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Microenvironment factors promoting the quality of vitrified cat oocytes

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ABSTRACT

In oocyte cryopreservation programs, vitrification has overthrown conventional slow freezing both in veterinary and human medicine. In animals, its feasibility in field conditions makes it the preferred technique for the safeguard of genetic resources from zoo or wild animals, including threatened felids, for which the domestic cat is an excellent model. However, many cellular injuries, such as cytoskeleton, mitochondria and meiotic spindle alterations, DNA damage, zona pellucida hardening and cumulus cell loss, might occur following vitrification. After warming, although the exact mechanisms are still unclear, degeneration is a frequent outcome for cat vitrified oocytes. For immature (germinal vesicle) gametes, in vitro maturation after warming is a challenge, and cleavage after fertilization barely reaches 15–30%, while for mature (metaphase II) cryopreserved gametes it can get to 30–50%. Anyway, the progression to late embryos stages is often impaired, and improvements are needed. Standard cryopreservation protocol and the use of conventional in vitro culture systems after warming may not be enough for vitrified oocytes to recover and demonstrate their full developmental potential. Physical or chemical factors applied to oocytes undergoing vitrification, as an enrichment to the vitrification step, or to the culture microenvironment, could create more favorable conditions and promote vitrified oocyte survival and development. From the use of three-dimensional culture systems to the regulation of metabolic activities and cellular pathways, this review aims to explore all the possibilities employed so far, including the studies performed by our own lab, and the future perspectives, to present the most effective strategies for cat oocyte vitrification and the best time for their application (i.e., before, during, or after vitrification-warming).

1. Introduction

Besides allowing an improvement of animal reproductive efficiency, assisted reproductive technologies (ARTs) have become an important tool for biodiversity preservation [1,2]. More and more species are threatened with extinction or experiencing a decrease in population size. Germplasm cryopreservation, and especially gamete cryopreservation, has become a crucial part of biodiversity preservation programs, since it allows to store genetic material for future use and to plan breeding with animals that are distant both in time and space. Among the most endangered mammalian taxa are the felids, and for some of them, such as the Iberian lynx, some conservation programs have already been implemented successfully [3,4]. However, assisted reproduction protocols are not efficient for most endangered species [5] and the use of closely related domestic species is useful to design specific procedures to be transferred to the threatened ones. For felids, the domestic cat is an excellent model [6].

Among germplasm cryopreservation options, gamete banking is probably the one that offers the best balance between efficiency and flexibility. Gonadal tissue preservation could offer the chance to store abundant germ cells, but it is still technically challenging, especially with regards to development of such gamete precursors into mature gametes after thawing, either in vivo post grafting or in vitro after culture [7]. Instead, gamete banking already proved to be successful, to some extent. In addition, storing male and female gamete separately offers the chance to better exploit genetic diversity and plan appropriately for population management in the future [8], while, for instance, embryo cryopreservation does not allow the same flexibility in the generation of offspring, since the combination of the male and female gametes has already been decided. Male gametes can be retrieved from ejaculates from living subjects, or from isolated gonads, and in particular from the *cauda epididymis* in case of castration or death, but this topic lies outside the scope of the present work. Female gametes, instead, can be surgically retrieved from living animals (also after hormonal stimulation) or from isolated ovaries after spaying or death. Most of the times, the animals are not hormonally stimulated, especially if the gamete collection

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takes place from isolated ovaries. In this case, it is unlikely to obtain mature (metaphase II, MII) oocytes, which would be ready to be fertilized, therefore, immature (germinal vesicle, GV) oocytes are usually retrieved. The GV oocytes can be either matured in vitro and then cryopreserved or they can be cryopreserved as GV right after collection. Efficiency of cryopreservation at different stages of oocyte maturation can vary, as described below.

The most used cryopreservation techniques, for oocytes, are slow (or controlled rate) freezing and vitrification. Although vitrification was developed later, it gained popularity for cat oocytes during the years (Fig. 1) thanks to its speed, ease of application and field-feasibility. Both techniques, however, currently do not allow to achieve satisfactory results. After freezing-thawing or vitrification-warming, oocyte struggle to mature and/or develop into embryos. In vitro maturation (IVM) after warming is a challenge for the GV oocytes, and cleavage after in vitro fertilization (IVF) usually reaches 15–30% [9–15] (Table 1), while for mature cryopreserved gametes it can reach 30–50% [16–18] (Table 2). However, progression of embryos to late in vitro stages is often impaired [19] (Tables 1 and 2), and improvements to cryopreservation protocols are needed. Even if the viability after warming is usually as high as 90% [19,20], many oocytes degenerate during the following in vitro culture, but the mechanisms causing injuries are still largely unclear [21]. Different approaches were experimented, such as acting on the oocytes prior to cryopreservation or using chemical or physical enrichment during the cryopreservation procedure itself or after thawing or warming, nonetheless maturation and embryonic developmental rates remained poor compared to fresh oocytes.

The purpose of this paper is to review cat oocyte vitrification, the strategies attempted so far to improve the outcomes and the time for their application (i.e., before, during, or after vitrification-warming), as well as analyzing other possible approaches for future trials.

2. Fundamentals of oocyte vitrification

While slow-freezing involves a slow, controlled, temperature decrease in a programmable freezer, with ice formation in the samples, vitrification brings about the solidification of small volumes of viscous solutions in a glass-like state thanks to a high cooling rate, which is usually achieved by direct plunging of the samples in liquid nitrogen [22,23]. For slow-freezing, after exposure to cryoprotectant solutions, the oocytes are loaded into straws and cooled to equilibrate in the programmable freezer. After equilibration, "seeding" (i.e., induced formation of ice in the straw to avoid excessive reduction in the freezing point due to the increasing concentration of solutes) needs to be performed, usually at −7 °C. Thereafter, the programmed slow cooling can proceed and be followed by final immersion of straws into liquid nitrogen [24].

On the other hand, if cryoprotectant-rich vitrification solutions (commercial or laboratory-made) and liquid nitrogen are available, vitrification can be performed everywhere, without any specialized equipment. Different carriers or devices have been developed to reach the "Minimum volume vitrification" goal and to support the oocytes during the cryopreservation procedure, especially during the immersion in liquid nitrogen after exposure to increasing concentration of cryoprotectants. Some of these are commercially available, while others are custom-made, and they can be classified as "Tubing techniques" or "Surface techniques". Tubing techniques usually allow a high cooling rate in a closed system, which is safer (concerning nitrogen-derived contamination) and easier to handle, and include for instance plastic straw, open pulled straw (OPS), closed pulled straw (CPS), superfine OPS, CryoTip and sealed pulled straw [22]. Surface techniques usually allow greater reduction of vitrification volume and high(er) cooling rates, but they are usually open systems, and they include electron microscope grid, Cryotop, Cryoloop, hemi-straw, solid surface, vitrification spatula, plastic blade and Vitri-Inga [22]. Among them, Cryotop is particularly easy to use and handle (oocytes can be loaded on a small film strip attached to a hard plastic holder, protected by a plastic tube cap during storage [25]), and it allows the vitrification of an ultra-low volume of solution (until 0.1 μL), which is also beneficial for the achievement of rapid cooling and warming rates. Nowadays it is probably the most used vitrification carrier for human and animal oocytes, including feline's [12,14,15,18,26–32].

Due to the architecture of each carrier and the volume of cells that can be loaded, cooling and warming rates vary [33], and this might have an influence on vitrification outcomes. However, besides its practical advantages, vitrification should probably be chosen over slow-freezing for domestic animal oocyte cryopreservation, since it has been suggested to be more effective for lipid-rich mammalian oocytes and embryos [34], including cat's. Focusing on oocyte vitrification, which is the topic of this Review, the biggest achievements have been a pregnancy from vitrified immature oocytes [9] and live births from vitrified mature oocytes [18,26], but protocols still need to be optimized to improve the efficiency of the technique, especially for immature oocytes. Indeed, efficiency of vitrification is limited by several cryoinjuries that might occur due to the extreme conditions that cells experiment during the cryopreservation procedure, such as the temperature decrease and the exposure to toxic cryoprotectants and consequent osmotic stress [23,35].

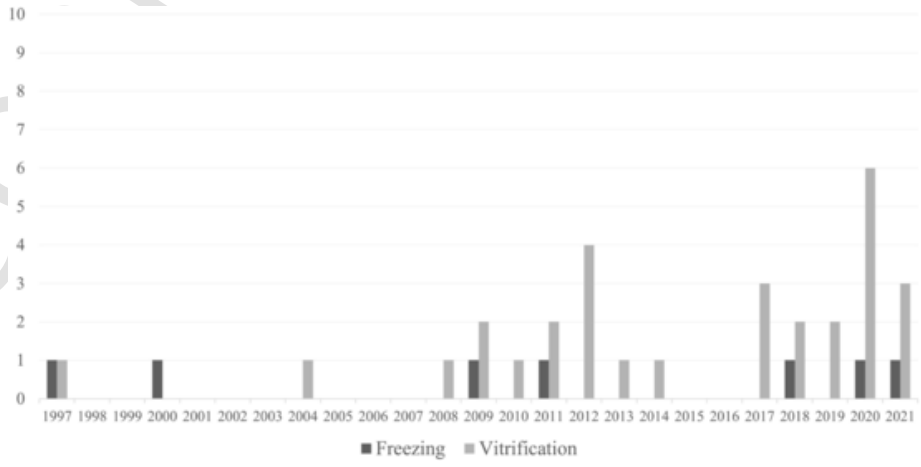

Fig. 1. Number of PubMed (https://pubmed.ncbi.nlm.nih.gov/) records related to feline oocyte cryopreservation per year, from the first publication until 2021. A) Number of records for cat oocyte slow-freezing. B) Number of records for cat oocyte vitrification. The queries "oocyt* AND (freez* OR frozen) AND ("cat" OR feline OR felid OR felis)" and "oocyt* AND (vitrif* OR ultra-rapid OR ultrarapid) AND ("cat" OR feline OR felid OR felis)" were searched on May 30th, 2022, and relevant records were manually selected.

Table 1
Overview of in vitro embryo production results achieved with domestic cat immature vitrified oocytes.

Vitrification support	Embryo production method	Embryo development			Reference
		Cleavage %	Morulae %	Blastocysts %	
Plastic gutter	IVF	17.7 ± 2.5	0	0	Comizzoli et al., 2009 [10]
OPS	IVF	18.6	10.4	4.3	Cocchia et al., 2010 [13]
Plastic gutter	IVF	31.0 ± 7.9 (out of matured oocytes)	0	0	Comizzoli et al., 2011 [11]
OPS	IVF	24.8	47.6 (out of cleaved embryos)	30.2 (out of cleaved embryos)	Tharasanit et al., 2011 [9]
Cryotop	ICSI	28.6	30	N.A.	Fernandez-Gonzalez & Jewgenow, 2017 [12]
OPS	IVF	33.2 ± 7.5	22.3 ± 3.8 (out of cleaved embryos)	16.2 ± 5.2 (out of cleaved embryos)	Arayatham et al., 2017 [21]
0.25 mL straw with the end cut in half	PA	9	N.A.	2	Snoeck et al., 2018 [56]
Cryotop	IVF	18.39 ± 16.67	1.79 ± 3.07	0.95 ± 2.52	Colombo et al., 2019 [14]
Cryotop	IVF	67.2 (out of matured oocytes)	1.6 (out of matured oocytes)	0	Colombo et al., 2020 [29]
Cryotop	ICSI	25 (out of matured oocytes)	25 (out of cleaved embryos)	0	Sowińska et al., 2020 [30]
Cryotop	IVF	73.2 (out of matured oocytes)	12.2 (out of matured oocytes)	2.4 (out of matured oocytes)	Colombo et al., 2021 [15]
Self-closed metal tubes	ICSI	20 (out of matured oocytes)	0	N.A.	Fernandez-Gonzalez et al., 2021 [68]

OPS = open pulled straw; IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection; PA = parthenogenic activation; N.A.: Data not available.
Papers are ordered by 1) publication date, 2) first Author's surname (alphabetically).
For the studies where more treatments were compared, the one which gave the best results was hereby reported.

Table 2
Overview of in vitro embryo production results achieved with domestic cat in vitro matured vitrified oocytes.

Vitrification support	Embryo production method	Embryo development			Reference
		Cleavage %	Morulae %	Blastocysts %	
Straw	IVF	20.0 ± 7.8	4.2 ± 2.4	3.7 ± 2.5	Murakami et al., 2004 [16]
Cryoloop	IVF	32.2	15.8	11.8	Merlo et al., 2008 [17]
Cryotop	IVF	53	13.3 (out of cleaved embryos)	10 (out of cleaved embryos)	Pope et al., 2012 [18]
	ICSI	68	11 (out of cleaved embryos)	0 (out of cleaved embryos)	
Cryolock	IVF	44.7 ± 4.1	N.A.	14.5 ± 6.9	Galiguis et al., 2014 [26]
Cryotop	IVF	47.7	N.A.	14.3 (out of cleaved embryos)	Herrick et al., 2016 [95]
Cryotech	PA	46	13 (out of cleaved embryos)	0	Nowak et al., 2020 [96]
Cryotop	ICSI	21.9 (out of matured oocytes)	33.3 (out of cleaved embryos)	0	Sowińska et al., 2020 [30]

IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection; PA = parthenogenic activation; N.A.: Data not available.
Papers are ordered by 1) publication date, 2) first Author's surname (alphabetically).
For the studies where more treatments were compared, the one which gave the best results was hereby reported.

3. Cryopreservation-induced damages and vitrification at different stages of oocyte maturation

Although not every cryoinjury can be observed grossly at the microscope, vitrification is known to cause morphological abnormalities in some oocytes (Fig. 2), among which the most common are probably changes in ooplasm shape and granulation, partial (or, rarely, total) loss of cumulus cells and (rarely) zona pellucida fractures [20].

Concerning mechanisms of oocyte injury, chilling injury, that causes irreversible alterations in membranes (including the oolemma), in lipid-droplets and in microtubules of mitotic/meiotic spindles [36,37], is the first issue. Cytoskeleton may also be affected by the high concentration of cryoprotectants and subsequent osmotic stress, that lead to shrinkage and alteration in cellular shape. In addition, a so-called fracture damage may also appear following vitrification with consequences on the zona pellucida, which may lose its integrity [38]. On the other hand, the same structure could undergo hardening due to premature exocytosis of cortical granules induced from calcium oscillation triggered by cryoprotectants [39]. Zona hardening can negatively influence sperm penetration and fertilization. Intracellular organelles can also be affected by vitrification. Beside cytoskeletal damage, which may also lead to multiple aster formation and to negative consequences on oocyte fertilization and following embryo development [40], mitochondria function and distribution may be affected, as well as the nucleus and the DNA. Fragmentation and chromosomal abnormalities may arise, as well as alterations in gene expression (particularly in genes related to oxidative stress, apoptosis, cell cycle and sperm-oocyte interaction) [41] and epigenetic modifications [42]. Around the gamete, cumulus cells might also be affected if cumulus-oocyte complexes are vitrified. Cumulus cells might physically and/or functionally detach from the oocyte due to the sensitivity of the cytoskeleton components of transzonal projections (i.e., the connections between the oocyte and its cumulus) to low temperatures and cryoprotectants [43]. This could be detrimental for oocyte maturation and fertilization, especially if immature gametes are vitrified [44]. Finally, activation of apoptotic pathways is reported after vitrification of oocytes [29,45–47] and cryopreservation of other cells [48–52], and this might be the type of cell death that leads to oocyte degeneration after warming.

Some studies specifically investigated consequences of vitrification on cat oocytes, where few cryopreservation-induced damages have actually been identified and reported and many intracellular mechanisms remain to be investigated. For instance, thanks to the use of a fluorescent MitoTracker® probe, after vitrification of immature and mature oocytes, no changes in distribution of mitochondria were found, while function and aggregation were altered [53]. Cytoskeleton was also found to be altered in immature and mature vitrified oocytes [31,54], and immature oocyte vitrification affected communication with cumulus cells [54]. A proteomic study reported that, after vitrification of im-

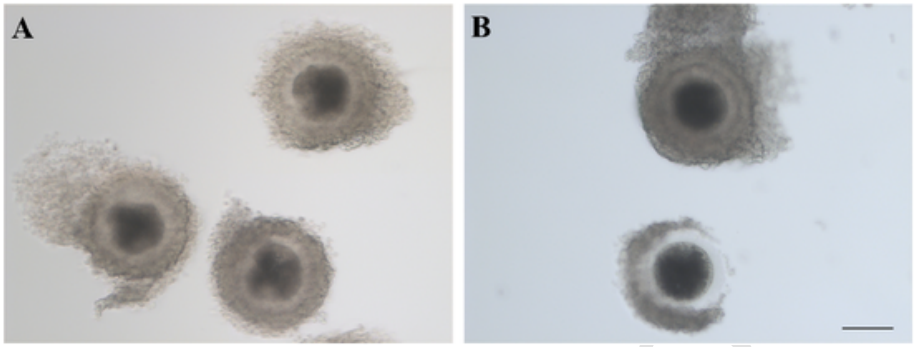

Fig. 2. Representative pictures of domestic cat oocytes after vitrification and of vitrification-induced damages. (A) Vitrified-warmed oocytes showing changes in ooplasm shape and granulation. (B) One morphologically normal vitrified-warmed oocyte (top) and one showing partial loss of cumulus cells (bottom). Scale bar 100 μm.

mature oocytes, warming and IVM, membrane and nuclear proteins were downregulated, while apoptosis and DNA repair proteins were overexpressed compared to fresh control oocytes [55]. Furthermore, alterations in the permeability of gap junctions hemichannel were hypothesized to occur following vitrification which may lead to loss of small essential metabolites, ionic imbalance and penetration of small, potentially toxic, molecules [56]. Apoptosis was also hypothesized to have a role [21], and indeed our recent study highlighted that vitrification causes an increase in caspase activity and DNA fragmentation in immature cat oocytes [29]. Altogether, these alterations, which sometimes have a different severity based on the maturation stage of the preserved oocytes (GV vs. MII), could contribute to the unsatisfactory outcomes of cat oocyte vitrification.

The best maturation stage to cryopreserve oocytes has been debated for a long time, and it still is a matter of interest [30]. Although the efficiency has usually been considered higher for mature oocytes (human [57]; cat [58]), preservation of immature gametes could offer some advantages. Following the consideration that MII oocyte preservation gives better results, whenever immature oocytes are retrieved and a specialized laboratory wherein IVM can be carried out is available, the preferred option should be to proceed with IVM. However, IVM is not completely efficient in the domestic cat, and the oocytes that will not mature will be lost. In addition, comparing the efficiency of GV and MII oocyte cryopreservation using post-warming development as an endpoint might be challenging, since MII oocyte have been already somehow pre-selected by the maturation process, and only the competent ones were matured and preserved [58]. This was also supported by a recent report in domestic cat oocytes, where embryo development rates did not differ between GV and MII vitrified oocytes when the percentages of cleavage and morulae were calculated on the number of mature or cleaved oocytes, respectively [30].

The reason why immature oocytes perform poorer than their mature counterparts could be explained by their peculiar features. Immature oocytes are usually preserved as cumulus-oocyte complexes since they need the cumulus cells to mature properly [59], but as mentioned earlier, the cumulus often suffer from a post-warming loss of connection and functionality, which is detrimental for maturation outcomes. Mature oocytes, on the other hand, do not need cumulus cells so much. In addition, the permeability of the oolemma, which varies along maturation, is more favorable to cryopreservation in MII oocytes [60]. Finally, although it was believed that the meiotic spindle of mature oocytes was more cryo-sensitive than the membrane-surrounded GV [61], the nuclear material of MII oocyte does not seem to be particularly affected. However, when an animal dies in the field, it is unusual to have the possibility to mature its oocytes in vitro, since there are few ARTs laboratories close to wildlife habitats, parks or zoos. Therefore, preservation of immature oocytes is often the only chance to save the gametes of wild valuable animals, which can be vitrified on site [62].

4. Application of microenvironmental factors to improve vitrified oocyte outcomes

Likely because the efficiency of vitrification particularly needs to be improved for immature oocytes, and because GV oocytes are often the only source of gametes that can be preserved, this paragraph will focus on the microenvironmental factors or modifications that were applied on GV oocytes, in different phases of the vitrification flow (i.e., before, during or after vitrification), in an attempt to improve their post-warming outcomes.

However, it is worth mentioning that a sort of microenvironmental modification has also been applied on mature oocytes, specifically to understand whether the cryosensitivity of cat oocytes was due to their high cytoplasmic lipid content and whether delipidation could improve their developmental competence [26]. Pre-vitrification centrifugation of mature cat oocytes allowed lipid polarization, and such treated oocytes showed higher post-warming embryo developmental rates [26].

The main microenvironmental factors applied so far for immature oocytes and their outcomes are summarized in Table 3. Studies focusing on the definition of type and concentration of cryoprotectants and their exposure time have not been considered as microenvironmental factors, since cryoprotectants are part of every vitrification protocol. Instead, studies experimenting different enrichments or modifications to culture or vitrification environments have been included, and specifically those that attempted:

- the modification or enhancement of the oocyte features, *before vitrification*, to make it more resistant to cryopreservation;
- the change of microenvironment *during vitrification-warming,* to provide more suitable conditions to accomplish an efficient cryopreservation process;
- the creation of enriched microenvironment or the addition of compounds with putative beneficial effects *after warming,* to help vitrified oocytes to recover from, or better survive to, vitrification-derived damages.

4.1. Before vitrification-warming

These first strategies take place before exposure to cryoprotectant solutions or equilibration, between oocyte collection and the beginning of the vitrification protocol. Physiologically, cat oocytes contain a large GV with highly decondensed chromatin, which could have a role in cryosensitivity of feline gametes, whereas a more compacted GV could increase the cryotolerance. The use of the histone deacetylase enhancer resveratrol, indeed, was able to reduce the size of the GV and to guarantee an increase in the cryo-resistance of immature oocytes, which was observed as an increase in maturation rates and early embryo development [10]. The same strategy was also combined with GV transfer in a

Table 3
Overview of the microenvironmental factors employed so far to improve the in vitro outcomes of cat immature vitrified oocytes.

Strategy	Time of application			Main outcomes	Reference
	Before vitrification	During vitrification-warming	After warming		
Germinal vesicle (GV) compaction using resveratrol to reduce GV cryosensitivity	X			Improved maturation, cleavage and 8–16 cells embryo rates	Comizzoli et al., 2009 [10]
Protection from cryoinjuries with follicular extracellular vesicles and their biomolecules content	X	X		Improved meiotic resumption (germinal vesicle breakdown - metaphase II)	de Almeida Monteiro Melo Ferraz et al., 2020 [27]
GV compaction, vitrification, GV transfer in a fresh cytoplast	X		X	Improved cleavage, morula and blastocyst rates	Comizzoli et al., 2011 [11]
Use of slush nitrogen to improve cooling rates		X		No improvements in maturation or embryo developmental rates	Fernandez-Gonzalez & Jewgenow, 2017 [12]
Use of the connexin mimetic peptide Gap26 to close hemichannels that might open during vitrification-warming and lead to loss of essential metabolites and entrance of toxic substances		X		Improved maturation rate. Blastocyst development	Snoeck et al., 2018 [56]
Self-pressurized vitrification in metal tubes to improve cooling rates		X		No improvements in maturation or embryo developmental rates	Fernandez-Gonzalez et al., 2021 [68]
Control of apoptotic pathways using the pan-caspase inhibitor Z-VAD-FMK		X	X	Not significant increase in maturation rate. No differences in embryo development	Colombo et al., 2020 [29]
Inhibition of rho-associated coiled-coil containing protein kinase 1 (ROCK1), involved in cytoskeleton and apoptosis regulation			X	Improved cleavage rate	Arayatham et al., 2017 [21]
Enriched culture conditions after warming (3D co-culture with fresh oocytes)			X	No differences in embryo development. Blastocyst development	Colombo et al., 2019 [14]
Enriched culture conditions after warming (liquid marble microbioreactor)			X	No differences in maturation rates	Colombo et al., 2019 [86]
Enriched culture conditions after warming (follicle-like structure, i.e., 3D culture of granulosa cells)			X	No differences in maturation rates	Colombo et al., 2020 [28]

Papers are ordered by 1) time of application of the microenvironmental factors, 2) publication date.

fresh cytoplast (i.e., an oocyte deprived of its nucleus to be used as "Recipient cytoplasm") to supply vitrified GV with a better microenvironment to accomplish maturation [11]. This resulted in higher maturation, cleavage and morula/blastocyst rates compared to oocytes vitrified without GV compaction or transfer [11]. More recently, another study employed follicular fluid extracellular vesicles to check whether they could deliver their content to the oocytes chosen for vitrification and the vesicle effect on oocyte meiotic competence [27]. Extracellular vesicles contain many regulatory molecules, including miRNAs, that could influence the metabolism and gene expression of cumulus-oocyte complexes. One hour coincubation of oocytes and extracellular vesicles, followed by vitrification-warming in the presence of extracellular vesicles, led to the delivery of the content of the vesicles to the oocytes and was beneficial for meiosis resumption rates [27].

In the pre-vitrification environment, future perspectives for further improvements may include the identification of specific markers of cryo-resistance. First, this could help in selecting the best gametes to be cryopreserved, and then it could also prompt the design of strategies aimed at improving the cryotolerance of less-resilient gametes. Surely, one of the main players in cat oocyte cryosensitivity is the presence of abundant lipids in the cytoplasm [63], and acting on them could change the outcomes of vitrification. In pigs, for instance, the use of cholesterol-loaded cyclodextrins, which can transfer cholesterol in the oolemma and make the gamete more cryo-resistant [64], was beneficial for survival and IVM rates, although not for embryo development [65]. Instead, contrasting results were obtained in bovine oocytes [64,66]. Another strategy to improve gamete resistance is based on the belief that sublethal environmental stress can stimulate cells to adapt to the new condition and it makes them more resistant to other stressors, such as cryopreservation. This theory prompted the application of high hydrostatic pressure prior to vitrification in in vitro matured human oocytes [67]. Treated oocytes showed lower oxidative stress, but no significant differences were found in embryo development compared to conventionally vitrified oocytes used as control [67].

4.2. During vitrification-warming

In the cryopreservation step, microenvironmental modifications alter some factors during exposure to cryoprotectant solutions or equilibration time or during the vitrification itself (plunging into liquid nitrogen and achievement of the glass-like solid state). Cryopreservation is a stressful process that, besides the aforementioned damages, could also lead to activation, or opening, of unpaired oolemma hemichannels. Hemichannels that are not assembled into gap junctions usually stay in the membrane in their closed configuration, but stressors may cause their opening. Open hemichannels might lead to the loss of essential metabolites and the entrance of toxic substances, which can be detrimental to the vitrified oocytes and be a cause for their death [56]. The use of hemichannels-closing compounds might therefore be useful to limit vitrification damages. A peptide known as Gap26, which mimics the connexins that form hemichannels and can bind to open hemichannels to close them, was indeed beneficial when added to vitrification-warming solutions [56]. Vitrified oocytes treated with Gap26 showed a higher IVM rate than untreated control and were the only vitrified group where blastocysts were obtained after parthenogenic activation [56].

Some strategies have also been attempted to act on vitrification dynamics and improve the cooling rate during the vitrification procedure. Unfortunately, the use of a slush nitrogen bath to plunge oocyte-loaded Cryotops did not lead to any improvement [12], although the application of negative pressure to liquid nitrogen successfully lowered its temperature until −206 °C/−210 °C. The use of closed metal tubes for self-pressurized vitrification, where higher cooling rates can be obtained exploiting the high pressure achieved by plunging closed tubes containing the samples directly into liquid nitrogen and the thermal conductivity of metals, led to similar results [68].

Moving forward to the following phase, the addition of chemical factors to both the vitrification-warming and post-warming microenvironment was evaluated. Since cryopreservation is known to induce apoptosis, the inhibition of the apoptotic enzymes caspases was at-

tempted in our study by the use of the tripeptide Z-VAD-FMK (Benzyloxycarbonyl-Val-Ala-Asp Fluoromethyl ketone) [29]. The supplementation of Z-VAD-FMK to vitrification-warming solutions and to the culture medium for the 24 h incubation after warming was beneficial for the apoptotic markers, which showed a decrease in caspase activity and DNA fragmentation compared to control vitrified oocytes [29]. The same treatment also led maturation rates of vitrified oocytes close to those of fresh oocytes, while it did not have any influence on embryo development [29].

Future developments might include every kind of modification of the vitrification-warming protocol itself. Of particular interest for their natural origin, antifreeze proteins could be a possibility to improve the procedure. Produced by some animals, plants, fungi and bacteria to survive at low temperatures, these polypeptides can control ice growth and lower the freezing point of water, with ice-stabilizing and anti-recrystallization properties [69]. Their use for the vitrification of murine oocytes brought about improvements in survival, cleavage, blastocyst rate and quality [70]. In addition, the use of antioxidants should also be investigated, during vitrification and after warming, since both cryopreservation and in vitro culture are known to cause oxidative stress [71,72]. Several compounds, often of natural origin (e.g., vitamins, plant-derived molecules, melatonin), were studied in different species, and an improvement in the developmental competence of vitrified oocyte was often achieved [73–79].

4.3. After vitrification-warming

After warming, the natural fate of immature vitrified oocyte is to undergo IVM, hence, the maturation microenvironment has been often involved in the design of ameliorative strategies. Both chemical and physical factors have been manipulated, with changes in medium composition or in the physical supports used for oocyte culture, respectively. Although this study was mentioned earlier, it is worth highlighting here that the insertion of vitrified GV to a fresh cytoplast through GV transfer was also beneficial [11]. The recipient cytoplast could have worked as a microenvironmental enrichment for the GV, after warming.

Starting with chemical factors, a first study assessed whether the inhibition of the rho-associated coiled-coil containing protein kinase 1 (ROCK1), which is involved in cytoskeleton and apoptosis regulation, could have an effect on vitrified oocyte outcomes [21]. The addition of the inhibitor to the maturation medium resulted in improved cleavage rates, and the treatment also allowed blastocyst development, although not to a significantly higher extent than untreated controls [21].

One of the targets chosen for the latter study, the cytoskeleton, has also a particular importance in view of the physical environment where oocytes are grown. Oocytes are usually cultured in two dimensions, as in Petri dishes or multi-well plates, but these supports do not mimic the physiological environment where the oocytes grow and mature. They might cause abnormalities in subcellular structures and events, such as changes in nuclear shape and alterations in gene expression, loss of membrane receptors and changes in the response to stimuli, and cell flattening on the culture surface, with negative consequences also on the cytoskeleton [80]. This issue might be particularly severe for vitrified oocytes, whose cytoskeleton is already stressed by the cryopreservation procedure. Luckily, cell biologists developed three-dimensional in vitro culture systems that can provide an in vivo-like environment and were already successfully used for fresh oocytes in different species. The use of co-cultures with different cells is also considered advantageous to enhance the quality of the culture microenvironment, in order to recreate cell-to-cell interactions and allow exchanges of beneficial soluble factors between co-cultured cells (e.g., oocyte-secreted factors, OSFs, during IVM [81,82]). The application of three-dimensional and enriched culture systems for vitrified oocytes was experimented by our group following the results obtained with fresh gametes [83–85]. Three-dimensional barium alginate microcapsules were used for the IVM of vitrified oocytes co-cultured with fresh oocytes and, after IVF, for the culture of deriving embryos, leading to blastocyst development [14]. Another non-flat microenvironment, known as liquid marble microbioreactor, was applied only for the IVM of vitrified oocytes, and results were similar to those of control oocytes cultured in two-dimensional conditions [86]. To further increase the similarity of the culture environment to its in vivo counterpart, we later designed three-dimensional follicle-like structures by encapsulation of feline granulosa cells in barium alginate. Such cultured granulosa cells maintained their functionality (i.e., hormonal secretion), but their use as an enriched microenvironment for the IVM of cat oocytes did not influence meiosis resumption [28].

In this context, future directions should involve the improvement of the cytoplasmic maturation of vitrified oocytes and the design of more physiological culture systems. Ooplasmic transfer (i.e., injection of ooplasm of a donor oocyte into the cytoplasm of the recipient vitrified oocyte) could be a way to provide vitrified gametes with a stronger cellular machinery to accomplish proper maturation and early embryo development. Supplementation of exogenous mitochondria could also be beneficial [87,88] for their roles, which include energy production and protection from oxidative stress [89]. Secondly, a more physiological post-warming in vitro culture could better resemble the in vivo environment where the oocytes mature and supply them with the best conditions, avoiding the exposure to damaging factors. Following this view, a semi-in vivo approach was experimented in the bovine model. Vitrified-warmed oocytes were injected into preovulatory follicles to mature, and embryo development was assessed after insemination, unfortunately without improvement compared to a whole in vitro system [90]. In the lab, the game-changer could be the use of microfluidic systems, in which the combination of 3D architectures, different types of cells and a fluid flow would allow creating a dynamic culture environment [91]. Obvious improvements in the exchange of nutrients, gases and metabolites, as well as in the physical support provided to the oocytes, could be obtained [92]. Furthermore, the optimization of microfluidic chips to create an “All-in-one” system, where the whole in vitro embryo production could be performed without unnecessary oocyte and embryo manipulation [93,94] would be interesting to investigate for the delicate vitrified-warmed oocytes. Finally, the identification of vitrification-induced damages (e.g., the exact molecules or pathways that are altered by vitrification-warming) might give us the chance to target more precisely the specific causes of vitrified oocyte degeneration. The final goal would be to use specific compounds to rescue cryopreserved samples, avoiding their death during in vitro culture and promoting their development.

5. Conclusions

Although continuous progresses are being made, there is still a strong need to enhance the in vitro outcomes of cat vitrified oocytes. When specific genetic pools have to be preserved, when equipped laboratories are not available to immediately apply in vitro ARTs such as IVM, or when there is no male counterpart for fertilization, oocyte cryopreservation is the only chance to preserve such valuable germplasm. The identification of markers or cryo-resistance and of vitrification-induced damages will be crucial in order to develop new vitrification and culture strategies. Only a joint effort to choose the most cryo-resilient oocytes, prepare them appropriately for cryopreservation, optimize the vitrification-warming protocol and supply the most suitable post-warming culture conditions will hopefully lead to significant improvements.

CRediT authorship contribution statement

Martina Colombo : Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Isa Mohammed Alkali :**

Writing – review & editing. **Gaia Cecilia Luvoni :** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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