), and only two reports have had offspring from vitrified oocytes (18, 19).

This thesis presents the strategies and protocols used for oocyte vitrification in different species to explore their outcomes and to optimize the vitrification protocol of equine oocytes by improving the embryo development rate of vitrified equine oocytes. We also evaluated a designed three-step vitrification protocol through the resumption of metaphase II (MII) after IVM and subsequent blastocyst development

2. Literature review

2.1. Ovarian anatomy of the mare

2.1.1. External structure

The equine ovary is kidney-shaped and has two surfaces (lateral and medial), two borders (attached and free), and two poles or extremities (cranial or tubal and caudal or uterine). On the free border, there is a very prominent depression (ovulation fossa); this is the only area from which ovulation occurs, interfering with multiple ovulations and limiting the number of embryos to just one or sometimes two and rarely three (34). The ovary's poles are rounded, and its cranial or tubal pole is attached to a portion of the fimbriae of the oviduct. In contrast, the caudal or uterine pole is attached to a point just caudal to the end of the uterine horn by the proper ligament of the ovary (35). Equine ovaries are larger compared to other domestic species (36). The average size is 50 x 30 x 30 mm, but considerable variations occur with changes in follicular activity and stage of the reproductive cycle, including preantral follicles and corpora lutea (37).

2.1.2. Internal structure

The relationship between cortical and noncortical areas of the ovary is peculiar in the mare (35). By definition, the cortex of an organ is the outer portion or external layer, while the medulla is the softer, vascularized area in the center (36). In the mare, the medulla or vascular zone is superficial, and the cortical region with follicles is in the interior of the organ. The cortex reaches the surface only at the ovulation fossa on the free border and the *corpus luteum* does not project from the greater surface of the ovary as in other species. In mares, a projection (ovulation papilla) may be seen in the ovulation fossa, especially in the newly-forming *corpus luteum* (35).

2.1.3. Function

The ovaries have gametogenic (development of gametes) and endocrine (production of hormones) functions. The two major endocrine structures on the equine ovaries are follicles and the *corpus luteum*. Follicles are fluid-filled structures that play a dual role

(production of oocytes, estrogens, and inhibin), whereas the function of the *corpus luteum* is endocrine only (production of progesterone) (36). Ovarian activity in the mare is very dynamic, involving changes from the development of a large pre-ovulatory follicle to the formation of a *corpus hemorragicum* then a *corpus luteum* during the estrous cycle (38); to no follicular activity during anestrus in which around 85% of mares will cease ovarian cyclicity (39). During this period of hormonal transition between the estrous cycle and anestrus periods, ovaries may have multiple and large follicles. Follicles are classified into primary, secondary, and tertiary structures and during the estrous cycle, the dominant follicle will approximately grow at a rate of 3 mm per day (37). The developing dominant follicle becomes responsive to follicle-stimulating hormone (FSH), reaching a size at the ovulation of around 40 mm in diameter (40). As ovulation approaches, the preovulatory follicle becomes soft and sensitive (41). Luteinizing hormone (LH) is responsible for the final maturation of the follicle and the induction of ovulation. The increase of LH is observed 24-48 h before ovulation, resulting in the peak of LH after ovulation, as shown in figure 1 (35). About 50% of ovulations result in the formation of an immediate *corpus luteum* and 50% of ovulations result in the formation of a *corpus hemorrhagicum* (41). The *corpus luteum* is located centrally in the equine ovary and, in non-pregnant mare, will be lysed at the end of diestrus (14-15 days post ovulation), in association with a growing population of follicles (34).

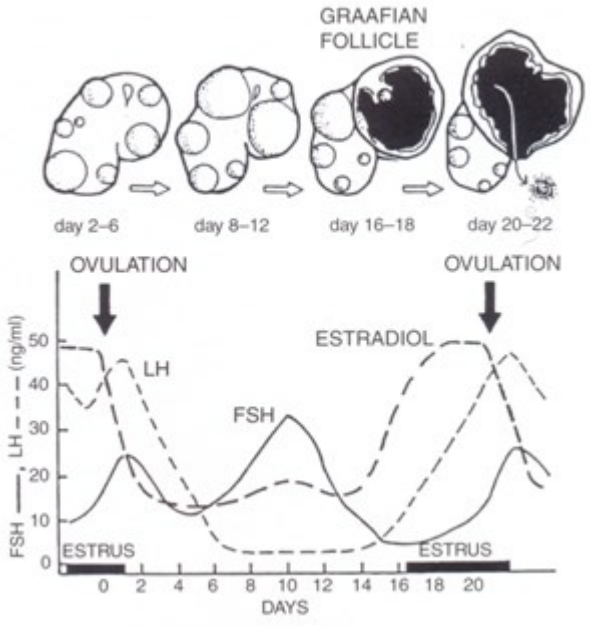

Figure 1. Hormone Patterns during estrus in mares (42).

2.2. Assisted reproductive techniques in the horse

In Thoroughbred artificial reproductive techniques are forbidden when the progeny is to be registered in the Weatherbys General Stud Book (the original register of Thoroughbred). On the other hand, ART in non-thoroughbred horses has been increasingly used in the equine industry; currently, most mares are inseminated successfully with fresh, cooled, or frozen semen, while a few are covered naturally (43). Frozen semen is shipped worldwide, expanding the choice of stallions available to mare owners (7). Furthermore, other technologies have developed in recent years; these include embryo transfer, with a pregnancy rate after transfer of about 75% (44), ICSI of *in-vitro* matured oocytes recovered from live or post-mortem mares with blastocyst rates up to 54% (45), nuclear transfer (3), and the vitrification of small blastocysts, associated with good pregnancy and foaling rates (76 and 71%, respectively) (46).

The use of the different ART for *in-vitro* embryo production by ICSI to obtain more than one foal per year has gained acceptance in commercial equine breeding programs (47). ART can be used to overcome reproductive problems in mares; for example, when the owner does not want the mare to be pregnant during physical activity like sports, the mare

is too valuable to risk the potential dangers during pregnancy or foaling, or the mare has lameness, pelvic damage, or maternal behavior problems (7). Moreover, *in-vitro* embryo production allows the reproduction and storage of valuable genetic material outside of the breeding season or to schedule a breeding program, rendering the technologies commercially attractive (5).

The collection of oocytes, followed by *in-vitro* oocyte maturation, ICSI, and *in-vitro* embryo culture, is an effective method for producing foals in mares that cannot become pregnant or provide an embryo under standard reproductive management (47). There are two main approaches to collect oocytes: one from live mares and the other from postmortem mares (9). The first approach is the aspiration of all the immature follicles from the ovaries from donor mares without ovarian stimulation with a recovery rate of 50% (48). The main disadvantage of immature follicle aspiration is that it is complicated, and the practitioner needs extensive training. The second approach is the aspiration of oocytes from excised ovaries from post-mortem mares (49). Some reports have shown a high recovery rate of oocytes (mean of 18 oocytes/mare; range 0–35) by scraping the wall of follicles with a bone curette(4); the disadvantage of this method is that it is a time-consuming process, but it is feasible to perform for valuable mares (6). Maturation is one of the final steps in the development of an oocyte and it is defined as the resumption of meiosis that occurs just before ovulation and subsequent fertilization (50). It has been reported that *in-vitro* maturation protocols in horses can achieve rates of 42% of mature oocytes (49). The optimum duration of maturation for blastocyst production varies according to the period that the oocyte spends within the ovary before it is recovered. Oocytes collected from the ovary within 1 hour after death require a more extended culture period to reach an optimal blastocyst formation rate (48).

In the few published IVF reports in horses, there has been a low success in fertilization rates ranging from 2% to 33% (51, 52). The failure is related to the inability of the spermatozoa to penetrate the *zona pellucida in-vitro.* On the other hand, methods for ICSI have been well developed in horses, as described above. Although ICSI has many drawbacks, including the need for expensive equipment, expertise in micromanipulation, oocyte and embryo handling and culture expertise, it also has significant advantages because it may be used with semen having low sperm numbers or quality (53). Currently, equine ICSI using frozen semen has become a standard procedure for oocyte fertilization.

In stallions, freezing semen is a commonly used technique to ensure the preservation of valuable male genetic material indefinitely. Semen can be collected from stallions as ejaculates (54), from the epididymis after castration (55), or post-mortem (16). In mares, however, there is currently no efficient method for gamete preservation (7). Oocyte cryopreservation is still in its infancy, and only two publications are available reporting the birth of a foal from previously-cryopreserved oocytes (18, 19). Oocyte cryopreservation is a promising technique that would allow more flexibility in the application of ART in clinical practice, in the research field and for the conservation of female genetic material.

2.3. Principles of cryopreservation

Nowadays, animal breeding has reflected an increased interest in cryopreserving oocytes and embryos of valuable animals, such as pigs, cows, and sheep, to increase meat and milk production (56). The United Nations suggests that the world population is predicted to grow to 9.15 billion by 2050, increasing as well the consumption of meat up to 20% per capita calorie intake (57). Additionally, the transportation of valuable animals is both expensive and logistically challenging to perform. Hence, the prospect of transporting frozen gametes instead of living animals is attractive (58).

Besides the facilitation and cost-effectiveness of transporting gametes instead of live animals, cryopreservation would also minimize the risk of most disease transmission within and between species (59). Additionally, ART, such as pre-implantation genetic diagnosis and sex determination techniques, could be adopted effectively (60). This would be economically favorable to agricultural breeding companies that own genetically valuable individuals. Cryopreservation also can save certain endangered species and native animal breeds (61, 62).

Cryopreservation involves cells or whole tissue preservation exposed to a sub-zero temperature in liquid nitrogen (LN_2) (-196°C). At such temperature, biological activity is effectively stopped, and the functional status of the cells may be preserved indefinitely (63). However, several physical stresses damage the cells at these low temperatures. For this reason, freezing protocols use a combination of dehydration, freezing point depression, supercooling, and cryoprotectants in an attempt to prevent cell damage (64, 65); such as spermatozoa (66), embryos (67), and more recently, oocytes (27). While the

cryopreservation of spermatozoa (12, 68) and embryos (46, 69) has undergone significant advances these last years, oocyte cryopreservation has only recently seen consistent success in some species (22, 33).

The cryopreservation protocols for oocytes can be divided into two categories: (1) slow freezing/rapid thawing and, (2) rapid cooling/warming or vitrification (Figure 2). Initial attempts to freeze oocytes employed the same slow-freezing methods considered the gold standard for embryo cryopreservation (27, 63). However, the slow-freezing oocyte protocols have resulted in relatively low survival and pregnancy rates (70, 71). With the recent advances in the vitrification technique, the efficacy of oocyte cryopreservation has significantly improved. Several groups worldwide have reported high survival and pregnancy rates using vitrified oocytes from different species (14, 33, 72).

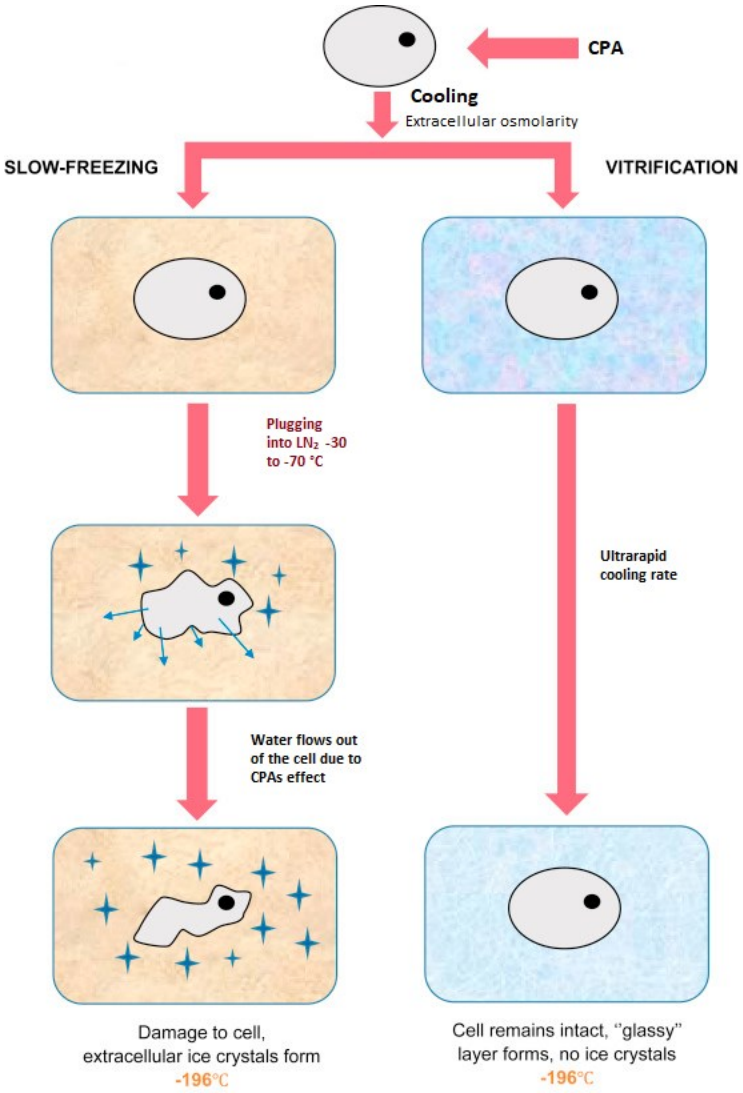

Figure 2. Comparison of ice crystal formation between slow-freezing and vitrification (56).

2.3.1. Slow freezing

Cryopreservation by slow freezing is a process in which extracellular water crystallizes (73). This method allows cells to be cooled to relatively low temperatures while minimizing intracellular ice crystal formation and simultaneously attempting to reduce the detrimental influences of increased solute concentrations and osmotic stress (74). Freezing induces water transformation into ice, leading to the separation of water from dissolved substances (64). The presence of intracellular ice crystals can be lethal to the oocytes during slow freezing (70). The method has given acceptable results for oocytes of species that are not

sensitive to freezing, such as humans (75) and mice (76). However, the oocytes of cows (77), pigs (78), and horses (19) are more sensitive to slow freezing techniques and have resulted in poor results.

In slow freezing techniques, oocytes are gradually exposed to relatively low concentrations of permeating CPAs such as glycerol (79), DMSO (24), EG (70), and propylene glycol (PG) (80) and non-permeating CPAs such as sucrose, glucose, trehalose, or fructose (81). During freezing, CPAs' effect increases the extracellular space, leading to a hypertonic medium due to the increment of solutes. Therefore, water leaves the cell, resulting in cell shrinkage (82). Cells are then loaded in small volumes into 2-mL straws, with cooling rates of 1°C/min from -5 to -9°C and they are kept in the straws for several minutes to equilibrate. After equilibration, ice crystallization is induced by a process known as “seeding,” which results in heterogeneous ice nucleation (63). After seeding, the cooling rate is reduced to 0.3–0.5°C/min until a lower temperature is reached (usually between -30 and -150°C). These procedures typically take several hours. Once the desired temperature is reached, the straws are plunged into LN_2 for storage (27).

2.3.2. Vitrification

This process's physical definition is the glass-like solidification of solutions at low temperatures without the formation of intracellular ice crystals (56). Vitrification protocol in embryos was first described in 1985 (83); this protocol was achieved with a solution containing DMSO, PG, and EG; but the solution was toxic to embryos and oocytes (83, 84). With the rapidly growing success of oocyte vitrification in human ART, IVF clinics around the world have changed from the traditional slow-freezing technique to the vitrification method as routine (15). Vitrification has been described as inexpensive because it does not require special equipment compared with slow freezing (26), making it an effective alternative technique to use in various fields. Once vitrified, cells can be stored for extended periods with no noticeable deterioration (85). In cryopreservation by vitrification, both intra- and extracellular compartments vitrify after cellular dehydration (73). While this approach improves the cells' viability, a high concentration of cryoprotectant is required to prevent ice crystal formation (13). Therefore, cryopreservation strategies are based on two main principles that determine vitrification's success to avoid cell injury and death: viscosity and cooling-warming rates (86).

The intracellular medium's viscosity is defined by the concentration and behavior of various CPAs and other additives during vitrification (87), and it plays an essential role during dehydration of the cell. A way to prevent ice crystal formation is to remove as much of the intracellular water as possible. However, removing the water results in cell injury and death due to osmotic shock (88). Therefore, the viscosity of high concentrations of CPAs will make water solidify without the formation of ice crystals (80). The high viscosity encountered during freezing in vitrification solutions, due to the high concentration of CPAs, and the glass transition temperature, reduce the chance of ice nucleation and crystallization (89). A successful vitrification protocol considers proper dehydration of the oocytes to reduce intracellular ice formation (90).

High cooling rates is the second principle to decrease detrimental physiological effects in vitrification protocols (91). Such rates could be achieved using minimum volume methods, facilitating the vitrification with less concentrated cryoprotectants (92). It has been reported that a small droplet of vitrification solution (around 1µL) can prevent ice crystal formation and fracture injury (27). Different techniques have been used to minimize the volume of vitrification solutions and submerge samples swiftly into LN_2, achieving cooling rates of approximately 2500°C/min (93); moreover, there are reports of suitable carrier systems, such as the open pulled straw, which reached cooling rates of 20,000°C/min (22). A 0.25mL conventional straw was initially used for the vitrification of oocytes. The cooling rate was around 2,500ºC/minute, and the warming rate was 1,300ºC/minute (93). Vajta *et al.* (22) developed open pulled straws (OPS) to hold bovine oocytes with a small amount of vitrification solution (1µL), reaching cooling and warming rates of 16,700°C/minute and 13,900°C/minute, respectively. This technique has been widely used, achieving successful rates of several species (94). Other cryo-devices have been reported, including the cryoloop called the Cryotop™ (95), and special carriers, such as the micro drops (96), mall nylon coils (97), and nylon mesh (9), which have achieved higher cooling rates, permitting the use of less concentrated solutions with less toxicity to the cell. Moreover, it is essential to note that when cells are immersed directly in LN_2, the process results in extensive boiling because cells are warmer objects. Evaporation occurs, and coated vapor surrounds the cells, creating an insulating layer that decreases the cooling rate. Minimizing the volume surrounding the cell, avoiding LN_2 vapor formation,

and establishing direct contact between the cryoprotectant and the LN_2 increase the cooling and warming rates during cell vitrification (98).

Ultra-rapid cooling rates have led to decreased CPA concentrations and have made vitrification a competitive alternative to conventional slow freezing. However, the solution's viscosity and composition, rather than the cooling or warming rate, has been the most critical factor determining the success of cryopreservation (99). Today, vitrification is a popular method for the cryopreservation of many different cell types, tissues, and organs. But the extent of cryoinjury and developmental rates are highly variable, depending on the species (9, 15, 33, 100).

2.3.3. Warming

There are different terms employed in cryopreservation: thawing is specifically the melting of ice, commonly used for vitrification, but inaccurate Thawing can occur rapidly, with temperature changes that exceed 360°C/min. Detrimental effects in the cell result from the recrystallization during thawing or osmotic stresses (63, 74). Warming is a more accurate description than thawing in the context of vitrified systems (80). Another concept is the critical warming rate that suppresses ice formation during warming, which depends strongly on the total solute content of the system and the chemical nature of the solute (23). During warming, full ice development is much more rapid. Hence, warming rates required to avoid significant devitrification are far higher than the cooling rates initially required to achieve vitrification(90). The solutes used for vitrification are generally the same as for warming. Warming solutions based on sugars help todisplace more water and have a more substantial effect on solution viscosity, thus reducing the critical warming rate (101).

The use of a high extracellular concentration of sucrose (e.g., 1 mol/l) counterbalances the high concentration of the cryoprotectant agents in the cell, reducing the difference in osmolarity between the intra and extracellular compartments (33, 99). Canesin *et al*. (17) found that in equine oocytes, warming solutions without sugar are as effective as the standard high concentrations of sucrose in warming solutions that had been reported earlier (80). This approach has a practical advantage during the manipulation of oocytes

because it simplifies finding and transferring oocytes from the warming solution to the maturation medium.

2.4. Oocyte damage caused by vitrification

During cryopreservation, cells are exposed to mechanical, thermal, and chemical stressors, which can compromise cell function and cell death because of disturbances in the homeostatic state (56). The two major causes of cellular damage in cells are mechanical damage resulting from the formation of ice crystals (83) and chemical damage due to cryoprotectants (102). To summarize, we can classify the detrimental effects to oocytes during vitrification as follows: osmotic intolerance due to osmotic swelling or shrinking; toxicity due to permeable cryoprotectants, cold-shock injury due to the sensitivity of oocytes to the reduction of temperature; and injury associated with intracellular ice crystal formation as shown in figure 3 (103).

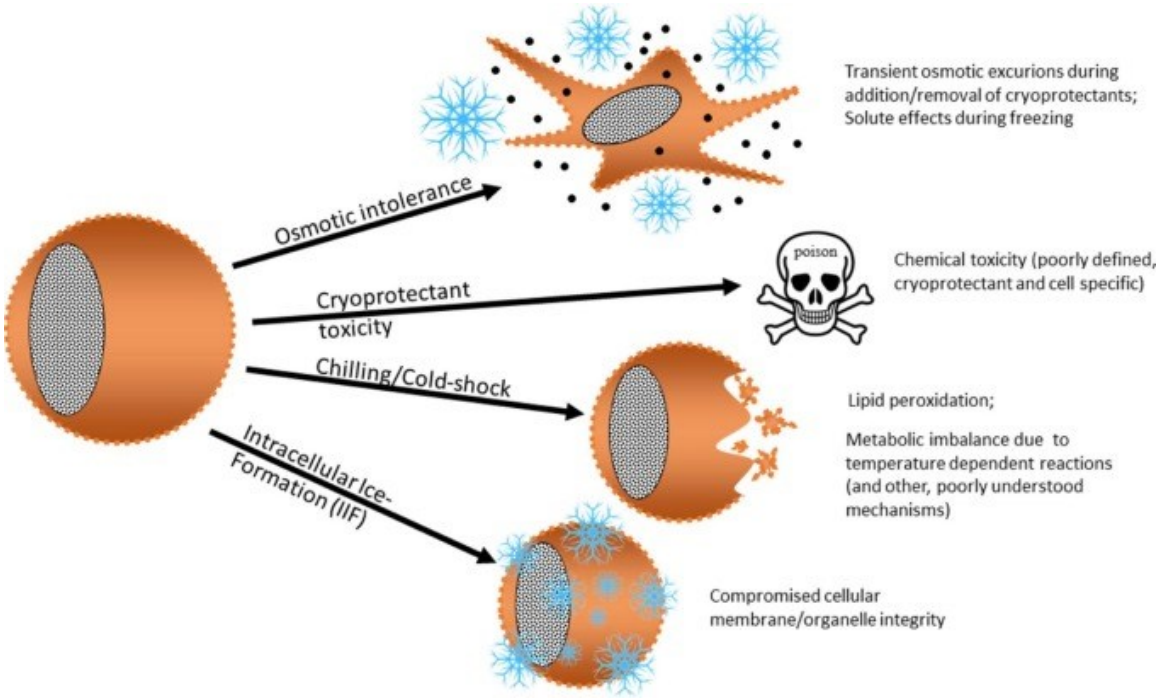

Figure 3. Schematic representation of the potential mechanisms of damage that can occur during cryopreservation (103).

2.4.1. Osmotic stress

The exposure of cells to solutions containing CPAs before cooling can be damaging due to an osmotic effect. Most of the commonly used permeating CPAs have lower plasma membrane permeability coefficients than water (104). This relationship results in cells experiencing osmotically-driven volume variations during cryoprotectant addition and removal from the cell (105). The cell membrane's water permeability dictates the rate at which water flows out of a cell (106). The permeability of cells to water depends on several factors, including temperature, CPAs, and cell type (82). Oocytes are quite different from sperm cells and embryos. In general, it has been demonstrated that oocytes are more sensitive to cryodamage (22) than embryos (107). Oocytes have several characteristics that reduce their membrane permeability to both water and cryoprotective agents compared to other cells, making them sensitive to osmotic effects; one of these characteristics is the low permeability coefficient of the plasma membrane. Mouse oocytes do not express aquaporins at a functional level, while bovine oocytes only express a small number of aquaporins to move water molecules. Therefore, water moves through the plasma membrane of oocytes predominantly by simple diffusion, in contrast to mouse morulae and blastocysts, in which the water mainly moves by facilitated diffusion via water channels (108) . The aquaporins are a family of water channels consisted of proteins and expressed at the plasma membranes of cells involved in fluid transport (109). Cryoprotective agents move through the oocytes and early embryos, similar to the water movement (104). The permeability to DMSO, EG, and PG of oocytes is low comparing with blastocyst permeability (82). Furthermore, the permeability value of PG is higher than other cryoprotectants in the oocytes. This might be related to its higher hydrophobicity compared to other cryoprotectants (104).

Another characteristic that makes the plasma membrane of oocytes different from that of embryos is the increment of intracellular free calcium produced following fertilization, facilitating the permeation of water and CPAs (110). Additionally, the concentration of sub-membranous polymerized filamentous actin of embryos increases and its conformation changes, which facilitates the penetration of water and CPAs (111). Finally, the higher strength of the cell membrane increases embryos' osmotic tolerance during warming, allowing them to resist osmotic changes better than oocytes, while in oocytes, the

presence of *zona pellucida* and cumulus cells can present obstacles to the penetration of CPAs (112).

Finally, the effects of cumulus cells on oocyte survival after freezing and warming are controversial. Nevertheless, the presence of granulosa cells may offer some protection against osmotic changes and stresses induced during the process of addition and removal of the cryoprotectant during the vitrification process (113); the optimal times for equilibration are different for the oocyte than for the cumulus cells (114). Additionally, the immersion of oocytes in hypertonic CPAs causes cell shrinkage. This shrinking may disrupt the granulosa cell processes and gap junction communication with the oocyte so that physiological interactions between the oocyte and the granulosa cells cannot be preserved (113).

2.4.2. Chilling injury

One of the principal consequences of chilling injury is the intracellular ice formation during cryopreservation when there is not enough time for water to exit the cell. As a result, large ice crystals form within the intracellular compartments. These crystals cause breaks in the cell membranes and damage cell organelles (80). Therefore, the cell dies due to intracellular ice formation, caused by the interaction of cooling velocity and water loss (115). If the cell can dehydrate before intracellular nucleation, intracellular ice crystals do form, but these crystals are much smaller (116). Vitrification reduces damage caused due to ice crystal formation during the cooling process because of a substantial rise in viscosity, which results in the formation of a solid glasslike form. This solid “glassy” layer is amorphous, meaning that it can readjust and take the shape of the cell, enabling the cell to maintain its structure and remain intact (83). Moreover, the use of permeating CPAs is helpful for the replacement of intracellular liquid and decreasing ice formation (102).

The oocyte needs to maintain the integrity of several structural features to undergo fertilization and further development. These structures include the plasma membrane, the *zona pellucida*, the cortical granules, the microtubular spindle, microfilaments, and condensed chromosomes (20). Post-warmed oocytes can show *zona pellucida* or cytoplasmic membrane fractures, resulting in various cytoskeletal and chromosomal modifications (22). A common problem in the cryopreservation of oocytes, which makes

them particularly susceptible to intracellular ice formation, is their large size and spherical shape that gives a smaller surface area relative to their large internal volume (117). Previous experiences with the cryopreservation of various cell types have led to the understanding that as cell size increases, difficulty in cryopreservation also increases (101); moreover, due to their large size and spherical shape, it is more likely for a large volume of water to be trapped inside the cells during cooling (118). This makes the dehydration and penetration of cryoprotectants challenging to achieve. On the other hand, it has been reported that some oocytes, depending on the species, can fully or partially repair themselves (27, 119, 120).

Another feature that determines the chilling sensitivity during cryopreservation is the intracellular lipid composition (56). There is a threshold level below which cell membrane function is weakened due to a phase transition in membrane fats. The phase transition is the change from a liquid state to a solid-state at a specific freezing point. The temperature at which this lipid phase transition occurs is inversely proportional to the amount of unsaturated fatty acids within the membrane (121). Thus, by altering their lipid membranes' composition, different organisms can adjust this threshold temperature (122). Therefore, cells' ability to survive at low temperatures is partly due to the increase in the ratio of unsaturated fatty acids within the cell membrane and lipid content, and low cryotolerance during vitrification has been linked to high lipid content (9, 30, 32, 56).

Mammalian oocytes have high lipid content, which gives them a dark appearance. Differences in the color of the cytoplasm have been correlated to the amounts of lipids within the cytoplasm (dark color and opaqueness of cytoplasm) (Figure 4) (123). In mammals, primary lipid storage consists of saturated and monosaturated fatty acids, which are part of the cytoplasmic membrane and organelles (124). Lipids are thought to interact with oocyte cytoskeletons, disrupting the arrangement of microtubules and microfilaments that form the cytoskeleton. These interactions are irreversibly affected during cryopreservation (123). Lipid content varies from species to species and also varies according to the level of maturity. For instance, porcine and equine oocytes (9) present a darker appearance than mouse and bovine oocytes (124). Mouse and bovine oocytes (14) are considered more cryostable than equine (9) and porcine oocytes(32) .

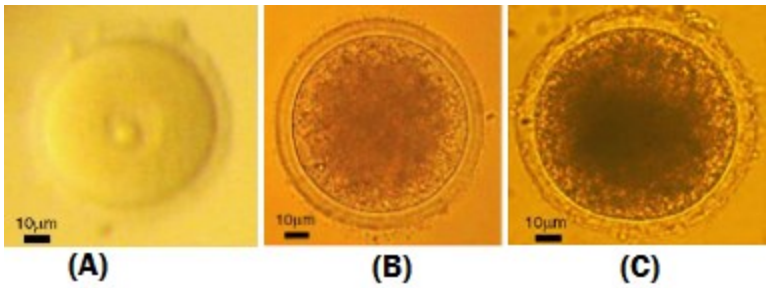

Figure 4. Lipid droplets of stained immature oocytes (dark zones in the cytoplasm). A) murine, B) bovine, and C) porcine (124).

Effect on development

The maturation of oocytes is a highly energy-intensive process. Adenosine triphosphate (ATP) is essential for the ability of motor proteins to capture and transport chromosomes along the spindle (125). During oocyte maturation, mitochondria accumulate around the nucleus before the germinal vesicle (GV) breakdown (126). Vitrification reduces mitochondrial function by altering the integrity of mitochondria (mt) deoxyribonucleic acid (DNA), and the ability to develop into the blastocyst stage in embryos and oocytes (127). The availability of functional mitochondria has been recognized as a critical determinant of embryo development competence since defects at the structural and mtDNA levels have been identified in compromised oocytes/embryos (128).

Free radicals are formed during the cryopreservation of tissues, resulting from changes in temperature and osmotic damage in cells (129). The enzymatic system is composed of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and peroxiredoxins, which detoxify reactive oxygen species (ROS) directly. Non-enzymatic systems contain mainly vitamins A, C, and E (130). The activation of cellular oxidative defense systems occurs in response to damage produced upon vitrification. They can cause structural and functional alterations of cells due to their oxidative property (129). Oocytes are incredibly vulnerable to oxidative stress, not only from the cellular metabolism but also from external influences, such as light, rapid change of oxygen concentration, and temperature changes (40). With the oxidative propriety of ROS, it could oxidize proteins, lipids, and DNA, causing structural and functional alteration of the cell (129). When the oxidative stress reaches a certain level, it may induce the release of cytochrome C and other apoptogenic factors from mitochondria, which eventually activate programmed cell death (131).

2.4.3. Toxicity of CPAs

In addition to the injury of osmotic shock and chilling injury, oocytes must also tolerate toxic levels of CPAs generally used in vitrification protocols. PG and DMSO were found to be the most toxic CPAs, whereas glycerol and ethylene glycol were much less toxic (99, 104). The detrimental effects of CPAs depend on three factors: their concentration, the temperature at which the cells are exposed, and the length of time for which the cells are held (82). Survival also depends on the cell type and its ability to overcome physicochemical changes during the process (108). As a result, oocytes can only be exposed to a minimal vitrification media volume for a short period (less than a minute) (132). All the CPAs and other additives have different toxicities, penetration rates, and transition temperatures (99). The combination of varying CPAs is often used to increase viscosity, transition temperature, and reduce the level of toxicity (92).

Effect on the metaphase II spindle

In vitrified oocytes, the chemicals used during cryopreservation, CPAs, increase abnormalities in spindle and chromosome configuration, resulting from the depolymerization of meiotic spindles and the disappearance of microtubule-organizing centers. Therefore, the fertilization of oocytes with disrupted spindles can lead to aneuploidy, disgeny, and arrested cleavage (113, 133). The meiotic spindles of oocytes consist of microtubules constructed by polymerization of tubulin dimers of α- and β-tubulin. Microtubules start from microtubular organizing centers at both poles and anchor chromosomes at the kinetochores. The synapsed chromosome pairs in metaphase I (MI), then the sister chromatids in MII have to be pulled to the opposite ends of the dividing oocytes along the mitotic spindle, which is guided by the extending and shortening of the meiotic spindles (Figure 5) (134). The tubulin dimer polymerizes and depolymerizes at various stages of a cell cycle. The meiotic spindles are crucial for the events following fertilization during the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle (135).

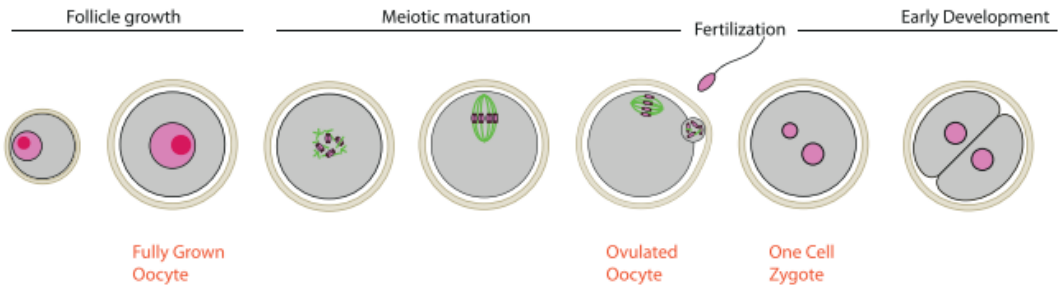

Figure 5. Schematic representation of the major steps in the progression of nuclear maturation in mammalian oocytes (134).

Various meiotic stages exhibit different sensitivity to freezing (136). Oocytes may be cryopreserved at the immature GV stage (11) or at the mature MII stage (100), depending on the species. Some reports have demonstrated that freezing immature oocytes is better because the meiotic spindle has not being formed and the DNA is confined within the nucleus (137, 138); however, immature oocytes are thought to be more sensitive to anastomotic stress and feature lower cell membrane stability than MII stage oocytes (114, 139).

In many species, studies have used matured oocytes at the MII stage (93, 100, 140). In species most susceptible to freezing, such as pigs and horses, vitrified MII stage oocytes generally demonstrate low developmental competence in terms of blastocyst formation rates and cell numbers in blastocysts (20, 141). Oocytes at the MII stage have undergone both cytoplasmic and nuclear maturation, including extrusion of the first polar body and alignment of chromosomes on a meiotic spindle. In contrast, the chromatin of GV oocytes is still in the diplotene phase of prophase I without a meiotic spindle (134). At the MII stage, the oocyte DNA is condensed into chromosomes that are aligned along the equatorial region of the meiotic spindle and are susceptible to disruption (140).

Researchers have demonstrated that oocyte exposure to cooling and CPAs can cause depolymerization and the disorganization of spindle microtubules (20, 107). Spindle damage in oocytes can have a fatal consequence, preventing the extrusion of the second polar body and resulting in abnormal chromosomal numbers in embryos (27, 123, 140). On the other hand, numerous reports have demonstrated that the metaphase spindle microtubules that depolymerize during oocyte cryopreservation by vitrification can

repolymerize, forming normal structural and functional spindles post-cryopreservation given time and the proper temperature (133, 140, 142).

Effect on cortical granule exocytosis

Intracellular calcium (Ca++) has been implicated as a second messenger in somatic cells and in the events that follow sperm penetration of the oocyte. Upon fusion, the sperm induces a series of calcium transients through a sperm-specific phospholipase C (143). Calcium is responsible for initiating cortical granule fusion with the oolemma, resulting in the release of their contents into the surrounding *zona pellucida* (144).

The exposure of the CPAs may increase intracellular Ca++ and trigger premature exocytosis of cortical granular material, leading to *zona pellucida* hardening and making sperm penetration and fertilization impossible (145). The use of ICSI overcomes the effects of zona hardening, resulting in higher fertilization rates (146). Although this potential problem has been alleviated from a practical perspective, zona hardening reflects that the oocyte has started to undergo activation has not been addressed (110). This might explain in part why oocytes do not develop well following cryopreservation (145).

Three commonly used cryoprotectants (EG, PG, and DMSO) induce an immediate increase in Ca++, but the magnitude and duration of the Ca++ transient are different depending on the cryoprotectant, with ethylene glycol resulting in the smallest and shortest increase and propanediol causing the most protracted elevation (147). The literature also says that DMSO affects Ca++ stores directly (145).

2.5. Vitrification protocols

It was discovered in the 1940s (66) that the addition of glycerol for protection against cryodamage greatly enhanced the survival of cryopreserved cells. This gave rise to the investigational concept of cryoprotectants. Some authors have mixed EG with other permeating agents, such as DMSO or PG, to reduce the concentration of a single cryoprotectant and to decrease the individual specific toxicity (27, 148). Additionally, the strategy for lowering toxicity by applying rapid exposure to a high CPA concentration has been widely used (149, 150). Vitrification media are currently prepared from buffered

media (e.g., TCM-199) with a stable pH between 7.2 and 7.4 and CPAs that prevent ice crystal formation after freezing by increasing cellular dehydration and viscosity (63). Typically, combinations of permeating and non-permeating CPAs are used (148, 151). Permeating CPAs are of low molecular weight, such as glycerol, EG, PG and DMSO. They penetrate the cell and form hydrogen bonds with intracellular water molecules, preventing crystallization (102, 152)**.** The non-permeating CPAs are of low molecular weight and include sucrose, glucose, trehalose, and fructose. They remain extracellular and draw free water out of the cell by osmosis, resulting in intracellular dehydration (153). The last component of vitrification media is a high molecular weight, non-permeating polymer used to reduce the concentration of CPAs necessary for vitrification, reducing the toxicity of the solution and protecting the *zona pellucida* against cracking. Standard solutions are FBS or bovine serum albumin, polyethylene glycol, polyvinylpyrrolidone, Ficoll, and polyvinyl alcohol (25, 116, 154).

Ethylene glycol, a mono-ethylene glycol with the molecular formula $C_2H_2(OH)_2$, is an important component of vitrification solutions, which forms from ethylene oxide hydrolysis, characterized by low toxicity and rapid permeation of the cell (99). DMSO is a polar compound ($C_2H_6SO_2$) widely used as a cryoprotectant in different species (29, 151, 155). The direct hydrolysis of propylene oxide produces propylene glycol ($C_3H_8O_2$). 1,2-propylene glycol and 1,3-propylene glycol are formed simultaneously through the sequential addition of propylene oxide (104). Since water is not viscous, it can only be vitrified by quite rapid cooling rates or by using high concentrations of permeable CPAs, which are small molecules that penetrate cells and limit the amount of intracellular and extracellular water that converts into ice during cooling (99, 150). But, the use of the lowest possible concentration of permeable CPAs to minimize problems with toxicity is effective by replacing penetrating CPAs with carbohydrates (90). Trehalose and sucrose are the most common carbohydrates used as non-permeating CPAs in the cryopreservation protocols of murine, bovine, equine, porcine, and ovine oocytes and/or embryos (32, 99, 156, 157). Other sugars are not commonly used as components of vitrification solutions in oocytes but are widely used to preserve organs, tissues, and cells or as components of a culture media (158). For instance, raffinose, fructose, and glucose have been used for sperm cryopreservation (159).

The addition of carbohydrates has facilitated the dehydration of embryos and works as an essential component of osmotic buffers (160). These solutions are used to increase the effective osmolality of the extracellular medium because sugars are unable to cross the cell membrane naturally, enhancing cellular dehydration by osmotic pressure, thus limiting the extent of swelling (158). It has been suggested that sugars can preserve the structural and functional integrity of membranes at low levels of water activity (99, 101). And it has further been reported that the critical cooling rate required to avoid ice crystallization in solutions with penetrating cryoprotectants was altered following the addition of sugar (101). Thus, the different types of sugars should be analyzed during the design of vitrification solutions.

Since vitrification is a method that requires a relatively high concentration of cryoprotectants, stepwise addition of cryoprotectants is used to decrease the toxic effects and minimize damage due to osmotic shock, resulting in higher maturation, fertilization and blastocyst formation rates (161, 162). Some strategies can be applied; the most common is the two-step protocol in which oocyte is exposed to a PVS as a first step, containing DMSO, EG, or PG (permeating CPAs); depending on the protocol, the oocyte may or may not remain for a relatively brief exposure in this first step (9, 19). The low concentration of cryoprotectants in the first solution is less toxic than the VS. Oocytes in the first solution shrink initially and gradually re-expand to their original volume. The second step is a relatively prolonged incubation in VS, containing higher concentrations of permeating CPAs and non-permeating CPAs (sucrose or trehalose) to dehydrate the cells (Figure 6) (9, 80, 163). The oocyte is then loaded onto a cryo-device with a small volume of vitrification medium before being plunged into liquid nitrogen (80).

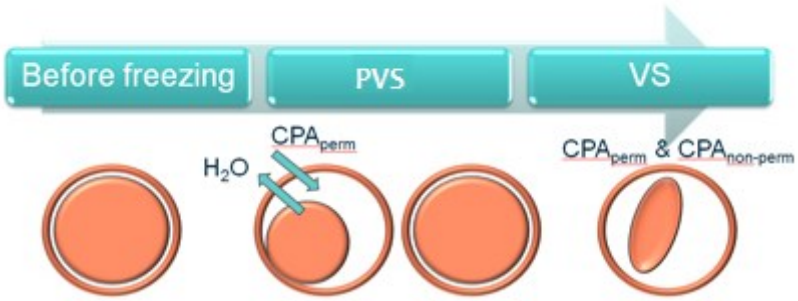

Figure 6. Schematic presentation of the vitrification procedure (164).

The procedure employed to load the CPAs into the oocytes will vary between the authors. Some are time-specific (19), some require that the processes be conducted at room temperature (17), and some are performed at higher temperatures (25). One-step may, because of the possibility of poor permeation of the cryoprotectants, result in intracellular ice formation during cooling or warming (27, 161, 162). Extensive discussions are unfolding about which vitrification container is the best, but it has been also claimed that the vitrification method thoroughly depends upon the technical skill of the one who performs the vitrification (142).

2.5.1. Vitrification protocols in species other than horses

The first report of oocyte cryopreservation with a viable pregnancy in humans originated from slow-rate freezing-thawing, containing phosphate-buffered saline, FBS and dimethyl sulphoxide (27). Several studies have compared slow-freezing techniques (156, 165, 166) and vitrification (167-169) in human embryology. Vitrification has been suggested to be more suitable for the cryopreservation of human oocytes by applying a higher concentration of cryoprotectants and a rapid cooling speed to prevent ice crystal formation (21). Some of the first reports using vitrification protocols with ethylene glycol and sucrose had a survival rate from 65% to 90% and a blastocyst formation rate of around 30% (170).

Several modifications in protocols for vitrification were proposed to improve the survival of frozen–warmed oocytes, employing an array of different vitrification systems, including electron microscope grids, open pulled straws, open hemistraws and cryoloops (167, 168, 171, 172). Significantly increased success rates were reported with the cryotop method performed with MII oocytes. This cryotop method consisting of 15% ethylene glycol, 15%

dimethyl sulfoxide, and 0.5 M sucrose was highly reproducible, reporting 91% survival, 81% cleavage, 50% blastocyst and 41.9% pregnancy rates (168). Significant points of consideration among the different protocols are the time and temperature of oocyte exposure to the vitrification solution, the concentration of the solution surrounding the oocytes, the carrier to hold the oocyte during vitrification and the temperature and media using during warming (101).

Data on mouse oocytes has demonstrated the superiority of vitrification over slow freezing in every parameter measured. A two-step vitrification protocol with a CPA solution composed of DMSO, EG, and sucrose achieved blastocyst rates of 67%, when the resultant mouse blastocysts were transferred, embryos derived from vitrified oocytes implanted at the same rate as blastocysts from non-cryopreserved oocytes (88% and 87%, respectively) (173). Recently, the viability of oocytes after a two-step vitrification protocol, with a CPA solution composed of EG, DMSO, trehalose and FBS, was reported to be more than 90%, and no significant difference of blastocyst formation was observed between the fresh and vitrified oocytes after IVF (174).

On the other hand, oocytes from cattle are extremely sensitive to cold temperatures, and slow freezing dramatically affects blastocyst formation (4.5%) (71). Although the cryopreservation of bovine oocytes remains a challenge, some of the highest blastocyst formation rates among domestic animals have been obtained from this species. A 25% blastocyst rate formation of vitrified matured oocytes has been reported using a two-step protocol with an open pulled straw and a solution composed of EG, DMSO and sucrose (22). Methods of vitrification of bovine embryos have significantly progressed in recent years. Currently, live offspring have been born from vitrified oocytes using a three-step protocol, achieving a maturation rate of 50% and a pregnancy rate of 36%. The protocol was as follows: oocytes were suspended in a PVS1 composed of 3% EG for 5–10 min for initial oocyte cryoprotectant exposure; groups of 5-6 oocytes were placed in a PV2 composed of 10% EG + 10% DMSO for 30 s; subsequently, oocytes were suspended in a VS composed of 20% EG + 20% DMSO + 17.1% sucrose, supplemented with 0.1% polyvinyl alcohol; finally, oocytes were transferred to a 2 µl VS droplet (the total exposure time to the VS was 25 s) and oocytes were loaded by capillarity into open-pulled glass micropipettes (14).

In efforts to improve development competence during oocyte vitrification in bovine, modification of the devices, such as an electron microscopy grid (10), has been approved to facilitate the holding of the cells. Moreover, it has been reported that with bovine oocytes, the survival, as well as the cleavage and blastocyst development rate, was not significantly different following vitrification and warming with or without cumulus cells (175). Additionally, the three-step protocol with EG/ sucrose and ficoll reported fewer abnormalities in the oocytes than the single-step exposure; this method was used to enhance the feasibility of nylon-mesh holder for vitrification of immature oocytes (176).

Currently, a limited number of studies are available on vitrification of small ruminant oocytes (177-179). In sheep, a two-step vitrification protocol using EG, DMSO and trehalose with a nylon loop resulted in a blastocyst development rate of 29.4% after several trials where low developmental rates were obtained following immature and mature oocyte vitrification (29). Poor success in ovine oocyte cryopreservation has been attributed to damage to enzymes such as mitogen-activated protein (MAPK) kinase, critical for oocyte maturation and subsequent embryo development (114).

Regarding the porcine species, oocytes are particularly susceptible to cellular damage by freezing because the meiotic spindle of porcine oocytes is quite sensitive to cryopreservation, resulting in impaired development at MII (107). Because porcine oocytes contain more lipid droplets than other species, a recent study combined removing cytoplasmic lipid droplets with microtubule stabilization and found that vitrified porcine immature oocytes could develop to the blastocyst stage and maintain the ability to develop into fetuses (180). After the improvement of a vitrification protocol with a reasonable survival rate (approximately 50%) and a blastocyst rate of 2% (30); the first study of successful piglet production from vitrified immature oocytes was reported with a two-step vitrification method with EG, PG, cytochalasin B, bovine serum albumin (BSA) and trehalose. However, the blastocyst rate was still very low (5.2%) (32). Improving the survival and development of oocytes after cryopreservation has also been based on optimizing the conditions of in vitro culture with granulosa cells in a two-step vitrification protocol with EG and sucrose, achieving a blastocyst rate of 43% after parthenogenetic activation (181).

For livestock management, although viable oocytes could survive after cryopreservation, the number of offspring resulting from cryopreserved oocytes is much lower than that from cryopreserved embryos (56). Despite recent advancements, the cryopreservation of the oocytes of most mammalian species remains a challenge. Despite the increasing work on the vitrification of oocytes, more research is needed to explain the species-specific mechanisms which lead poor embryo development following vitrification.

2.5.2. Vitrification protocols in horses

Vitrification is the most commonly used cryopreservation technique reported for equine oocytes due to the failure of slow freezing (157, 182). Although bovine oocytes have been reported to tolerate cryopreservation better at the MII stage than the GV stage, vitrified equine oocytes after IVM display considerable spindle damage (20). Moreover, it has been demonstrated that, although initial cumulus morphology does not affect the proportion of vitrified GV oocytes that reach MII (22%) during IVM, it markedly affects the quality of the MII spindle (113).

Different approaches have been reported in the literature to standardize the vitrification protocol for equine oocytes. Pregnancies of two live-born foals have resulted after the fertilization of *in-vivo* matured oocytes following the vitrification technique using a three-step protocol with EG, DMSO, sucrose, and ficoll, thus providing a blastocyst rate of 12% (18). In the mentioned protocol, cryoprotectants were loaded in three steps: 5% DMSO and 5% EG for 30 s; 10% DMSO and 10% EG for 30 s; and 20% DMSO, 20% EG, with 0.65 M sucrose for ~20 s, before being loaded onto a nylon loop. Also, Ortiz-Escribano *et al*. (19) demonstrated that immature equines with corona radiata oocytes could be vitrified successfully, using a high concentration of CPAs and a short time of exposure to the equilibration and vitrification solutions. For the first time, they obtained a foal after transferring an *in-vitro* produced blastocyst, which was derived from an immature vitrified oocyte with a two-step vitrification protocol using solutions with DMSO, EG, sucrose, and BSA. Although the maturation rate was 42%, the blastocyst development rate remained low (7%).

Historically, vitrified equine oocytes have improved their MII rates (28−46%); however, approximately 50% of oocytes reaching the MII stage exhibit spindle abnormalities and

low developmental competence. Much of the equine oocytes' damage affects the mitochondria and gap junctions between the oocyte and surrounding cumulus-corona radiata cells (113). These interactions are critical for successful maturation and developmental competence. As in other species, equine oocytes are rich in cytoplasmic lipid droplets, rendering them highly sensitive to chilling and making necessary species-specific optimization of the exposure time and concentration of CPAs (9). In pigs, it was shown that the combination of PG and EG provided better embryo development after the vitrification of immature oocytes (32). Therefore, Canesin *et al.* (17) reported a short vitrification protocol with EG, PG, trehalose, and FBS, achieving maturation rates in 20% of vitrified immature equine oocytes and 15% blastocyst formation, which was higher than previous studies on vitrified-warmed immature equine oocytes.

The literature says that the exposure time of the oocytes to cryoprotectants may be shortened to avoid toxic effects (132); however, as mentioned by Canesin *et al.* (17) this measure did not prevent the ice crystal formation in equine oocytes. Therefore, the key to the success of the vitrification of oocytes is to find a balance between the use of a minimal concentration of cryoprotectants without compromising their cryoprotective actions to avoid ice crystal formation (56). Currently, there is no universal oocyte vitrification protocol in horses. Protocols use different ways of exposing the oocytes to CPAs. In those protocols, oocytes are placed into a PVS, whose composition is like the VS but with a lower concentration of permeable cryoprotectants to avoid any anticipated toxicity before placement in the VS (17). Only a few foals have been born from mature or immature oocytes cryopreserved by vitrification (18, 19). These empirically derived protocols for oocyte cryopreservation can be improved, and different strategies used in other species have been evaluated to increase blastocyst development in vitrified equine oocytes (Table 1).

PV1	PV2	VS	%Oocytes matured	% Oocytes cleaved	% Blastocyst development	Live offspring	References
5% EG / 5% DMSO	10%EG /10%DMSO	20%EG/20% DMSO/0.6M sucrose	-	-	12	2	Maclellan *et al,* 2002
10% EG / 10% DMSO	-	20%EG/20% DMSO/0.5M sucrose	42	34	1	-	Tharasanit *et al, 2006*
10% EG / 10% DMSO	-	20%EG/20% DMSO/0.5M sucrose	40.2	41.6	7	1	Ortiz-Escribano *et al.,* 2017
2% EG / 2% PG	-	17.5%EG/17.5%PG/0.5 M trehalose	20.6	85	15	-	Canesin *et al*, 2018

Table 1. Overview of vitrification treatments reported in equine oocytes.

Since vitrification requires relatively high concentrations of CPAs, stepwise addition of cryoprotectants may reduce their toxic effects and minimize damage due to extreme cell-volume expansion. As this is an additional step, there is less intracellular water to extract, reducing the osmotic effects and, in turn, decreasing the total exposure time to the cryoprotectant (101, 183). The addition of steps in a vitrification protocol, including the three-step vitrification protocol, has been reported to result in less damage to oocyte for both cytotoxicity and osmotic effect (161, 162). In terms of optimizing vitrification procedures, one goal is to determine the optimal combination of the exposure time in PVS and VS (184). The second goal is to find the combination of CPAs most suitable for equine oocytes. In vitrification protocols of equine oocytes, the most common combinations of permeable cryoprotectants are either EG/DMSO or EG/PG (19, 113, 157, 182, 185). Canesin *et al*. (9) hypothesized that, due to the similar composition of lipid content between equine oocytes and pig oocytes, they might have the same response to cryoinjury. This would mean that successful methods with EG/PG in pig oocytes (33) may improve the viability of equine oocytes. Therefore, further research is necessary to standardize a vitrification protocol for immature equine oocytes and elucidate which combination of permeable CPAs (EG/PG or EG/DMSO) is the best for equine oocyte vitrification.

3. Hypothesis and objectives

As noted above, assisted reproduction in the equine industry is greatly hampered by the low success rates that currently characterize cryopreservation. Further research into this technology is essential if progress is to continue. An essential step toward this goal is the standardization of vitrification protocols for immature equine oocytes and elucidate, which combination of permeable CPAs could be more effective than the inefficient procedures currently in use. The following hypotheses and objectives have been formulated to address this need:

Our **hypothesis** is that a three-step vitrification protocol in immature equine oocytes using EG/DMSO will improve the rates of post-fertilization development of equine embryos.

Our **first objective** is to assess post-warming viability and maturation of immature equine oocytes vitrified in a three-step solution with two different cryoprotectants (EG/PG or EG/DMSO). The **second objective** is to assess blastocyst development after ICSI of oocytes vitrified using the best treatment identified in the first objective, comparing with cryoprotectant-exposed treatment without freezing

4. Methodology

4.1. Ovary and oocyte collection

4.1.1. Postmortem collection

Ovaries were obtained from a local slaughterhouse and transported to the laboratory at the Université de Montréal in insulated containers at room temperature (~22 °C). Oocytes were collected and transferred to a holding medium within the initial 7 hrs after the death of any given mare.

The recovery of cumulus-oocyte complexes (COCs) was performed through a vacuum pump procedure (49). After removing extraneous tissue from the ovaries, each follicle visible on the exterior was incised, and the follicular fluid was drained. The walls of each follicle were scraped with a 14G needle attached to a sterile bottle via a tube and a vacuum pump, with the pressure set to aspirate approximately 40 mL of fluid per minute, as shown in figure 7. The needle and tubing were intermittently flushed by immersion of the needle in a commercial oocyte recovery medium (EquipPro OPU® Recovery Medium; MOFA Global, Verona, WI, USA). Once all visible exterior follicles had been processed, the ovary was cut into slices, approximately 1 cm in thickness, to expose the interior follicles, on which the same process was performed. Follicles greater than 25 mm in diameter or having viscous, dark yellow follicular fluid were not scraped. Ovaries with evident pathological characteristics were discarded.

After all the follicles were processed, the collection bottle contents were poured through a cup-type embryo filter (Immuno Systems, Inc., Spring Valley, WI, USA). The bottle was then rinsed three times with a fresh recovery medium, and the rinsed fluid was poured through the filter. The contents of the filter cup were then rinsed into a 100-mm Petri dish with a fresh recovery medium. COCs were located under a dissecting microscope and were transferred to a 35-mm Petri dish containing EquiPro OPU® recovery medium. Oocytes were held in the dish at room temperature (~22°C) until all searching was completed. After collecting oocytes, any excess cumulus cells were removed through pipetting with a glass pipette, typically until only the corona radiata remained (Figure 8). Oocytes were then placed into a 1-mL borosilicate glass vial (Thermo Fisher Scientific,

Waltham, MA, USA) filled to near capacity with vigro (Vetoquinol, USA) holding medium and without exceeding more than 25 oocytes per vial. Vials were packaged, protected from light, and kept at room temperature until the next day.

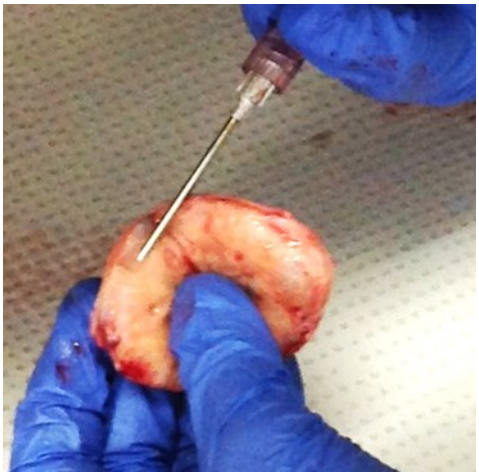

Figure 7. Recovery of COCs through a vacuum pump procedure.

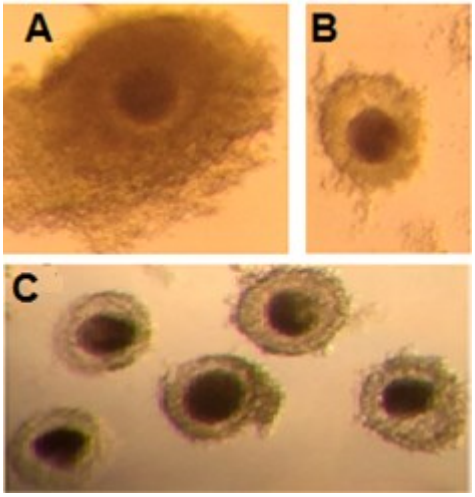

Figure 8. COCs before IVM with stereomicroscopy 50X. (A) Expanded oocyte; (B) Compacted oocytes; (C) Viewed oocytes with four to five layers of cumulus cells.

4.1.2. Transvaginal ultrasound-guided follicular aspiration (TVA)

Eleven quarterhorse type breed mares weighing 400–500 kg and aged 8–16 years were used as oocyte donors. The mares were housed outside in paddocks and were fed hay

ad libitum for the duration of the study. All experimental procedures were performed according to the United States government regulations, and Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training were approved by the Laboratory Animal Care Committee at Texas A&M University. All follicles greater or equal to 8 mm in diameter were aspirated by TVA in each mare approximately once every two weeks. There was no attempt to manipulate the cycle between aspiration sessions.

Mares were restrained in stocks with tails bagged and tied. The perineal region was cleaned with a povidone-iodine scrub. Before sedation, mares were evaluated to identify the number and size of follicles using transrectal examination. Mares with more than 6 follicles greater than 8 mm were subjected to TVA. Sedation was offered as follows: firstly, 5–7 mg detomidine (Dormosedan, Pfizer, Exton, PA, USA) intravenous (IV) prior to the procedure; 10 mg Butorphanol (Torbugesic, Overland Park, KS, USA) and 60 mg N-butyl hyoscine bromide (Buscopan, Boehringer Ingelheim, Ingelheim, Germany) were given IV immediately before the beginning of aspiration. Additional detomidine (1–3 mg, IV) was given as needed. A 2-L bag of complete flush solution Vigro (Vetoquinol, USA) with 5 IU heparin/mL that had been pre-warmed to 37°C was attached to the controlled flush set, using a vacuum pump set at 145 mm Hg. A 3-Mhz sector ultrasound probe was fitted within a transvaginal probe handle. A needle guide, lightly coated with a contact gel and covered with a plastic sleeve lubricated lightly with the same gel, was placed into the mare's vagina. A 12-gauge double-lumen oocyte aspiration needle (Cook Veterinary Products, New Buffalo, MI, USA) was inserted through the needle guide. One operator held the ovary transrectally while the other operator manipulated the ultrasound probe transvaginally to visualize follicles on the ovary for follicular puncture (Figure 9). Each follicle was flushed four times with a flush solution. The aspirated fluid from all follicles was collected into 500-mL sterilized plastic bottles; a separate bottle was used for each mare. After aspiration was completed, mares were treated with flunixin meglumine (Banamine, Schering-Plough Animal Health, Union, NJ, USA), 500 mg IV, and they were returned to their herd immediately after recovering from sedation.

Follicles greater than 30 mm in diameter were aspirated separately to avoid including expanded granulosa or oocytes from preovulatory follicles in the immature-follicle aspirate. The aspirated fluid was filtered through an embryo filter, and the COCs were recovered.

As they were located, the COCs were placed in a 35-mm polystyrene Petri dish containing Syngro holding medium (Vetoquinol, USA).

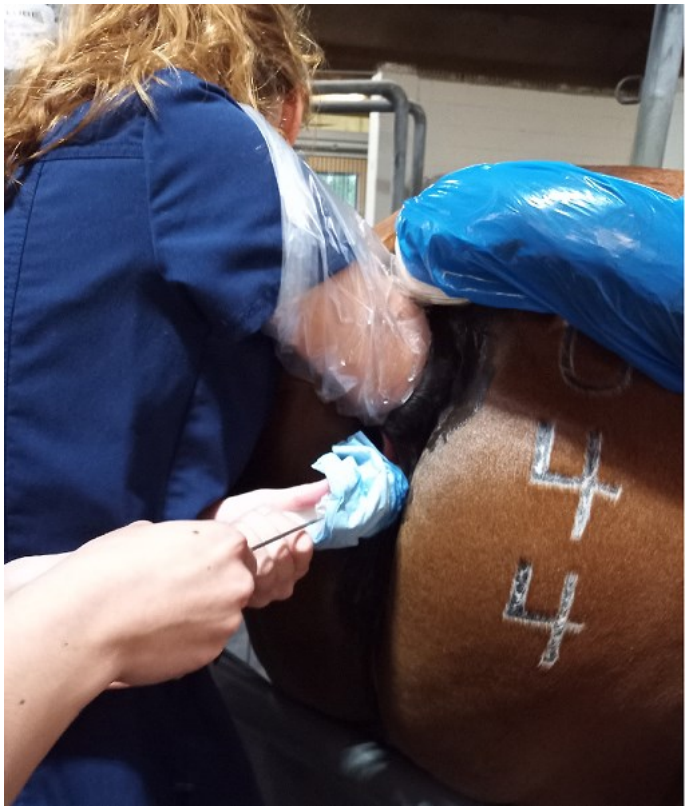

Figure 9. Transvaginal placement of probe and rectal palpation.

After collecting the oocytes, any excess of the cumulus cell was removed, typically until only the corona radiata remained. Oocytes were then placed into a 1mL borosilicate glass vial (Thermo Fisher Scientific, Waltham, MA, USA) filled to near capacity with Syngro holding medium (Vetoquinol, USA) without exceeding more than 25 oocytes per vial. Vials were packaged, protected from light and kept at room temperature until the next day.

4.2. Experimental design

4.2.1. Vitrification study 1. Effect of different vitrification protocols on maturation rate

COCs were collected from equine ovaries obtained from a local slaughterhouse, as described in the oocyte collection section. The day after collection, all COCs were

collected from vials and they were placed in a base solution. They were then randomly allocated to vitrification treatments and the control group (see table 2). The control group was transferred from the base solution to IVM without freezing or exposure to CPA. The concentrations of CPAs used in the three-step vitrification protocols were:

- PV1: 5% EG (Sigma Aldrich, 102466)/PG (Sigma Aldrich, P4347)/ 5% PG or 5% EG/DMSO (Sigma Aldrich, D2650)
- PV2: 10% EG/PG or 10% EG/DMSO
- VS: 17.5% EG/PG/ 3 M sucrose or EG/DMSO 17.5%/ 3 M sucrose (Sigma Aldrich, s1888).

COCs were then subjected to warming, IVM, and staining. The rates of maturation to MII were analyzed among groups.

GROUP:	CONC. (%) PV1	TIME (S) PV1	FINAL CONC. (%) PV2	TIME (S) PV2	CONC. (%) VS	TIME (S) VS
EG/PG VS-10	5	40	10	40	17.5	10
EG/PG VS-20	5	40	10	40	17.5	20
EG/PG VS-30	5	40	10	40	17.5	30
EG/PG VS-40	5	40	10	40	17.5	40
EG/PG VS-50	5	40	10	40	17.5	50
EG/DMSO VS-10	5	40	10	40	17.5	10
EG/DMSO VS-20	5	40	10	40	17.5	20
EG/DMSO VS-30	5	40	10	40	17.5	30
EG/DMSO VS-40	5	40	10	40	17.5	40
EG/DMSO VS-50	5	40	10	40	17.5	50
CONTROL	0	0	0	0	0	0

Table 2. Treatments of vitrification study 1.

4.2.2. Vitrification study 2. Effect of a vitrification protocol with freezing and without freezing on maturation, cleavage, and blastocyst rates.

For this experiment, COCs were collected through TVA in live mares from Texas A&M University, USA, as described in the oocyte collection section. The COCs were held overnight in a commercial embryo-holding medium. The day after collection, all COCs

were collected from vials and they were placed in a base solution. They were then randomly allocated to treatments (see table 3). The control group was transferred from the base solution to IVM without freezing, warming, or exposure to CPA. The CPA-exposure group was exposed to CPAs and warming solutions as described in the vitrification and warming section but without being exposed to freezing.

- Vitrification treatment
- CPA-exposure without freezing
- Control: non-exposure to CPA or freezing

After exposure to the warming solution, the oocytes were cultured for IVM and then subjected to ICSI and embryo culture. The rates of maturation to MII, cleavage, and blastocyst formation were compared among groups.

GROUP	CONC. (%) PV1	TIME (S) PV1	FINAL CONC. (%) PV2	TIME (S) PV2	CONC. (%) VIT	TIME (S) VIT
VITRIFICATION	5	40	10	40	17.5	10
CPA-EXPOSED	5	40	10	40	17.5	10
CONTROL	0	0	0	0	0	0

Table 3. Treatments of vitrification study 2.

4.3. Vitrification

The day after collection, all COCs were placed in a base solution composed of 20% FBS (Wisent INC, 080150) + M199/Hanks' salts (Gibco, 12350-039). Ten COCs were then transferred to a 150-µL droplet of base-solution. With a fine glass pipette filled with PVS1, the oocytes were placed in either 5% EG/DMSO or 5% EG/PG (PVS1 DMSO or PVS1 PG) diluted in M199/Hanks' salts + 20% FBS. The COCs were held in PVS1 for 40 seconds. Immediately after, employing the same glass pipette filled with PVS2, the COCs were transferred to a 150-µL droplet of PVS2 DMSO or PG composed of 10% EG/DMSO or 10% EG/PG diluted in M199/Hanks' salts + 20% FBS and were held for 40 seconds. The oocytes were taken from each droplet 6 seconds before the 40 seconds total (PV1 to PV2 and PV2 to VS). The COCs were then transferred to 150-µL of VS, composed of

17.5% EG/DMSO or 17.5% EG/PG + 3 M sucrose diluted in M199/Hanks' salts + 20% FBS for different times (20s, 30s, 40s and 50s) depending on the treatment (VS-10, VS-20, VS-30, VS-40, and VS-50). Finally, before the time ended (approximately 15 seconds), the COCs were placed in a vitrification mesh (1x 0.5 cm) with a filter underneath, the liquid was blotted off the mesh, and it was plunged into LN_2 at the end of the exposure. We modified the VS-10 treatment because of the difficulties in manipulating during the short exposure time; after PVS2 solution exposure, the COCs were transferred to a droplet placed directly on the mesh without a filter. After 10 seconds, the fluid was blotted off with the aid of the filter and the mesh was plunged into LN_2 immediately after. All meshes were placed inside of a cryovial and stored in LN_2. The cryovial was capped and kept in LN_2 until warming, as described in the warming section.

4.4. Warming

For the warming process, the oocytes were placed on a heated surface. The cryovial containing the vitrification mesh was removed from the storage tank and placed into LN_2 in a Styrofoam box positioned on the work surface near the microscope. The cryovial was opened under LN_2, and the mesh was removed from the vial and quickly placed into a 35-mm Petri dish of 20% FBS + M199/Hanks' salts at 38°C. Oocytes remained in the Petri dish for 5 minutes. After this time, the Petri dish was removed from the heating surface to search for the oocytes. The COCs were then placed in a four-well dish of 20% FBS + M199/Hanks' salts until all groups were warmed. The oocytes were then transferred directly to IVM (17).

4.5. *In-vitro* maturation

After warming, the COCs were washed twice in Earl's salts (Gibco, 11150-059) maturation medium with 5 mU FSH/mL (Sioux Biochemical Inc., Sioux Center, IA), supplemented with 10% FBS, and 25 µg gentamycin/mL (Wisent INC, 450-134). 8-10 COCs were then cultured in a 100-µL medium drop in a 35-mm Petri dish covered with mineral oil (Sigma-Aldrich, M5310). For the preparation of the culture dish, three 50-µL droplets of maturation medium were placed in a 35-mm Petri dish; 3.5 mL of mineral oil was added to cover the droplets, and finally, 50 µL of the maturation medium was added to the droplets to

complete the 100-µL droplet (Figure 10). The oocytes were kept in the incubator at 38.5°C in 5% CO_2 atmosphere for 34 hours (9).

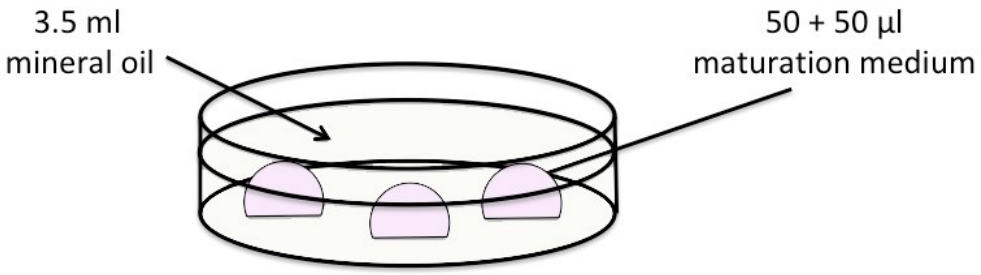

Figure 10. Schematic representation of the IVM plate preparation.

4.6. Assessment

4.6.1. Vitrification study 1: Fluorescent microscopy of oocytes

This study used fluorescence microscopy to evaluate the chromatin configuration of equine oocytes after *in-vitro* maturation. The COCs assigned to chromatin evaluation following maturation were denuded from cumulus cells by repeated pipetting with a fine glass pipette in 0.5% hyaluronidase (Sigma-Aldrich, H4272) solution with M199/Hank's salts. After the cumulus cells were removed, the oocytes were fixed for a minimum of 12 hours in a buffered formalin solution. The denuded oocytes were stained with Hoechst 33258 (Cayman chemical, 16756) and then placed on a slide with a drop of mounting medium (permafluor Thermo Fisher Scientific, 4-030-FM). A coverslip with petroleum jelly on the corners was placed on the top of the oocytes, and the edges were sealed with fingernail polish. The oocyte preparations were evaluated with a fluorescence microscope with a 365nm exciter filter (PALM MicroBeam Zeiss). The chromatin configuration of the oocytes was classified as GV (including stages from condensed chromatin through prometaphase I); metaphase I; metaphase II (including all stages from anaphase I through metaphase II); and degenerated (abnormal chromatin configuration such as multiple chromatin foci, hair-like strands of chromatin, chromatin distributed throughout oocyte, or no chromatin seen) (Figure 11) (40).

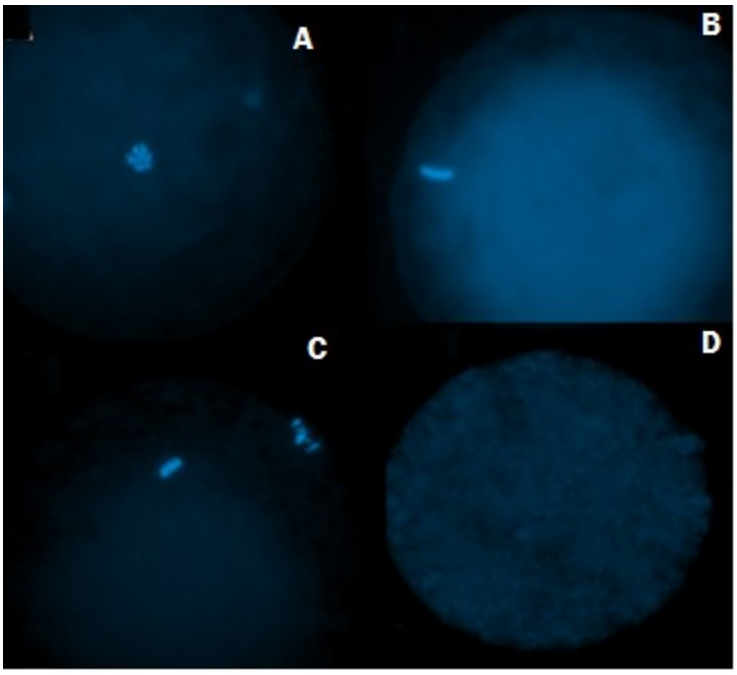

Figure 11. Chromatin configuration classification after Hoechst staining 40x. (A) GV; (B) MI: Metaphase I; (C) MII: Metaphase II; and (D) Degenerating.

4.6.2. Vitrification study 2: ICSI and embryo culture.

Following maturation, the oocytes were denuded of cumulus by pipetting in 0.05% hyaluronidase in 10% FBS + M199/Hanks' salts, and those with a polar body were held in Earle's medium and 10% FBS in an atmosphere of 5% CO_2 in air at 38.2°C before being used for ICSI with frozen-thawed sperm. The spermatozoa were prepared for ICSI via a swim-up procedure; 200 µl of thawed semen was layered under 1 ml of GMOPS (Vitrolife, Englewood, CO, USA) with 10% FBS and incubated at 38 °C for 20 min. After incubation, the medium's top 0.6 ml was collected and centrifuged at 327g for 3minutes. The supernatant was removed, and the sperm pellet was re-suspended in the remaining medium. The sperm immobilization droplet contained 7% PVP (7:3 ratio of 10% PVP solution (PVP, LifeGlobal, Guilford, CT, USA) and G-MOPS/FBS), and the motile sperm were identified after swimming down into the droplet. Sperm injection was carried out with the Piezo drill, with the stage warmer set at 32°C. The oocyte was held in separate 5-µL droplets of M199/Hanks' salts with 10% FBS. A motile sperm in the sperm droplet was aspirated tail-first into the injection pipette (7–8µm outer diameter). As the mid-piece entered the pipette, the sperm was immobilized by pulses of the Piezo as the pipette was moved so that the edge of the pipette made contact with the mid-piece. The sperm was

then drawn entirely into the injection pipette, and the pipette was moved to the oocyte droplet. The oocyte was positioned according to the polar body's location at either 6:00 or 12:00, depending on where we observed the clearest cytoplasm aspect closest to the holding pipette. With the second injection pipette, a piece of *zona pellucida* was removed under pulses from the Piezo drill. The sperm was approached to the tip of the injection pipette. The pipette was introduced through the hole in the zona, and it was advanced into the oocyte until it was close to the opposite side of the oocyte (i.e., advanced through ~80% of the oocyte diameter). The Piezo pulse broke the oolemma, and the sperm was ejected into the cytoplasm. Injected oocytes were held in 10% FBS + Earl's 5% CO_2 in air at 38.0 °C for 30 minutes and were then placed in an embryo culture medium.

After the post-injection holding period, the injected oocytes were cultured in a commercial human embryo culture medium (Global medium, LifeGlobal, Guilford, CT, USA) supplemented with 10% FBS, in droplets under oil, at a ratio of 5 µL medium per embryo, with a minimum of one embryo and a maximum of three embryos per droplet. The embryos were rinsed first in a 100-µL droplet of the same medium before being placed in the 15-µL droplet. They were then incubated in a humidified atmosphere of 6% CO_2, 5% O_2, and 89% N_2 at 38.2°C. On Day 5, cleavage was evaluated. Structures that were not single cells were considered cleaved embryos. Due to the relatively late day of cleavage assessment, some structures that were interpreted as being cleaved embryos may have undergone cellular fragmentation. Cleaved embryos were transferred to 15-µL droplets of DMEM/F-12 (Sigma-Aldrich) supplemented with 6 mL/L NaOH, 2.5 mM glycyl-glutamine (Sigma-Aldrich), and 10% FBS, under oil and cultured in an environment of 5% CO_2, 5% O_2, and 90% N_2 at 38.2°C. From day 7 to day 10, blastocyst development was evaluated. Presumptive blastocysts were fixed and stained as described above for oocytes on the day that they were recognized. Embryos were classified as blastocysts if they contained more than 64 nuclei, and they had started organizing the outer presumptive trophoblast cells (9).

4.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The dependent variables in this study were binomial (present-absent) and were expressed as percentages based on the total number of oocytes tested in each treatment. We compared each treatment to the control using Fisher's exact test. For each comparison, percentages were calculated over all replicates. Results are expressed as mean ± SEM of the replicates. Four replicates were performed in the first experiment and six replicates were performed in the second experiment. For the first experiment, the maturation rate was defined as the number of oocytes classified as MII divided by the number of oocytes evaluated after the maturation culture; for the second experiment, the maturation rate was defined by the number of oocytes with an intact plasma membrane and a visible polar body divided by the number of oocytes evaluated after the maturation culture. The cleavage rate was defined as the number of apparently cleaved embryos on Day 5 divided by the number of sperm-injected oocytes. The blastocyst rate was defined as the number of confirmed blastocysts divided by the number of sperm-injected oocytes. Values with a $P < 0.05$ were considered significant.

5. Results

5.1. Vitrification study 1. Effect of different vitrification protocols on maturation rate.

The vitrification protocols were designed based on the results of the supplementary data (M&M), which showed that immature equine oocytes could maintain viability and meiotic competence following vitrification through the use of a three-step vitrification protocol, composed of permeable and non-permeable CPAs; moreover, we found that the exposure of oocytes to the disaccharide sucrose gave better maturation rates, rather than the disaccharide trehalose.

Immature equine oocytes were used to evaluate the effect of two different combinations of CPAs (EG/PG vs. EG/DMSO) and five different treatments (VS-10, VS-20, VS-30, VS-40, and VS-50) on the maturation rate of vitrified-warmed equine oocytes. The maturation rate of VS-50 of both treatments EG/PG and EG/DMSO was significantly lower compared with control ($p<0.05$); moreover, VS-40 EG/PG treatment showed a significantly lower maturation rate compared with control (20% vs. 48% respectively, $p<0.05$), while VS-40 EG/DMSO treatment did not show significant difference with control (27% vs 48% respectively, $p>0.05$). The highest maturation rate observed in vitrified oocytes was 42% with VS10EG/DMSO treatment, as showed in figure 12.

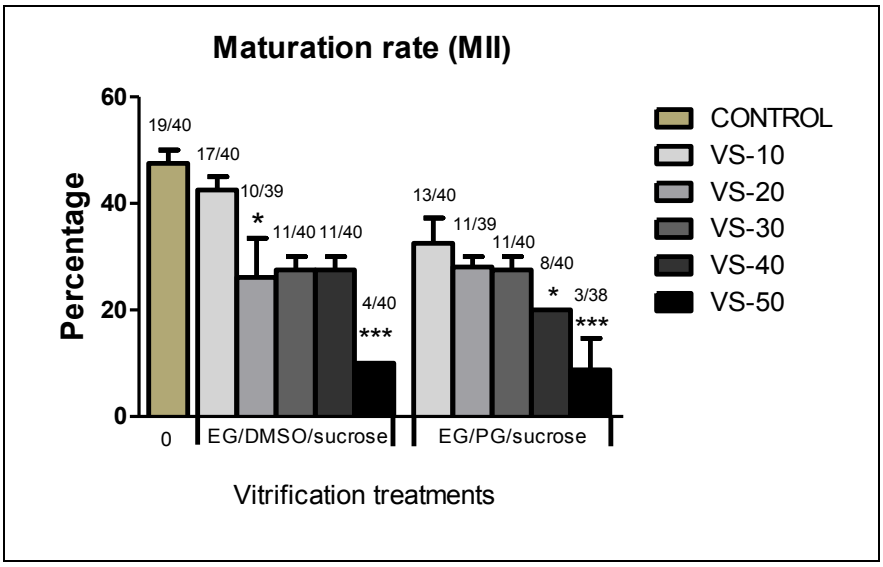

Figure 12. Maturation rate comparison between PG and DMSO, evaluating different times in the VS solution for each treatment. For each group, percentages with (*) are significantly different (*: $p < 0.05$, ***: $p < 0.001$) from control. No (*) indicates no significant effect of treatment compared with control.

5.2. Vitrification study 2. Effect of a vitrification protocol with freezing and without freezing on maturation, cleavage, and blastocyst rates.

According to the results from vitrification study 1, we selected the treatment with the highest maturation rate to evaluate maturation with polar body extrusion, cleavage and blastocyst rates. Total exposure of oocytes to CPAs without freezing did not affect the rate of maturation to MII using the three-step protocol with EG/DMSO/sucrose (50%) in comparison to the control (48%; $p>0.05$) as shown in table 4. However, no blastocysts were obtained in CPA-exposed treatment in contrast to 28% blastocyst in the control group. Furthermore, the maturation rates of the vitrified group did not differ significantly from the control (37% *vs* 48%; $p>0.05$). However, no blastocysts were obtained as for the CPA-exposed treatment. The morphological structure of oocytes after IVM is shown in figure 13, in which the oocyte of each treatment presents a complete *zona pellucida* and a polar body.

Treatment	Intact cytoplasm	MII	Cleavage	Blastocyst
Control	58% (30/52)	48% (25/52)	92% (23/25)	28% (7/25)
CPA-exposed	61% (34/56)	50% (28/56)	74% (20/27)	0% (0/27)*
Vitrification	57% (29/51)	37% (19/51)	65% (11/17)*	0% (0/17)*

Table 4. Rates of in-vitro maturation to MII with polar body extrusion, cleavage, and blastocyst formation after ICSI of vitrified-warmed and CPA-exposed immature equine COCs. For each concentration, percentages with (*) are significantly different ($P<0.05$) from control. No (*) indicates no significant effect of treatment compared with control.

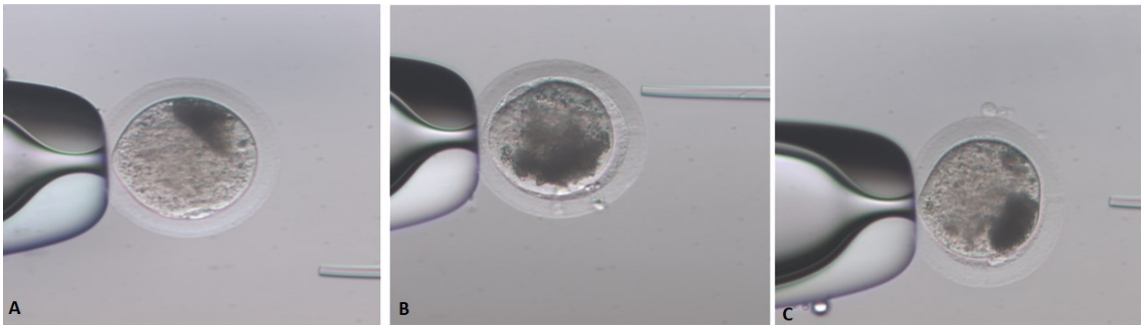

Figure 13. In-vitro *matured equine oocytes. A) Control, B) CPA-exposed, C) Vitrified.*

6. Discussion

6.1. Three-step vitrification protocol

According to previous literature, equine oocyte vitrification protocols are derived from empirical approaches that use techniques described in other species (22, 30, 168). During vitrification, the oocyte undergoes osmotic damage, chemical toxicity, and the formation of intracellular ice crystals. If the procedures are not optimally designed, it could reduce oocyte viability because of the irreversible plasma membrane damage and/or disruption of the organelles (182). Several statements have reported different efforts to improve the survival rate of vitrified equine oocytes through the resumption of MII after IVM and subsequent blastocyst development (17-20, 88).

For our first vitrification study, we designed a vitrification protocol following the most recent approaches cited in equine literature, including warming solution (17), maturation stage (9, 19, 20), interactions of cumulus cell with vitrified oocytes (17, 113), and concentration and duration of exposure of the PVS solution (17). Additionally, we introduced an extra step in our vitrification protocol, which has been previously reported for the vitrification of *in-vivo* matured equine oocytes (18). We added the non-permeable cryoprotectant at the concentration that gave us the best maturation rates in supplementary data M&M. Finally, we compared the combinations of permeable cryoprotectants used in previous studies of vitrification of immature equine oocytes: EG/PG and EG/DMSO (9), evaluating different timing exposures of the VS.

Ortiz- Escribano *et al*. (19) reported maturation rates similar to the control group when they used a combination of EG/DMSO/sucrose regardless of the length of the vitrification protocol (40s or ~1 min). The vitrification protocol achieved maturation rates not significantly different from the control group's rates, but only a 7% blastocyst formation rate in the 40s protocol. On the other hand, Canesin *et al*. (17) reported a short vitrification protocol with EG/PG/trehalose (1min, 45s total), achieving maturation rates of 20% of vitrified immature equine oocytes and 15% blastocyst formation, higher than previous studies on vitrified-warmed GV-stage equine oocytes. However, it is not possible to compare different results due to the many variables, such as the type and concentration of cryoprotectants, the vitrification device, and the warming protocol involved. Extensive research has been

conducted in humans (186) and mice (174) to determine the CPA concentration levels and exposure time needed for vitrification and maintenance of an ice-free state upon warming. However, the optimal time for such exposure in immature equine oocytes is not known. Our study supported the findings by Ortiz- Escribano *et al*. (19) that immature equine oocytes can survive the vitrification process using a high concentration of CPAs and a brief exposure (less than 1 minute total) to the vitrification solutions. In the first experiment, the total time of exposure of oocytes to the three vitrification solutions went from around 90 seconds to 150 seconds. The maturation rates appeared to be significantly lower compared to control when oocytes remained in the VS solution for more extended periods for both the EG/PG and EG/DMSO combinations.

Analysis of subsequent embryo development following ICSI is the most common method used to assess the success of cryopreservation procedures. Only four studies have reported embryo development after vitrification of immature equine oocytes (9, 17, 19, 20). However, the efficiency of blastocyst production for those using a combination of EG/DMSO was low (9, 19), leading to the conclusion that with this system, cooling injury was the main factor reducing development competence. Based on the results of the first vitrification study, we decided to evaluate the treatment with the most similar maturation rate to the control group (EG/DMSO/sucrose VS-10), and we compared the effect of exposure of equine oocytes to CPAs (EG/DMSO/sucrose) in a vitrification protocol, with and without freezing, on development competence. These results showed that the vitrification and the cryoprotectant exposure had significant detrimental effects, resulting in no formation of blastocyst after ICSI.

Fracture damage occurs because of the mechanical effect of ice crystal formation during freezing, especially in relatively large cells such as oocytes, associated with damage to the plasma membrane and producing the destruction of the cell (182). Furthermore, during cryopreservation, there is a volumetric response to hypertonic conditions, in which the cells shrink in response to differences in osmotic pressure between intracellular and extracellular solutions. This shrinking can lead to cytoskeleton damage and fracture of the *zona pellucida* because of cell shape changes (17). In our work, we attempted to protect the immature oocytes from cryoinjury and osmotic shock for successful embryo development by incorporating several steps in our vitrification protocol. Firstly, with the study reported in the literature on volume dynamics in vitrified equine oocytes denuded

until *corona radiata*, in which 40 seconds was the optimum time for PVS (17); moreover, we used the concentration of PVS and VS based on previously published studies in equine protocols: 5% (18), 10% (18-20) and 17.5% (9). We explored the type and concentration of the non-permeable CPAs and type of permeable CPAs more suitable for IVM of immature oocytes (sucrose 0.3 M and EG/DMSO respectively); we attempted to increase the cooling rate by adopting a carrier system such as a stainless mesh that enables the oocytes to be loaded into a minimum drop volume (17, 187). Finally, we used a three-step vitrification protocol to balance the osmotic shock and toxicity of CPAs by shortening the duration of exposure of equine oocytes to a VS with a total duration of 90 seconds (PV1 40s/PV2 40s/VS 10s). We believe that we could avoid ice crystal formation and osmotic shock because oocytes maintained their morphological integrity, including membrane and *zona pellucida*. Besides, the maturation rate was not significantly different than control in the vitrified group. Nonetheless, we could not avoid the toxicity of CPAs, as evidenced by the failure to observe blastocyst formation in the treatment of only CPA exposure without freezing. Therefore, a three-step vitrification protocol of immature equine oocytes using DMSO increased oocyte survival rates and improved the frequency of meiosis resumption in comparison to the three-step PG protocol but did not result in high rates of post-fertilization development of equine embryos.

Improving the development competence of oocytes subjected to freezing and warming requires careful selection of less toxic cryoprotective agents. The use of rigorous optimization methods to design CPA addition protocols that simultaneously minimize CPA toxicity and osmotic shock may increase blastocyst formation after the fertilization of vitrified equine oocytes. Mouse experiments have also demonstrated that it should be possible to immerse oocytes into CPAs in less than 90 seconds (188). Short protocols have shown positive results in mouse oocytes, which could be loaded with DMSO in a remarkably short time and recovered very well (188, 189). Thus, in our first study, we expected that rapid CPA loading processes would significantly improve the cryopreservation outcome of equine oocytes by minimizing exposure to potentially toxic CPAs while limiting volume changes as well. However, we still had a markedly detrimental effect with the combination of EG/DMSO in the three-step vitrification protocol of 90 s total duration. To the best of our knowledge, no study has investigated the effect of the exposure of immature equine oocytes on blastocyst development after ICSI featuring a

three-step short vitrification protocol using EG/DMSO with and without freezing to reduce the osmotic shock and toxic effect of cryoprotectants.

6.2. Potential causes for experimental failure

Vitrification techniques were made possible with the use of high concentrations of DMSO, EG, and PG, as previously described (94, 100, 136, 172, 188). Oocyte physiology changes among the different species have been reported after cryopreservation, including perturbation of the intracellular calcium flux (145) and high rates of aneuploidy caused by meiotic spindle disruptions (140, 190), even in horses (20). Vitrification solutions composed of combinations of CPAs, principally EG/DMSO, are the most common types of solutions used for oocyte vitrification in humans and animals. Pedro *et al.* (104) suggested that EG is less toxic because of its lower permeability. Still, there is no information about the specific osmotic or toxic effects of each kind of cryoprotectant on the development competence of equine oocytes. Moreover, the combination of CPAs in vitrification solutions reflected increased protein destabilization tendencies because the total sum of CPAs might have a cumulative denaturing effect (191). In these experiments, although survival and maturation rates were similar to those of the control group, the systems used reflected a high level of toxicity, as evidenced by the observation that the oocytes that were not frozen and only exposed to CPAs stopped their development competence after cleavage.

Although meiotic spindle abnormalities have been observed after the warming of vitrified oocytes (20), a report in mice showed that the metaphase II oocyte spindle returned to conventional configuration with typical spindle and chromosome configurations after post-warming incubation (186). In our second vitrification study, the oocytes were drastically affected, as demonstrated by the complete absence of blastocyst formation after ICSI in both treatments, exposure to CPAs, and vitrification using EG/DMSO. In these results, despite the oocytes had maturation rates similar to control, development stopped after cleavage in both CPA exposure and vitrification treatments. The major concerns related to spindle damage in oocyte freezing are linked to the use of mature equine oocytes (20, 113, 182). Thus, attempts have been made to freeze immature equine oocytes in which the meiotic spindle is not yet formed. Vitrification of oocytes at the GV stage may preserve their ability to reach MII after IVM. Nonetheless, immature equine oocytes undergo significant damages during cryopreservation. Overall, equine oocytes have achieved

similar MII rates with our vitrification protocol compared to rates reported in the literature (42% vs. 21−46%, respectively) (17, 19, 20, 182). The successful vitrification protocol remains a question, as more than 50% of oocytes reaching the MII stage have exhibited spindle abnormalities and low developmental competence (113). Extensive spindle morphology and chromosome alignment evaluation are necessary to explain CPAs' toxicity in equine oocytes.

Studies have demonstrated that exposure to penetrating CPAs (including DMSO, PG, and EG) causes a transient rise in intracellular Ca++, which induces premature cortical granule exocytosis (144). It is also known that CPA exposure will lead to the hardening of the *zona pellucida* because of premature cortical granule exocytosis, producing fertilization failure (192). In other species, data indicate that the use of ICSI solves the effects of zona hardening to permit sperm access to the plasma membrane and fertilization (15, 145, 146). In the literature, the fertilization of vitrified equine oocytes has been accomplished by ICSI; however, the blastocyst rate formation was significantly lower than untreated oocytes (9, 17-20). In the second experiment, oocytes were fertilized by ICSI, but the groups exposed to CPA (vitrified oocytes and only CPA exposure) did not show blastocyst formation. It has also been reported that a massive increase in calcium might be detrimental for further embryo development due to the effect on cell cycle proteins and activating apoptosis (131). More studies are necessary to determine the causes that produce such adverse effects on the development of embryo after fertilization of vitrified equine oocytes.

7. Perspective

To date, attempts to develop cryopreservation procedures for equine oocytes have been based primarily on empirical approaches. Further information on biological aspects of equine oocytes and their interactions with the different solutions used for cryopreservation are required to predict optimal freezing protocol; for example, the ultrastructural evaluation of spindle, cytoskeleton, organelles, and cumulus cells communications. According to the present results, one of the most critical steps of oocyte vitrification is the concentration of permeating CPAs, such as DMSO and EG, resulting in severe perturbations in the cell of the CPAs toxicity. Consequently, the optimization of CPA loading procedures is important for maximizing the probability of success in oocyte cryopreservation.

It has been claimed that the first step should be the ‘‘vitrification solution effect’’ test to examine the effect of solution toxicity (87). Oocytes might be exposed to vitrification and warming solutions without undergoing the freezing process. In equine oocyte vitrification, although it has been recognized that membrane transport models are useful to guide the design of CPA loading methods (17), extensive studies in toxicity due to prolonged CPA exposure have not been considered. Outcomes used to assess the success of a given protocol have typically been survival, fertilization, and in some cases, embryo development (9, 17-19). It is important to identify any similarities or differences in treated oocytes compared to control to establish objective parameters for evaluating oocyte quality to develop and optimize a vitrification protocol that allows oocytes to be successfully fertilized (193). For example, chromosomal abnormalities and ultrastructural damage are one of the main adverse effects of cryopreservation due to the toxic effects of CPAs; therefore, detailed evaluation through fluorescence staining parthenogenetic activation has been suggested (113, 194). Also, transmission electron microscopy has been reported to be a valuable research tool that can be used to determine an oocyte’s cytoplasmic maturation status; this includes oocyte shape and dimension, *zona pellucida* texture, perivitelline space, appearance, oolemma integrity and density, mitochondria, mitochondria-smooth endoplasmic reticulum aggregate and mitochondria-vesicle complex number, and the presence of ooplasmic vacuolization (193). Moreover, vitrification solutions may also result in changes at the molecular level in oocytes. Therefore, heat shock proteins and manganese superoxide dismutase are two critical factors related to stress that can be evaluated (94).

The second step is to evaluate the different cryo-devices to load oocytes into a minimum drop volume with the selected most suitable solution for successful vitrification (87). A delicate balance must be maintained among CPAs to ensure successful vitrification; moreover,attention should be paid in methods to introduce and remove cryoprotectants to reduce the toxicity and ice crystal formation. Consequently, protocols that require maximal cell volume changes at a rate that the cells can tolerate should be used to reduce cellular membrane damage (195). Finally, we consider that the final step for evaluating a successful vitrification protocol is the survival and development of high-quality embryos.

8. Conclusion

The three-step vitrification protocol with EG/DMSO did not affect the acquisition of meiotic competence by immature equine oocytes compared to control. However, vitrified oocytes with EG/DMSO did not develop to the blastocyst stage. Moreover, cryoprotectant exposure drastically affected blastocyst formation negatively. Therefore, maturation to MII alone is not a useful indicator of the effectiveness of a vitrification protocol. Further investigation into the overall physiology of equine oocytes and their interactions with CPAs necessary to optimize the developmental capacity of embryos.

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10. Appendix Preliminary studies

10.1. Methodology

10.1.1. Preliminary study 1: The effect of different concentrations of two disaccharides on maturation rates.

COCs were collected from equine ovaries obtained from a slaughterhouse, as described in the oocyte collection section. Groups of 10 oocytes with three to four layers of *corona radiata* cells were exposed to three different concentrations of either trehalose (Sigma Aldrich, T0167) or sucrose. Two timings were tested based on the concentrations: 0.3 M for 30s, 0.5 M for 30s, and 0.65 M for 20s. The oocytes were placed in a 100-µL droplet of each disaccharide according to each timing. They were then immediately rinsed in a 100-µL droplet of M199/Hanks' salts + 10% FBS for the same schedule and transferred into a maturation culture. For the control group assessment, 10 oocytes were exposed to a 100-µL drop of M199/Hanks' salts + 10% FBS for 30 seconds and rinsed in an100-µL drop of M199/Hanks' salts + 10% FBS for 30s and they were then transferred to IVM. COCs were randomly allocated to the disaccharide treatments and then transferred to IVM. The rates of maturation to MII were compared, as described in the *in-vitro* maturation section.

10.1.2. Preliminary study 2: Effect of the results of various exposure times and concentrations for sucrose on oocyte survival and maturation rates.

All procedures were carried out at room temperature. COCs were collected from equine ovaries obtained from a slaughterhouse, as described in the oocyte collection section. Groups of 10 oocytes with three to four layers of *corona radiata* cells were exposed to three different concentrations of sucrose: 0.25 M, 0.5 M, 0.75 M, and 1 M. Three durations were tested for each concentration: 20s, 40s, and 60s. The oocytes were then transferred to 100-µL droplets of sucrose and were immediately rinsed in 100-µL droplets of M199/Hanks' salts + 20% FBS for 60 seconds. They were then transferred to a holding medium composed of M199/Hanks' salts + 20% FBS. After all treatments were performed, the oocytes were transferred to a maturation culture. For assessment of the control group, 10 oocytes were exposed to 100-µL droplets of M199/Hanks' salts + 20% FBS for 40 seconds and rinsed in 100-µL droplets of M199/Hanks' salts + 20% FBS for 60 seconds

and they were then transferred to IVM. The rates of maturation to MII were compared among groups, as described above.

10.1.3. Preliminary study 3: Effect of different warming temperatures with only non-permeable cryoprotectants on the maturation rates.

COCs were collected from equine ovaries obtained from a slaughterhouse, as described in the oocyte collection section. The excess cumulus cells surrounding the oocyte were then removed through pipetting until approximately four layers of cells surrounded the oocyte.

The vitrification procedure was initiated by transferring 8 COCs into 200-µL droplets of vitrification solution composed of 0.75 M sucrose diluted in 20% FBS. It was considered that oocytes could achieve their maximum dehydration rate with only the maximum concentration of sucrose reported in our last experiments, which resulted in the optimal maturation rate. The COCs were held for 60 seconds in a sucrose solution. They were then transferred in 20 µL of a medium onto a sterile stainless-steel 75-um mesh (1x 0.5 cm). The underside of the mesh was then placed on a sterile absorbent paper to remove the excess medium, and the mesh was immediately plunged into LN_2 and placed inside a cryovial under LN_2. The cryovial was capped and kept in LN_2 until warming.

For this experiment, oocytes were divided into two treatments during the warming process. The first half was warmed at room temperature and the other half at 38°C. The cryovial containing the vitrification mesh was removed from the storage tank and placed in LN_2 in a styrofoam container positioned on the work surface near the microscope. The cryovial was opened under LN_2, and the mesh was removed from the vial and quickly placed in a 35-mm Petri dish of 20% FBS + M199/Hanks' salts at either 38°C or room temperature, and then transferred to IVM.

10.1.4. Preliminary study 4: Effect of different timings on the final vitrification solution, comparing EG, PG, and sucrose with EG, DMSO, and sucrose.

COCs were collected from equine ovaries obtained from a slaughterhouse, as described in the oocyte collection section. After the denuding process, the COCs were placed in a BS composed of 20% FBS + M199/Hanks' salts. 10 oocytes were then transferred to a

35-mm polystyrene Petri dish containing a 150 µL droplet of either 5% EG/DMSO or 5% EG/PG pre-vitrification solution 1 (PVS1 DMSO or PVS1 PG) diluted in M199/Hanks' salts + 20% FBS. The COCs were held in PVS1 for 40 seconds. The COCs were then transferred to a 150 µL droplet of PVS2 DMSO, PG composed of 10%EG+10%PG, or DMSO diluted in M199/Hanks' salts + 20% FBS. They were held for 40 seconds. Finally, the COCs were transferred to a vitrification solution (VS), composed of 17.5% EG + 17.5% PG or DMSO + 3 M sucrose diluted in M199/Hanks' salts + 20% FBS for different amounts of time depending on the treatment (see table 5). For the assessment of control group, 10 oocytes were transferred to IVM without freezing or exposure to CPAs.

GROUP:	CONC. (%) PV1	TIME (S) PV1	FINAL CONC. (%) PV2	TIME (S) PV2	CONC. (%) VIT	TIME (S) VIT
EG/PG 1	5	40	10	40	17.5	30
EG/PG 2	5	40	10	40	17.5	40
EG/PG 3	5	40	10	40	17.5	50
EG/PG 4	5	40	10	40	17.5	60
EG/PG 5	5	40	10	40	17.5	70
EG/DMSO 6	5	40	10	40	17.5	30
EG/DMSO 7	5	40	10	40	17.5	40
EG/DMSO 8	5	40	10	40	17.5	50
EG/DMSO 9	5	40	10	40	17.5	60
EG/DMSO 10	5	40	10	40	17.5	70
CONTROL	0	0	0	0	0	0

Table 5. Treatments of preliminary study 4.

After exposure to the solution, the COCs were transferred in a droplet of VS onto a sterile stainless-steel 75um mesh (1x 0.5 cm). The underside of the mesh was then placed on sterile paper to absorb the excess medium, and the mesh was immediately plunged into LN_2 and placed inside a cryovial under LN_2. The cryovial was capped and kept in LN_2 until warming, as described in the warming section.

10.1.5. Preliminary study 5: Effect of oocyte manipulation after vitrification, comparing EG, PG, and sucrose with EG, DMSO, and sucrose.

COCs were collected from equine ovaries obtained from a slaughterhouse, as described in the oocyte collection chapter. The vitrification protocol was the same as the previous experiment, comparing the groups, as described in the table below. For the warming process, oocytes were warmed at 38°C. The cryovial containing the vitrification mesh was removed from the storage tank and placed in LN_2 in a Styrofoam container positioned on the work surface near the microscope. The cryovial was opened under LN_2, and the mesh was removed from the vial and quickly placed in a 35-mm Petri dish of 20% FBS + M199/Hanks' salts at 38°C; and then depending on the group, oocytes were placed in a four-well dish of 20% FBS + M199/Hanks' salts for 2 hours at room temperature or transferred directly to IVM. After 2 hours, the oocytes were transferred to IVM (see table 6). Control groups were not exposed to freezing or CPAs.

GROUP: EG/PG	CONC. (%) PV1	TIME (S) PV1	FINAL CONC. (%) PV2	TIME (S) PV2	CONC. (%) VIT	TIME (S) VIT	TIME AT RT BEFORE IVM (H)
EG/PG 1	5	40	10	40	17.5	30	2
EG/DMSO 2	5	40	10	40	17.5	40	2
EG/PG 3	5	40	10	40	17.5	30	0
EG/DMSO 4	5	40	10	40	17.5	40	0
CONTROL5	-	-	-	-	-	-	2
CONTROL6	-	-	-	-	-	-	0

Table 6. Treatments of preliminary study 5.

10.2. Results

10.2.1. Preliminary study 1: The effect of different concentrations of two disaccharides trehalose and sucrose on maturation rates.

Preliminary study 1 was conducted to determine the effect of different concentrations of trehalose and sucrose on meiotic and developmental competence of equine oocytes in

IVM. The goal of the study was to establish the best choice of sugar and its concentration for use as a base medium for vitrification.

The rates of maturation of oocytes exposed to sucrose were generally higher than the rate for those exposed to trehalose, considering 0.3 M and 0.65 M treatment (40% and 45% vs. 31% and 17%, respectively). However, within sucrose exposure, the maturation rate for 0.5 M was significantly lower than that of the control ($P<0.05$), whereas the 0.3 M and 0.65 M were not. Oocytes in the sucrose 0.65 M treatment had the highest MII rate (45%) of the different treatments, as showed in figure 14.

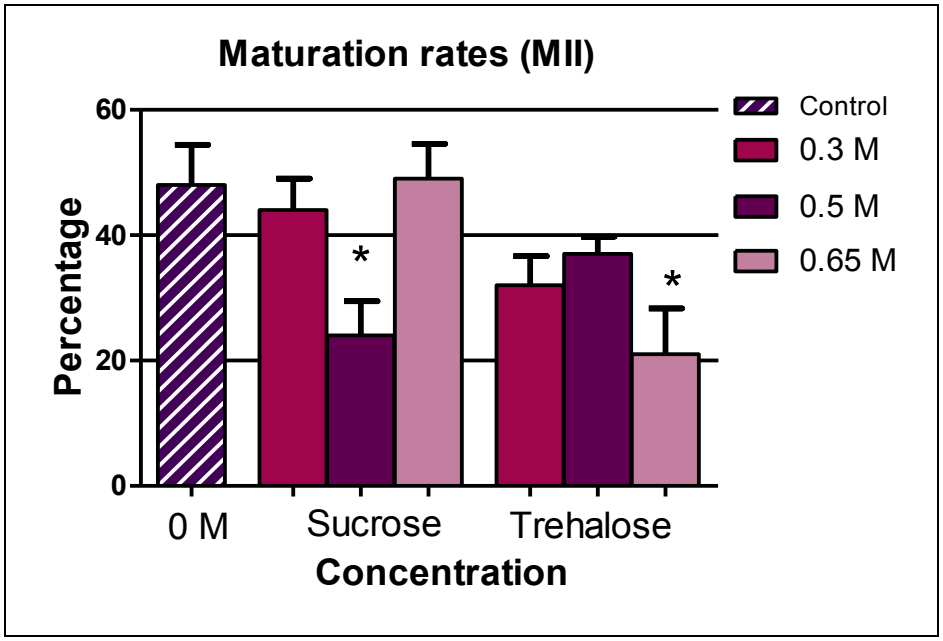

Figure 14. Maturation rate comparison between two different non-permeable cryoprotectants (sucrose and trehalose). For each concentration, percentages with (*) are significantly different ($P<0.05$) from control using Fisher's exact test. No (*) indicates no significant effect of treatment compared with control.

10.2.2. Preliminary study 2: Effect of the results of various exposure times and concentrations on oocyte survival and maturation rates.

Based on the results of the first preliminary study, sucrose was determined to be the best non-permeable cryoprotectant because of the maturation rate of the equine oocyte. A second preliminary study was then performed to look closer at the effect of sucrose at

several concentrations and durations on the meiotic competence of equine oocytes to establish the most effective concentration and timing for vitrification.

Although there was no significant change between the groups, 0.5 M displayed a marked decrease in maturation. Moreover, the highest time exposure, 60 seconds with 0.5 M, reflected a significant decline compared to the control (19% vs. 44%, P<0.05). In general, 0.25 M had a better maturation rate (39-47%), but it was not significant compared to the other groups, including the control. The maturation rate of the control group was 44%, as showed in figure 15.

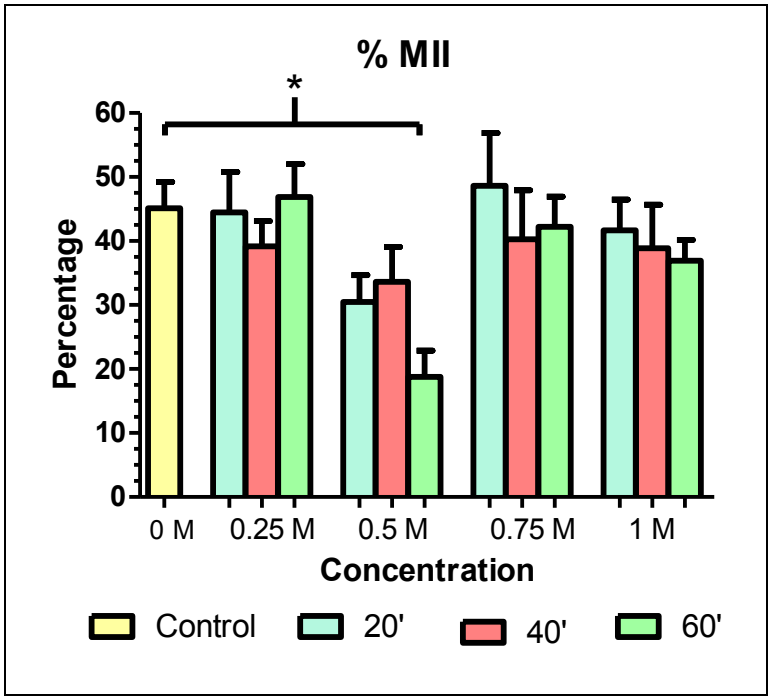

Figure 15. Maturation rate comparison between levels of concentration with sucrose exposure and different time exposures. For each concentration, percentages with (*) are significantly different (P<0.05) from control using Fisher's exact test. No (*) indicates no significant effect of treatment compared with control

10.2.3. Preliminary study 3: Effect of different warming temperatures with only non-permeable cryoprotectants on the maturation rates.

Based on the last results, we decided to use the best sucrose concentration to design a vitrification protocol with only non-permeable cryoprotectants. But this experiment only

showed the survival of one oocyte in a warming temperature of 38°C, which showed a GV stage, but did not develop into MII (see table 7).

Warming temperature	N	Maturation rates			
		GV	MI	MII (%)	Deg.
38°C	100	1 (1%)	0 (0%)	0 (0%)	99 (99%)
Room temperature	105	0 (0%)	0 (0%)	0 (0%)	105 (100%)
Control	30	3 (10%)	1 (3%)	13 (43%)	13 (43%)

Table 7. Rates of in-vitro maturation to metaphase II of vitrified-warmed oocytes.

10.2.4. Preliminary study 4: Effect of different timings on the final vitrification solution, comparing EG, PG, and sucrose with EG, DMSO, and sucrose.

Because of the failure of preliminary study 3, we decided to evaluate the effect of EG/DMSO and EG/PG combinations in a short three-step protocol. This experiment resulted in 10 vitrification treatments, in addition to the control group. In general, the MII maturation rate was higher for DMSO than for all PG groups, with the exception of the 70s group. While DMSO displayed a progressive decrease in maturation rate relative to the time in VS with the best groups in the 30s and 40s (22 and 24% respectively), PG did not reflect the same pattern, presenting the best groups in 40s and 70s (21-18% respectively). The maturation rates for all vitrification treatments (0-24%) were lower than for the control group. Although the maturation rate was not significantly lower for groups DMSO 30s and 40s and PG 40 and 70s compared with the control group ($P>0.05$); the maturation rate for the control was lower compared to all previous experiments (Preliminary study 1-3, in addition to the not reported data).

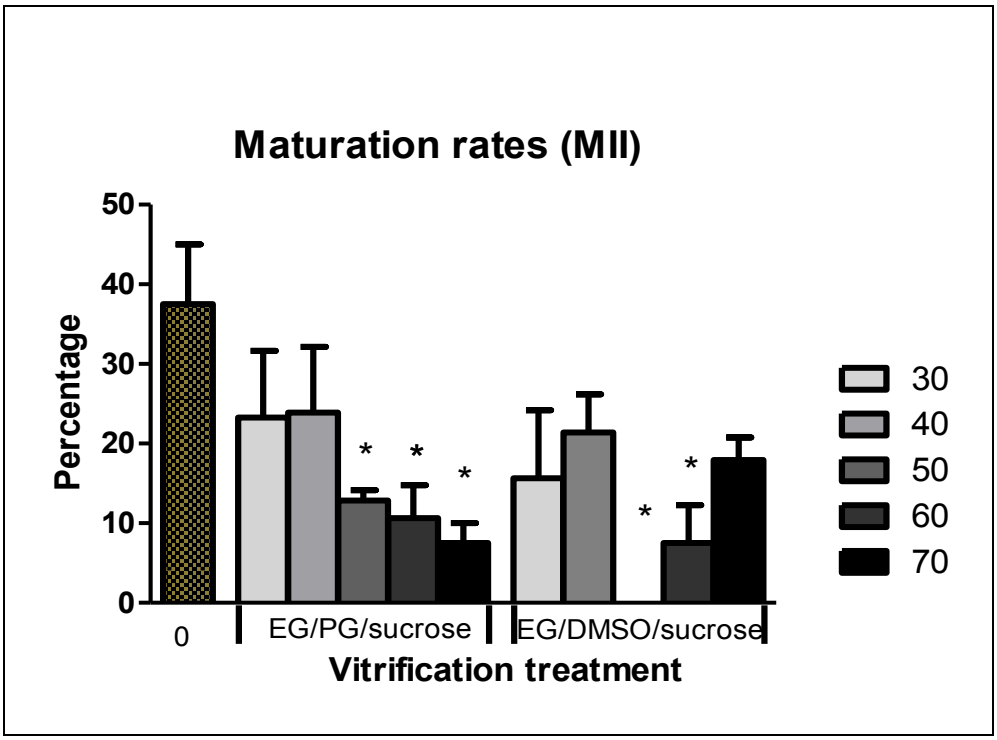

Figure 16. Maturation rates comparison between two different vitrification treatments, evaluating different time exposure in the VS of each treatment. For each group, percentages with (*) are significantly different (P<0.05) from control using Fisher's exact test. No (*) indicates no significant effect of treatment compared with control.

10.2.5. Preliminary study 5: Effect of oocyte manipulation after vitrification, comparing EG, PG, and sucrose with EG, DMSO, and sucrose.

Because of the results in the previous experiments in the control group, we aimed to evaluate the effect of vitrification on equines oocytes, comparing oocytes transferred directly to IVM after warming and transferred after 2 hours of being at room temperature.

A clear effect was observed between 0 and 2 hours on the maturation rate of vitrified equine oocytes. In all groups, including the control, there was a decrease in the maturation rate of oocytes transferred immediately to IVM after warming. The maturation rate at 0 hours for the control group was lower (32.5%) than the control group at 2 hours (42%). The maturation rate of the DMSO 0-hour group decreased significantly compared with the 2 hours DMSO group ($p<0.05$). In contrast, oocytes vitrified with PG did not showed significant difference between the treatments 0-hour and 2 hours, as illustrated in figure 17.

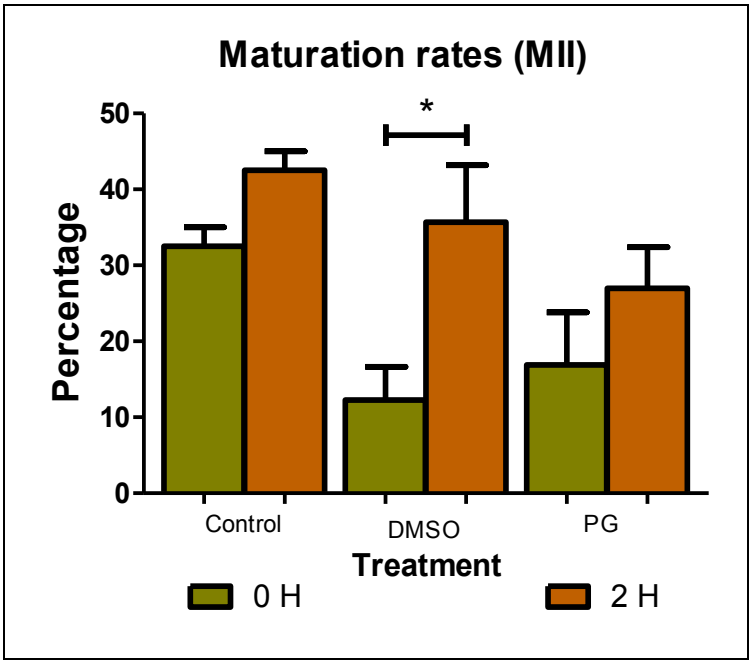

Figure 17. Maturation rate comparison between two different treatments (0h and 2h) with either DMSO or PG vitrification protocols. Percentages with (*) are significantly different ($P<0.05$) using Fisher's exact test. No (*) indicates no significant effect between treatments 0 h and 2 h.

10.3. Discussion.

Preliminary experiments were designed to investigate important characteristics of immature equine oocytes and standardize a vitrification protocol for immature equine oocytes. Base on the best results of literature, we chose immature oocytes, denuded until corona radiata, and a holding medium Vigro® to keep the oocytes overnight after the collection for all experiments (9). The first step was to determine the best type and concentration of non-permeable CPAs for immature equine oocytes. Historically, protocols for equine oocyte vitrification have derived from empirical approaches used in other species. As a result, equine oocyte protocols have used trehalose (17) or sucrose (19, 20, 113, 157, 182) at a concentration of 0.3 M and 0.5 M, respectively as cryoprotectants. But equine vitrification protocols have resulted in a low blastocyst rate. Therefore, in the first preliminary study, we exposed immature oocytes to different concentrations and timings of two disaccharides, and differences in the meiotic maturation between treatments were found. Trehalose was found to have a generally lower maturation rate than sucrose.

However, sucrose 0.5 M had a significantly lower maturation rate than 0.3 M and 0.65 M. The low maturation rate was consistent in the next experiment, which compared more timings and sucrose concentrations. Our results indicate that immature equine oocytes tolerate exposure to sucrose solutions ranging between 0.25 M and 1 M without significant effects on their maturation rate, except for 0.5 M, which was detrimental for the maturation rate. Furthermore, the duration of exposure is not a variable that affects oocyte maturation concerning non-permeable cryoprotectants. To our knowledge, there have not been any reports on the effects of dehydration using high concentrations of saccharides before the vitrification of immature equine oocytes.

In the first experiments, we did not find the basis of the different changes among treatments. We considered existing theories to explain the cryotolerance and developmental competence following osmotic, heat, or oxidative stress induced by permeable and non-permeable CPAs. When cells are subjected to various stress factors, they increase heat shock proteins (190). The exact function of these proteins in oocytes is still unclear, but it has been found that they are involved in the protection against apoptosis and ATP metabolism induced by a variety of stimuli (196). These preliminary studies with disaccharides demonstrated that the induction of osmotic stress with non-permeable CPAs caused significant changes, affecting the meiotic competence of IVM in equine oocytes. However, further investigations are needed to evaluate stress protein expression to select suitable CPAs and concentrations. Based on our results, we set sucrose as the non-permeable cryoprotectant in further vitrification protocols.

As previously stated, vitrification solutions generally contain high CPAs concentrations, increasing the osmotic stress with potentially chemically toxic effects on the cell. The effect of CPAs is shaped by several factors, such as the vitrification device used and the membrane permeability of the cell and the species (19, 33, 80). The addition of sugars to a vitrification media can influence the vitrification properties of the solution and assist in stabilizing the membrane structures because disaccharides act as osmotic buffers, reducing the osmotic shock and the toxicity of non-permeable CPAs by decreasing the concentration required to achieve successful cryopreservation (90, 99, 175). Additional mechanisms may also play a role in the cryoprotection offered by these two compounds. For example, trehalose has been reported to have a beneficial effect on the cryo survival of human oocytes after being microinjected into cells (197), and trehalose

supplementation of the maturation medium has stabilized cell membranes during vitrification and the warming of oocytes (178).

Since equine oocytes are quite sensitive to non-permeable CPAs, we decided to vitrify immature equine oocytes without permeable cryoprotectants. The vitrification of mouse oocytes without permeable cryoprotectants resulted in high blastocyst rates of 70%, demonstrating the ability of oocytes to survive vitrification (90). Our results did not lead to any survival of vitrified oocytes with only sucrose as the cryoprotectant. Although we prepared the base media using disaccharides, macromolecules (FBS), and the buffer medium M199, the ultra-rapid warming rate with a laser pulse was not available. Further studies on the topics of osmotic tolerance, warming rate, and warming solution are needed in order to optimize a vitrification protocol with only disaccharides.

Consequently, for the following protocols, two permeable and one non-permeable cryoprotectant were added to the vitrification media to optimize the vitrification protocols, which increased the survival of vitrified equine oocytes (17, 19, 182). For the design of our vitrification protocol, we used immature equine oocytes whose cumulus cells had been removed until *corona radiata*. We exposed the oocytes for 40 seconds to both PVS solutions because Canesin *et al*. (17) identified 40 seconds for PVS as the optimum time for immature equine oocytes denuded until *corona radiata* to be exposed to a PVS. Finally, we used the cryo-device and the warming solution described in previous literature, which has resulted in the highest rates of blastocyst formation after the vitrification of equine oocytes (9).

The preliminary studies demonstrated that immature equine oocytes could maintain viability and meiotic competence following vitrification through the use of a three-step vitrification protocol composed of permeable and non-permeable CPAs. However, the first vitrification technique had a markedly detrimental effect on meiotic competence after IVM, characterized by a low maturation rate with both combination of cryoprotectants, EG/PG and EG/DMSO. The total exposure of oocytes in the three vitrification solutions went from around 110 seconds to 150 seconds. In our studies, even though the 30s and 40s groups in VS did not display significant differences compared to the control, the maturation rates of the control groups were lower than previous experiments (35% vs. 50%, respectively),

confirming the difficulty in establishing vitrification protocols. With this data, we noticed that each detail of oocyte manipulation plays an important role in oocyte quality.

It is essential to optimize vitrification protocols to determine the variables that could be more detrimental for oocyte development. Because of the lower maturation rate obtained compared with previous experiments in the control group, we aimed to evaluate the effect of vitrification on equine oocytes, comparing oocytes transferred directly to IVM after warming and transferred after 2 hours of being at room temperature. Therefore, we decided to look deeper into the manipulation of oocytes. We found that the maturation rate of oocytes can often be compromised when they are placed in the incubator while the door is kept open. This also likely affects the CO_2 in the atmosphere and the temperature in the incubator and results in pH modification, affecting the maturation rates and blastocyst formation (45).

With this last result, we decided to modify our general protocol. We kept the oocytes at room temperature until the end of the vitrification experiments (~2 h), instead placed each group immediately in the incubator after vitrification. This is an important step for the management of a technique and establishment of successful protocols.