jove

Minimum Volume Vitrification of Immature Feline Oocytes

Martina Colombo[1], **Gaia C. Luvoni**[1]

[1] Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare 'Carlo Cantoni', Università degli Studi di Milano

Corresponding Author

Martina Colombo
martina.colombo@unimi.it

Citation

Colombo, M., Luvoni, G.C. Minimum Volume Vitrification of Immature Feline Oocytes. *J. Vis. Exp.* (160), e61523, doi:10.3791/61523 (2020).

Date Published

June 24, 2020

DOI

10.3791/61523

URL

jove.com/video/61523

Abstract

In wild animals' conservation programs, gamete banking is crucial to safeguard genetic resources of valuable individuals and rare species and to promote biodiversity preservation. In felids, most species are threatened with extinction, and domestic breeds are used as a model to increase the efficiency of protocols for germplasm banking. Among oocyte cryopreservation techniques, vitrification is more and more popular in human and veterinary assisted reproduction. Cryotop vitrification, which was at first developed for human oocytes and embryos, has demonstrated to be well-suited for cat oocytes. This method offers several advantages, such as the feasibility in field conditions and the speed of the procedure, which can be helpful when several samples need to be processed. However, the efficiency is strongly dependent on the operator's skills, and intra- and inter-laboratory standardization are needed, as well as personnel training. This protocol describes minimum volume vitrification of immature feline oocytes on a commercial support in a step by step field-friendly protocol, from oocyte collection to warming. Following the protocol, preservation of oocyte integrity and viability at warming (as high as 90%) can be expected, although there is still room for improvement in post-warming maturation and embryonic development outcomes.

Introduction

Cryopreservation has become a key step of assisted reproduction techniques (ARTs). In humans, it allows preservation of fertility or postponement of parenthood for medical or personal reasons. In animals, it is necessary to overcome distance and time in planned matings, especially in farm animals and pets, or to preserve genetic material of valuable subjects in conservation programs, particularly in wild endangered species. Gamete cryopreservation is the best choice when the individuals to be bred have not been chosen yet or in order to avoid ethical issues associated with embryo freezing, especially in human medicine[1]. Spermatozoa are relatively easy to preserve and give satisfactory outcomes after thawing, but oocytes, due to their structural features, might be more complex to store. Indeed, the low surface/volume ratio, as well as the presence of the zona pellucida surrounding the ooplasma, limits the movement of cryoprotectants and water across the cell[2]. Moreover, in domestic animals including felids, they are

characterized by a lipid-rich cytoplasm, which is thought to make them more sensitive to cryopreservation[3].

Most felids are threatened, and the domestic cat is used as a model to develop protocols for germplasm preservation thanks to the availability of gonads from routine ovariectomy. In wild animals, gonads can be obtained after elective surgeries or (more frequently) *post-mortem*, and immature (germinal vesicle) gametes can be retrieved. Hormonal stimulation aimed to obtain mature (metaphase II) oocytes is not as common as in human ARTs because of the ethical issues and the species- and individual-specific response to treatments[4].

Therefore, the development of cryopreservation strategies has focused on immature gametes, which can usually be retrieved after the unexpected or sudden death of rare individuals. From a biological point of view, there are some differences in the cryopreservation of immature or mature gametes, each having its advantages. Firstly, DNA is more protected in immature oocytes, whose germinal vesicle contains chromosomes surrounded by a nuclear membrane, while the meiotic spindle of metaphase II oocytes could be more vulnerable to cryoinjuries[5]. Secondly, cold-induced cytoskeleton damages might affect spindle rotation, polar body extrusion, pronuclear migration and cytokinesis, which could have different impacts according to oocyte developmental phase, influencing meiosis progression or post-fertilization events. Finally, and perhaps most importantly, whereas mature oocytes are ready to be fertilized, immature gametes rely on the support of the surrounding cumulus cells to go through nuclear and cytoplasmic maturation[6], and this is the reason why whole cumulus-oocyte complexes (COCs) are cryopreserved. However, the loss of cumulus cells and/or the loss of functional connection between the gamete and the surrounding somatic cells are probably the most detrimental effect of cryopreservation of immature COCs.

Among cryopreservation techniques, vitrification is one that can be applied more easily in field conditions. Compared to slow (or controlled rate) freezing, vitrification is faster and does not require specific equipment, such as a programmable freezer. In order to satisfy the three fundamental principles of vitrification (i.e., high viscosity, connected to high cryoprotectant concentration, small volumes and ultra-rapid temperature decrease), several media and supports especially have been developed and used in cats for both immature and mature oocytes. Beginning with simple straws[7], devices were then developed to reach the “Minimum volume” goal. Cryoloop[8], open pulled straws (OPS)[9], plastic gutters (modified straw)[10] and cryotubes[11] have been used, until a more efficient device (i.e., Cryotop) was employed[11], improving survival and meiosis resumption. Cryotop (**Supplemental Figure 1**) is a commercially available support which has become the elective open system for vitrification. Developed for the vitrification of human oocytes and embryos, it consists of a small film strip attached to a hard plastic holder, protected by a plastic tube cap during storage[12]. Thanks to its usability and to the extreme reduction in vitrification volume (as little as 0.1 µL), which also leads to extremely rapid cooling and warming rates, this vitrification support has been increasingly applied in several species, including the domestic cat, in which it has been used with a variety of media[13,14,15,16,17].

The purpose of this manuscript is to describe a collection-vitrification-warming protocol, with minor modifications from the one originally developed for human oocytes, which employs laboratory-made media and commercial supports for

minimum volume vitrification and can be easily applied in field conditions for the cryopreservation of immature feline COCs.

Protocol

The procedures hereby depicted did not undergo ethical approval since cat ovaries were collected at veterinary clinics as byproducts from owner-requested routine ovariectomy or ovariohysterectomy.

1. Oocyte collection

1. Before starting the experiments, prepare solutions for ovary and oocyte collection. Prepare ovary collection solution with phosphate buffered saline (PBS) with a mixture of antibiotics and antimycotics (100 IU/mL of penicillin G sodium, 0.1 mg/mL of streptomycin sulphate, 0.25 µg/mL of amphotericin B). Prepare oocyte collection solution (i.e., PBS/PVA) with PBS with 100 IU/mL of penicillin G sodium, 0.1 mg/mL of streptomycin sulphate and 0.1% (w/v) polyvinyl alcohol (PVA).
2. Store solutions for ovary and oocyte collection at 4 °C until use.
3. On the day of queens' spaying, a few hours before processing the samples, take part of ovary and oocyte collection solutions and let them warm up at room temperature (RT; 25 ± 2 °C).
4. When samples arrive to the lab, isolate the ovaries from the surrounding connective tissue and from the oviduct and wash them in fresh ovary collection medium in a Petri dish.
5. Fill one 35 mm Petri dish for each queen with about 3 mL of RT PBS/PVA, and one more dish to collect the oocytes.
6. Place a pair of ovaries in a Petri dish and mince the cortex with a surgical scalpel. Ensure all the follicles have been broken with the help of a stereomicroscope (magnification 8x).
7. Collect COCs with a pipette and move them in fresh PBS/PVA in the allocated Petri dish. Select good qualities COCs, with a homogenous, dark cytoplasm and surrounded by several compact layers of cumulus cells (grade I[18]).

2. Vitrification

1. Prepare equilibration and vitrification media.
    1. Prepare equilibration solution (ES) with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO) in Medium 199, with 20% fetal bovine serum (FBS).
    2. Prepare vitrification solution (VS) with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose in Medium 199, with 20% FBS (modified from [19]).
    3. Let ES and VS warm up at RT before use.
    NOTE: Cryoprotectants (e.g., EG, DMSO) are known to be cytotoxic. A strategy to counter their toxicity is to reduce the temperature at which the cells are exposed to them[20], and oocytes are usually exposed to cryoprotectants at RT. Thus, the whole procedure, from oocyte collection to vitrification, is carried out at RT in this protocol to avoid temperature fluctuations.
2. Prepare the vitrification dish (i.e., a special dish consisting of six conical wells divided in two rows, known as "Repro plate"). Prepare one row (three wells) for each vitrification support.
    1. Add 20 µL of ES on the bottom of the first well.

2. Add 300 µL of VS on the bottom of the second (i.e., 1VS) and third (i.e., 2VS) wells.

3. Equilibrate COCs in ES.

    1. With a small-bore pipette (e.g., a Pasteur pipette pulled on a Bunsen beak to make it thinner – diameter should be at least 200 µm), transfer one (or more) COC(s) on the bottom of the first well.

        NOTE: Choose the size of the pipette according to the number of layers of cumulus cells surrounding the oocytes, so to COCs dimensions, aiming to reduce as much as possible the volume of media which is transferred with COCs in each step. With the present protocol, trained operators can vitrify successfully up to eight feline COCs per each vitrification support.

    2. Slowly add 20 µL of ES on the border of the drop. Wait for 3 minutes.

    3. Slowly add other 20 µL of ES on the border of the drop. Wait for 3 minutes.

    4. Slowly add 240 µL of ES on the border of the drop. Wait for 9 minutes.

    5. While waiting, prepare a box with liquid nitrogen (LN_2), label a vitrification support with the experiment/cat identification code and leave it open. Put both of them near the stereomicroscope, so that they can be easily reachable while working.

        CAUTION! Wear personal protective equipment when handling LN_2.

    6. Alternatively, if several COCs have to be vitrified, begin the first equilibration step (see step 2.3.1 - 2.3.2) for another group of COCs (which will be loaded onto a new vitrification support).

4. Vitrify COCs in VS in less than 90 seconds.

    1. Using the same small-bore pipette, fill it with 1VS, take the COCs from the bottom of the first well (ES) and move them to the surface of the second well (1VS).

    2. Wash the pipette with 1VS.

    3. Take the COCs and move them in another area (on the bottom) of the well; mix the medium surrounding them with the pipette.

    4. Fill the pipette with 1VS in another area of the well, take the COCs, move them and mix the medium surrounding them with the pipette.

    5. Repeat step 2.4.4.

    6. Wash the pipette with 2VS.

    7. Take the COCs and move them on the bottom of the third well (2VS); mix the medium surrounding them with the pipette.

    8. Fill the pipette with 2VS in another area of the well, take the COCs, move them and mix the medium surrounding them with the pipette.

    9. Repeat step 2.4.8.

    10. Fill the pipette with 2VS in another area of the well, take the COCs and load them on the strip of the vitrification support, as close as possible to the tip. Aspirate excess medium to reduce the volume of VS as much as possible.

    11. Immediately plunge the vitrification support in LN_2, moving it. Close it with the help of some clamps, making sure it always remains immersed in LN_2.

        NOTE: Keeping the right timing is crucial due to cryoprotectant cell-toxicity. Because of their high concentration in vitrification media, cell exposure needs to be controlled, also by flawless execution of

the technique[21]. If samples are abundant, consider dividing them to process them more quickly and minimize the amount of time from oocyte collection to vitrification.

5. Store the loaded vitrification supports in a goblet and keep them in a storage LN_2 tank until warming.

   NOTE: All the previous steps of the protocol can also be applied in field conditions if LN_2 is available. A dry shipper will be necessary to transport the samples to the lab safely and then proceed there with the following phases.

3. Warming

1. Prepare warming media.
   1. Prepare thawing solution (TS) with 1 M sucrose in Medium 199, with 20% FBS.
   2. Prepare dilution solution (DS) with 0.5 M sucrose in Medium 199, with 20% FBS.
   3. Prepare washing solution (WS) with Medium 199 with 20% FBS.
   4. Before use, warm up TS to 38 °C and DS and WS at RT.
2. If necessary, prepare the culture medium for the subsequent use of warmed COCs (e.g., in vitro maturation, IVM).
3. Place the heating stage close to the stereomicroscope and turn it on (38 °C). Put the lid of a Petri dish to warm up.
4. When everything is ready, transfer the vitrification supports which must be warmed from the storage tank to a box with LN_2, and put the box near the stereomicroscope.
5. Prepare the "Repro plate". Prepare a row for each vitrification support that has to be warmed.
   1. Add 300 µL of DS on the bottom of the first well.
   2. Add 300 µL of WS on the bottom of the second (i.e., 1WS) and third (i.e., 2WS) wells.
6. Make a drop of TS (100 µL) on the lid of the Petri dish.
7. With clamps, open one vitrification support in the LN_2.
8. Put the TS drop under the stereomicroscope and with one fast movement take the vitrification support from the LN_2 and immerse its strip in the drop, moving it until all COCs detach. Remove the support from the drop as soon as it is empty, but leave the COCs in TS for 1 minute in total (from immersion until the following step).
9. Take a pipette similar to that used for vitrification and fill it with DS. Take the COCs from the drop (TS) and move them to the bottom of the first well (DS). Wait for 3 minutes.
10. Wash the pipette with 1WS. Take the COCs and move them on the bottom of the second well (1WS). Wait for 5 minutes.
11. Wash the pipette with 2WS. Take the COCs and move them on the surface of the third well (2WS). Wait for them to touch the bottom of the well.
12. Repeat step 3.11 using another area of the well.
13. Wash the pipette with culture medium and move the oocytes to the culture dish for the following use (e.g., IVM).

Representative Results

Following cat oocyte vitrification and warming according to the present protocol (**Figure 1** and **Supplemental Figure 1**), the vast majority of gametes survive. After vitrification, among other techniques, viability can be evaluated at the optical microscope as morphological integrity[22] or with the

use of vital stains. One of the latter is fluorescein diacetate/propidium iodide (FDA/PI), which allows the identification of viable (bright green fluorescence) and dead cells (red fluorescence). **Figure 2** shows a representative picture of vitrified-warmed cat COCs stained with FDA/PI right after warming. Data from the experiments showing the percentage of survival after vitrification are reported in **Table 1**, which depicts post-warming data of oocytes intended for in vitro maturation[17] or in vitro embryo production[16]. On the whole, in the two experiments used as examples here[16,17], 395 out of 435 oocytes survived, scoring an overall 90.8% post-warming viability.

However, some morphological changes can be noticed after warming (**Figure 3**). In gametes vitrified-warmed following this protocol, the most frequent morphological abnormalities are changes in ooplasm shape and granulation, partial (or, rarely, total) loss of cumulus cells and (rarely) zona pellucida fractures. On the other hand, as reported in our previous works on minimum volume vitrification with the same support, these vitrified COCs can mature[17] and develop into embryos in vitro[16], even if at lower rates than fresh COCs. In addition, they do not usually present zona pellucida hardening (which is another well-known consequence of cryopreservation), since in vitro fertilization (IVF) is successful[16].

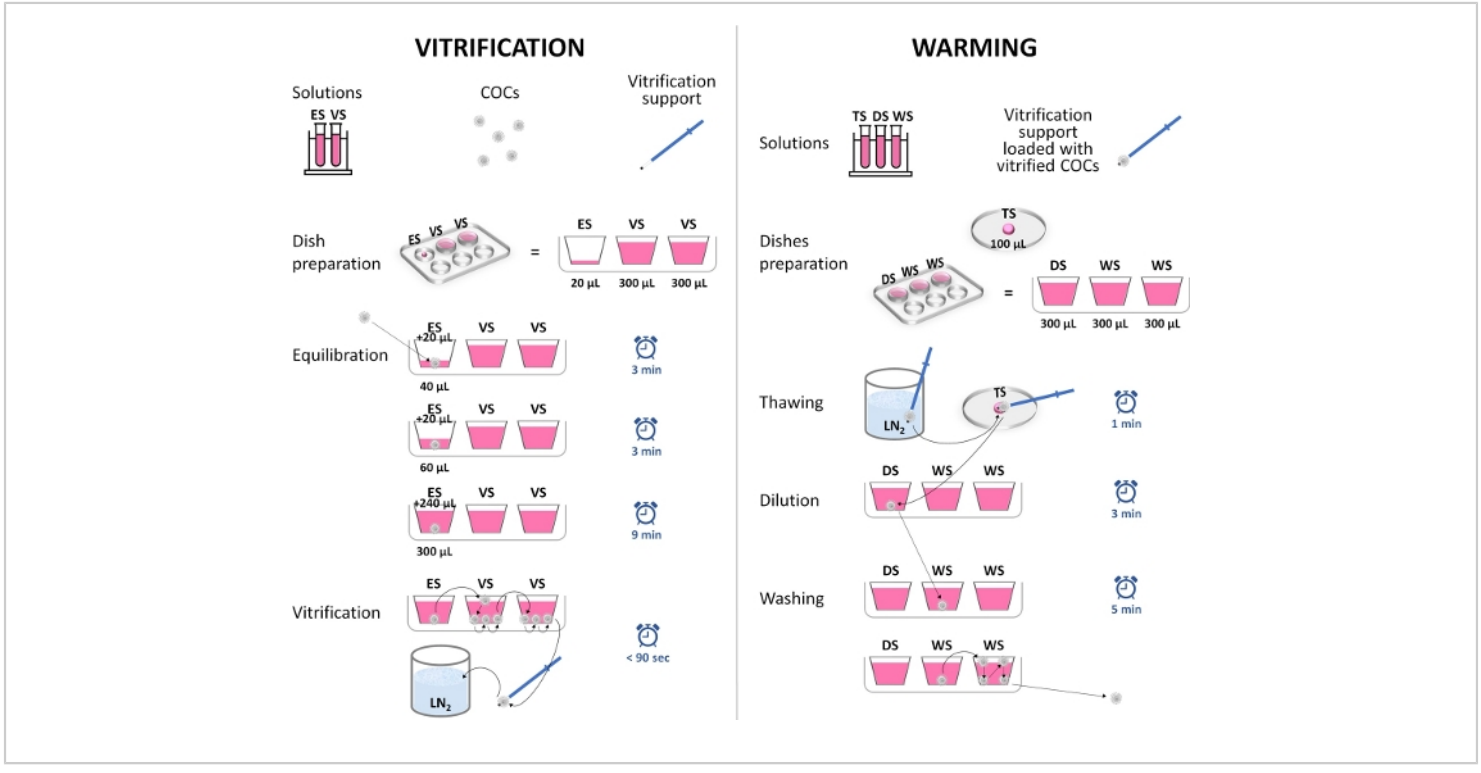

Figure 1: Schematic depiction of oocyte vitrification-warming protocol.

Please refer to the manuscript text for complete indications. COCs= cumulus-oocyte complexes; ES=equilibration solution; VS=vitrification solution; TS=thawing solution; DS=dilution solution; WS=washing solution; LN_2 =liquid nitrogen. Please click here to view a larger version of this figure.

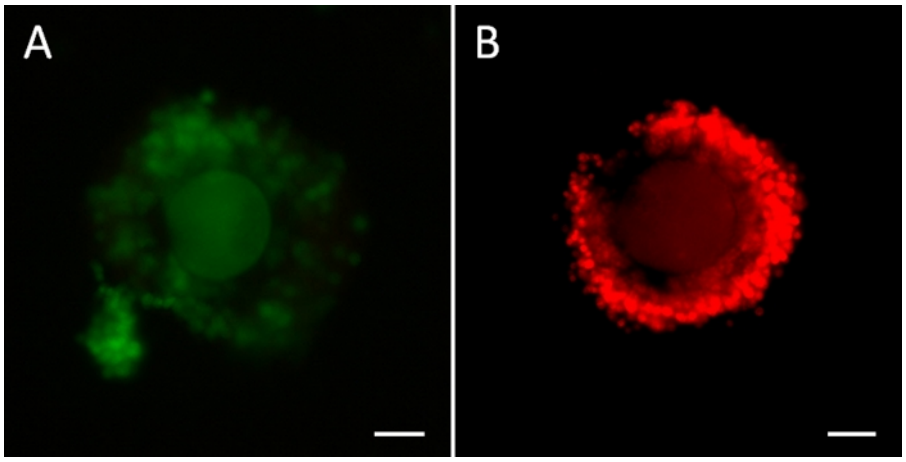

Figure 2: Viability of vitrified cat oocytes after warming.

Vitrified cumulus-oocyte complexes stained with fluorescein diacetate/propidium iodide (FDA/PI) show green fluorescence when viable (**A**) or red or weak fluorescence when dead (**B**). Excitation/emission wavelengths: 495 nm/517 nm for FDA; 538 nm/617 nm for PI. Scale bar: 50 µm. Please click here to view a larger version of this figure.

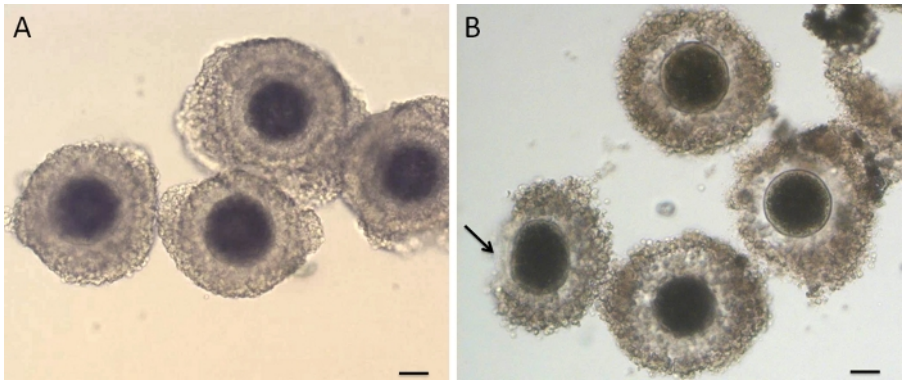

Figure 3: Light micrographs of fresh and vitrified cat oocytes.

(**A**) Fresh cumulus-oocyte complexes after collection, before vitrification. (**B**) Vitrified cumulus-oocyte complexes after warming, showing some vitrification-induced injuries (changes in shape and loss of cumulus cells, black arrow). Scale bar: 50 µm. Please click here to view a larger version of this figure.

Experiment	Group	Warmed oocytes (n)	Viable oocytes (n)	Viability (%)	Viability (mean % ± SD)
1†	1	13	12	92.31	91.54 ± 3.66
	2	47	44	93.62	
	3	52	45	86.54	
	4	26	24	92.31	
	5	41	40	97.56	
	6	21	19	90.48	
	7	50	44	88.00	
2‡	1	17	17	100.00	91.51 ± 9.16
	2	17	17	100.00	
	3	9	8	88.89	
	4	21	21	100.00	
	5	30	23	76.67	
	6	26	23	88.46	
	7	17	13	76.47	
	8	10	10	100.00	
	9	15	14	93.33	
	10	23	21	91.30	

Table 1: Representative results of viability in cat vitrified-warmed oocytes. Data from †Colombo et al. 2019[16] and ‡ Colombo et al. 2020[17] .

Supplemental Figure 1: Representative picture of the supports used for oocyte vitrification. The commercial support measures 13 cm and is easy to handle and store. Oocytes have to be loaded on the thin plastic strip at the top of the device, as close as possible to the black mark near the tip. Copyright: Kitazato Corporation. Please click here to download this figure.

Discussion

Oocyte cryopreservation is a crucial germplasm conservation technique, especially in taxa where many species are endangered, such as Felidae family. In this manuscript,

a simple and field-friendly protocol for the vitrification of immature cat oocytes was presented. Laboratory-made media, minimum volume vitrification supports and trained personnel are the key factors for the success of this method, which allows obtaining viable oocytes consistently and repeatedly, as shown by the representative results hereby reported.

Beginning with media preparation, the protocol should be followed accurately to ensure the best results, but the operator's skills are the major issue. Media should be prepared the same day of the vitrification procedure, or, if that is not possible, they should be prepared the day before and stored at 4 °C. In any case, before sample processing, enough time should be left to allow the solutions to warm up to RT. Oocyte collection and COCs selection are quick and simple procedures that are routinely performed by every ARTs laboratory. However, cryopreservation is the most delicate procedure. While the warming procedure is not so critical, if the protocol and the timings are followed, vitrification might be more complex. After equilibration, in which care must be taken just in respecting timings, the most challenging phase is likely to be the vitrification step (see 2.4. of the present protocol). Indeed, oocyte transfers and pipette washings need to be well-timed and, all together, performed in less than 90 seconds. For beginners, it is advisable to time the procedure with a stopwatch while training, while also for trained individuals it might be useful to have a timer beeping after 60 seconds as an alert that time is running up. In addition, this would allow a higher standardization, since operators vitrifying groups of 6-8 COCs should be close to the transfer of the oocytes from the second to the third well of the Repro plate (see step 2.4.7 of the present protocol) when the alarm goes off. Hopefully, with the aid of this article, a higher standardization between individuals and between laboratories could be achieved.

The major limitations of the protocol remain the intrinsic features of cryopreservation procedures themselves. As previously noted, vitrification exposes oocytes to non-physiological and potentially damaging conditions, even if it is performed flawlessly. The incubation with cryoprotectants and the decrease of temperature are the most critical events[20] and they need to be kept under strict control. Among the most common cryoinjuries, some are easily observable at the optical microscope (e.g., cumulus cell loss, alteration of cellular shape and size), while others are not visible and often affect subcellular structures (e.g., zona pellucida hardening, oxidative stress, cytoskeleton damages, meiotic spindle and DNA alterations)[23, 24]. Although mostly viable, COCs vitrified following this protocol often present some evident morphological anomalies after warming. However, there is no correlation between morphology and viability, which might also be hampered because of subcellular damages[3, 25], but morphological alterations are likely a consequence of cryoinjuries and partially a reason for the in vitro developmental outcomes of vitrified oocytes, which are quite poor if compared with that of fresh oocytes. The careful preparation of vitrification media and the precise observation of protocol timing contribute to obtain good post-warming viability and morphological integrity, but improvements in further in vitro maturation of oocytes and embryo development are still needed, since viability often decreases during the following culture[16].

Cold-induced injuries unfortunately occur with every cryopreservation method[24]. The minimum volume vitrification support used in this protocol has been developed to reduce the vitrification volume as much as possible,

resulting in better cooling and warming rates compared to other cryopreservation devices (-23,000 °C/minute and 42,000 °C/minute respectively, according to the manufacturer specifications). As a consequence, in human medicine, where clinical studies assessing both in vitro (i.e., fertilization and embryo development) and in vivo parameters (i.e., pregnancy rates and live births) are available, minimum volume vitrification is the most common choice for oocyte cryopreservation due to its incomparable results in terms of post-warming survival and, finally, live births[12] from vitrified mature human oocytes. In addition, compared to other vitrification devices, the one used in this protocol is easier to use and safer to store[12], since it is comfortable to handle and is protected by a cap during storage. For instance, the Cryoloop is also an efficient support in order to reach the minimal volume goal, but its structure (a loop supporting a film of solution on which the oocytes are loaded) is fragile and prone to accidental warming[12]. Straws (0.25 mL volume) or open pulled straws (OPS) can also be used, but they do not allow a significant reduction in the vitrification volume and are also challenging to fill and empty, as some oocytes might be lost[26]. Many other supports have been developed[2], but each of them has its drawbacks. Instead, compared to slow freezing, which previously was the most used cryopreservation technique, the main advantages of minimum volume vitrification on commercial supports are its feasibility and speed. As previously mentioned, vitrification does not require a programmable freezer to be performed, and while slow freezing of oocytes takes about one hour and half to be concluded[25], vitrification can be accomplished in about 17 minutes following the present protocol.

In conclusion, staff skills and inter-operator standardization might be the most challenging requirements when starting a vitrified oocyte bank, but they will be achieved with personnel training. This minimum volume vitrification protocol for cat immature oocytes gives consistent and repeatable results when performed by experienced operators. However, there are still chances for improvement in oocyte developmental rates, even if post-warming viability and integrity are satisfactory. With further optimization, the present protocol could also be applied in field conditions for wild felids, for which published data concerning oocyte cryopreservation is still scarce[27]. Widening the application of this method not only would increase the possibility to improve cryopreservation protocols, but would also allow establishing vitrified oocyte biobanks for fertility and biodiversity preservation in felids.

Disclosures

The authors have nothing to disclose.

Acknowledgments

This work was partly supported by EVSSAR (European Veterinary Society for Small Animal Reproduction) Grant 2016 and by Università degli Studi di Milano, Piano di Sostegno alla Ricerca 2019 (Linea 2 Azione A). We also wish to thank Dr. MariaGiorgia Morselli for her contribution to the experiments hereby depicted and to image acquisition.

References

1. Bankowski, B., Lyerly, A., Faden, R., Wallach, E. The social implications of embryo cryopreservation. *Fertility and Sterility.* **84** (4), 823–832 (2005).
2. Saragusty, J., Arav, A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction.* **141** (1), 1–19 (2011).
3. Luvoni, G.C. Gamete cryopreservation in the domestic cat. *Theriogenology.* **66** (1), 101–111 (2006).

4. Thongphakdee, A., Tipkantha, W., Punkong, C., Chatdarong, K. Monitoring and controlling ovarian activity in wild felids. *Theriogenology.* **109**, 14–21 (2018).
5. Parks, J.E. Hypothermia and mammalian gametes. *Reproductive Tissue Banking.* 229–261 (1997).
6. Carabatsos, M.J., Sellitto, C., Goodenough, D.A., Albertini, D.F. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Developmental Biology.* **226** (2), 167–179 (2000).
7. Murakami, M. et al. Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. *Cryobiology.* **48** (3), 341–348 (2004).
8. Merlo, B., Iacono, E., Regazzini, M., Zambelli, D. Cat blastocysts produced in vitro from oocytes vitrified using the cryoloop technique and cryopreserved electroejaculated semen. *Theriogenology.* **70** (1), 126–130 (2008).
9. Luciano, A.M. et al. Effect of different cryopreservation protocols on cytoskeleton and gap junction mediated communication integrity in feline germinal vesicle stage oocytes. *Cryobiology.* **59** (1), 90–95 (2009).
10. Comizzoli, P., Wildt, D.E., Pukazhenthi, B.S. In vitro compaction of germinal vesicle chromatin is beneficial to survival of vitrified cat oocytes. *Reproduction in Domestic Animals.* **44** (SUPPL. 2), 269–274 (2009).
11. Alves, A.E., Kozel, A.C., Luvoni, G.C. Vitrification with DAP 213 and Cryotop of ex situ and in situ feline cumulus-oocyte complexes. *Reproduction in Domestic Animals.* **47** (6), 1003–1008 (2012).
12. Kuwayama, M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology.* **67** (1), 73–80 (2007).
13. Pope, C.E. et al. In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. *Theriogenology.* **77** (3), 531–538 (2012).
14. Galiguis, J., Gómez, M.C., Leibo, S.P., Pope, C.E. Birth of a domestic cat kitten produced by vitrification of lipid polarized in vitro matured oocytes. *Cryobiology.* **68** (3), 459–466 (2014).
15. Fernandez-Gonzalez, L., Jewgenow, K. Cryopreservation of feline oocytes by vitrification using commercial kits and slush nitrogen technique. *Reproduction in Domestic Animals.* **52**, 230–234 (2017).
16. Colombo, M., Morselli, M.G., Tavares, M.R., Apparicio, M., Luvoni, G.C. Developmental competence of domestic cat vitrified oocytes in 3D enriched culture conditions. *Animals.* **9** (6), 329 (2019).
17. Colombo, M., Morselli, M.G., Apparicio, M., Luvoni, G.C. Granulosa cells in three-dimensional culture: A follicle-like structure for domestic cat vitrified oocytes. *Reproduction in Domestic Animals.* (2020).
18. Wood, T.C., Wildt, D.E. Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. *Journal of Reproduction and Fertility.* **110** (2), 355–360 (1997).
19. Cobo, A., Kuwayama, M., Pérez, S., Ruiz, A., Pellicer, A., Remohí, J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertility and Sterility.* **89** (6), 1657–1664 (2008).

20. Mullen, S.F., Critser, J.K. The science of cryobiology. *Oncofertility - Fertility preservation for Cancer Survivors.* 83–109 (2007).

21. Fahy, G.M., Wowk, B. Principles of cryopreservation by vitrification. *Methods in Molecular Biology.* **1257**, 21–82 (2015).

22. Apparicio, M., Ruggeri, E., Luvoni, G.C. Vitrification of immature feline oocytes with a commercial kit for bovine embryo vitrification. *Reproduction in Domestic Animals.* **48** (2), 240–244 (2013).

23. Brambillasca, F., Guglielmo, M.C., Coticchio, G., Mignini Renzini, M., Dal Canto, M., Fadini, R. The current challenges to efficient immature oocyte cryopreservation. *Journal of Assisted Reproduction and Genetics.* **30** (12), 1531–1539 (2013).

24. Moussa, M., Shu, J., Zhang, X., Zeng, F. Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. *Science China Life Sciences.* **57** (9), 903–914 (2014).

25. Luvoni, G., Pellizzari, P., Battocchio, M. Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. *Journal of Reproduction and Fertility. Supplement.* **51**, 93–98 (1997).

26. Calvo, J.J., Robert, D., Viqueira, M., Lombide, P., Calvo, J.J. *Vitrified and* in vitro *matured oocytes viability from adult housecat (Felis catus) in reproductive season. International Journal of Morphology.* **33** (4) (2015).

27. Nowak, A. et al. The viability of Serval (Leptailurus serval) and Pallas Cat (Felis manul) oocytes after cryopreservation using the Rapid-I Method. *Cryo Letters.* **40** (4), 226–230 (2019).