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Vitrification of Immature Feline Oocytes with a Commercial Kit for Bovine Embryo Vitrification

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Contents

The aim of this study was to evaluate the suitability of a commercial kit for bovine embryo vitrification for cryopreserving cat oocytes and to evaluate comparatively the effects of its use with slow freezing procedure on cryotolerance in terms of morphology and oocyte resumption of meiosis. Germinal vesicle stage oocytes isolated from cat ovaries were either vitrified ($n = 72$) using a vitrification kit for bovine embryo or slow frozen ($n = 69$) by exposing oocyte to ethylene glycol solution before being transferred to a programmable embryo freezer. After thawing and warming, oocytes were cultured for 48 h and then were examined for meiosis resumption using bisbenzimide fluorescent staining (Hoechst 33342). Fresh immature oocytes ($n = 92$) were used as the control group. The proportion of oocytes recovered in a morphologically normal state after thawing/warming was significantly higher in frozen oocytes (94.5%) than in the vitrified ones (75%, $p < 0.01$). Morphological integrity after culture was similar in vitrified (73.6%) and slow frozen oocytes (76.8%); however, only 37.5% of the morphologically normal oocytes resumed meiosis after vitrification compared to 60.9% of those submitted to slow freezing procedure ($p < 0.01$). Fresh oocytes showed higher morphological integrity (91.3%) and meiosis resumption rates (82.6%, $p < 0.002$) than cryopreserved oocytes, irrespective of the procedure used. These results suggest that immature cat oocytes vitrified with a kit for bovine embryos retain their capacity to resume meiosis after warming and culture, albeit at lower rates than slow frozen oocytes. Vitrification and slow freezing methods show similar proportions of oocytes with normal morphology after culture, which demonstrate that thawed and warmed oocytes that resist to cryodamage have the same chances to maintain their integrity after 48 h of culture.

Introduction

Cryopreservation of oocytes is an important tool in assisted reproduction programs as it allows long-term storage for future use as well as maintenance of biodiversity through genetic preservation (Luvoni 2006). However, the methods of cryopreservation used up to date still needs adjustments once they are often related to low survival and development rates (Ambrosini et al. 2006).

It is well known that during cryopreservation of oocytes, there is an inevitable morphological and functional damage, although the extent of injury may be variable depending on the species (Pereira and Marques 2008). While bovine oocytes are well freezable, cat oocytes present an elevate cryosensibility, mostly owing to their high lipid content (Luvoni et al. 1997; Pereira and Marques 2008). It is believed that the presence of intracellular lipid droplets could be responsible for uneven intracellular ice formation, which could affect the freezing–thawing process (Luvoni 2006). For this reason, efforts have been made to improve and simplify cryopreservation procedures to overcome these injuries and to preserve the developmental ability of the oocyte after thawing.

Different methods of cryopreservation have been employed in cat oocytes with distinct results. The standard method (slow freezing) implies slow cooling rate and the use of relatively low concentration of cryoprotectants, but does not prevent ice-crystal formation. On the other hand, vitrification avoids ice-crystal formation and it has become a reliable alternative to the traditional approach (Pereira and Marques 2008). Its increasing application to mammalian gametes and embryos is attributable to the simplicity of the procedure and the satisfying results obtained in gamete and embryo survival. Recently, kits for vitrification are available on the veterinary market, mainly for bovine embryo. These commercial products have the advantage to be practical, standardized and ease to perform.

Previous studies have reported conflicting results regarding oocyte cryopreservation at different maturation stages (Luvoni 2006). According to Luciano et al. (2009), the use of immature (germinal vesicle stage) oocytes has its justification once they are more easily obtained in significant numbers compared to mature oocytes that are mostly obtained after *in vitro* culture or from ovaries that have been submitted previously to *in vivo* hormonal treatment. Moreover, germinal vesicle (GV) stage oocytes are considered more resistant to cold-induced damage once the chromatin is still protected by a nuclear envelope and no spindle apparatus is present (Luciano et al. 2009), and a recent study investigating cat oocyte vitrification in open pulled straws (OPSs) demonstrated that vitrified GV oocytes were able to develop until blastocyst stage (Cocchia et al. 2010).

The purpose of this study was to evaluate the suitability of a commercial kit for bovine embryo vitrification to cryopreserve immature cat oocytes. Cryotolerance in terms of morphological integrity after cryopreservation (vitrification vs slow freezing) and subsequent meiosis resumption after *in vitro* culture were compared.

Materials and Methods

All chemicals in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated.

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Oocyte collection

Queen ovaries were collected after routine ovariectomy and transported to the laboratory at room temperature in Dulbecco's PBS supplemented with antibiotics (100 UI/ml penicillin G sodium salt + 100 μg/ml streptomycin sulphate) within 1–4 h. Ovaries were sliced with a scalpel blade to release the cumulus-oocyte complexes (COCs), and only those with intact corona radiata, compacted cumulus cells and medium to dark pigmented cytoplasm were selected for the experiments. Then, they were rinsed and held at room temperature in Dulbecco's PBS-antibiotic solution with 0.1% polyvinyl alcohol before being randomly divided into two groups: vitrification and slow freezing.

Cryopreservation of immature oocytes

Slow freezing

Slow freezing was performed accordingly to the protocol described by Luvoni and Pellizzari (2000). Briefly, COCs were exposed to 1.5 M ethylene glycol and 0.2 M sucrose solution at room temperature for 20 min. After equilibration in the freezing solution, oocytes were loaded into 0.25-ml straws (maximum of seven oocytes per straw) and placed in a programmable freezer (EF-1 Agrogen; Freiburg, Switzerland). The cooler rate was of −2°C/min from +25°C to −7°C. After manual seeding with chilled forceps, straws were cooled at a rate of −0.3°C/min to −30°C before being plunged into liquid nitrogen.

Vitrification

Vitrification was performed using a Syngro bovine vitrification kit® (Bioniche Animal Health, Pullman, WA, USA) following the manufacturer's instructions. Briefly, COCs were placed in 1000 μl vitrification solution 1 (VS1) for 5 min. While the oocytes were in VS1, a column of diluent was aspirated into the straw followed by air. After the 5-min period elapsed, oocytes were transferred in 15 μl drop (which was the amount to be aspirated into the straw) of VS2 solution for 60 s. Before the end of the 60 s, oocytes were drawn up by holding the 0.25-ml straws vertically over the drop followed by another column of air and a second column of diluents and were sealed (total period = 60 s). They were placed in a plastic goblet and maintained in liquid nitrogen vapours for 1 min. Then, goblet was plunged into liquid nitrogen for storage.

The exact composition of the vitrification solutions employed in the present study is not known because of the proprietary issues.

Thawing, warming and morphological assessment

Oocytes were thawed by exposing straws to air for 10 s followed by 30 s holding in a water bath at +38°C. Then, frozen COCs were recovered from the straws and equilibrated for 5 min within freezing solution, while straws with vitrified oocytes were held at the cotton plugged end and shaken five times like a clinical thermometer to mix diluents and vitrification solution. Oocytes were immediately recovered and transferred to a petri dish where they were washed two times in maturation medium, with a total period of exposure of less than a minute. After removal of cryoprotectant, vitrified and frozen oocytes were assessed for morphological damage post-thawing/warming. Oocytes with symmetrical shape with no signs of disruption, membrane damage, degeneration and surrounded with compacted cumulus cells were classified as normal (Fig. 1a), and those with damaged cumulus cells (partly or fully denuded), disruption of zona pellucida, leakage of cytoplasm or change in shape were classified as abnormal oocytes (Fig. 1b). Only those with normal morphology were subjected to *in vitro* maturation.

In vitro maturation

Cryopreserved oocytes were subjected to IVM. Oocytes were rinsed twice in the maturation medium and then cultured for 48 h in 100 μl drops (maximum 10 oocytes per drop) under oil at 38.5°C in 5% CO_2 in air. The maturation medium was modified Krebs-Ringer bicarbonate (mKRB) supplemented with antibiotics (100 IU/ml penicillin G potassium salt + 100 μg/ml streptomycin sulphate), 3 mg/ml BSA, 0.5 IU/ml FSH + 0.5 IU/ml LH (Pluset; Bio98, Milano, Italy) and 0.6 mM cysteine.

A group of freshly isolated COCs was simultaneously cultured for 48 h as described above and used as a control.

Assessment of morphology and meiotic maturation

After 48 h of culture, oocytes were evaluated again for morphological changes and then were denuded by gentle mechanical pipetting with a small bore glass pipette. To assess meiotic maturation, denuded oocytes were fixed and stained with a solution of sodium citrate/ethanol (3 : 1) containing 10 μg/ml of bisbenzimide (Hoechst 33342, Sigma-Aldrich); they were placed on a slide with a minimum amount of medium and covered by 10 μl of Hoechst solution. Elapsed 5 min of incubation in the

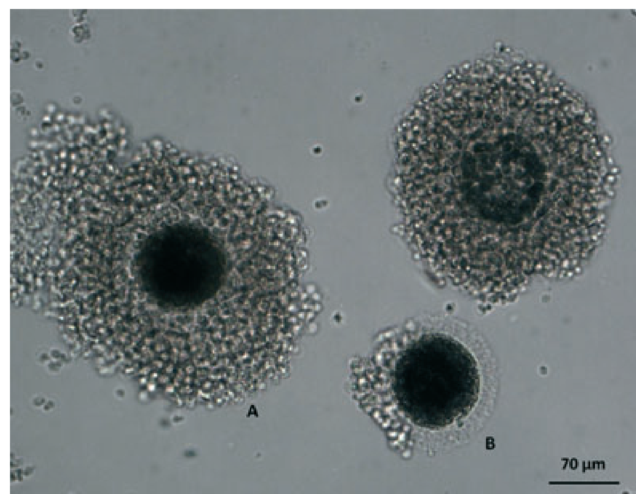

Fig. 1. Domestic cat oocytes after thawing (a) Normal morphology. (b) Abnormal morphology

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dark, the Hoechst solution was removed, and the oocytes were covered with an anti-fade reagent (Gel Mount™; Biømeda corp, Foster City, CA, USA). The oocytes were overlaid with a coverslip supported by four small droplets of acetic silicone sealant and then observed under a fluorescent microscope (Axiovert 100; Zeiss, Arese, Italy) at 400× magnification for the evaluation of meiotic status. Nuclear stages ranging from germinal vesicle breakdown (GVBD) to metaphase II (MII) were considered to have resumed meiosis. Oocytes in which nuclear stage was not identifiable or the chromatin was missing were considered degenerate.

Statistical analysis

The percentage of morphologically intact oocytes and the percentage of oocytes resuming meiosis (GVBD-MII) after cryopreservation and thawing were analysed by chi-square test. Values were considered to be significant at $p < 0.05$.

Results

A total of 169 oocytes were thawed/warmed and 141 were morphologically intact (Table 1); those were submitted to IVM and evaluated for resumption of meiosis (72 vitrified and 69 frozen). Untreated oocytes ($n = 92$) were used as control.

There was a significant difference between the percentage of morphologically normal oocytes after thawing/warming; frozen oocytes presented higher rates (94.5%) of intact morphology compared to the vitrified ones (75%, $p < 0.01$).

No significant differences were noticed between vitrified (73.6%) and slow frozen oocytes (76.8%) in morphologically normal form after IVM (Table 1), but morphological integrity was remarkably lower compared to that of fresh oocytes (91.3%; $p < 0.01$ and $p < 0.05$, respectively).

Table 2 presents the rates of meiotic resumption of cryopreserved oocytes and those used as control (untreated oocytes) after 48 h of culture. The percentage of vitrified oocytes that resumed meiosis was remarkably lower (37.5%) compared to slow frozen oocytes (60.9%; $p < 0.01$). However, meiotic competence of cryopreserved oocytes was significantly lower than that of fresh oocytes (control group = 82.6%; $p < 0.002$), irrespective of the procedure used.

Table 1. Morphological integrity of cryopreserved oocytes after thawing/warming and after 48 h of IVM

Treatment groups	Examined oocytes No.	Oocytes with normal morphology after thawing/warming* No. (%)	Oocytes with normal morphology after IVM** No. (%)
Fresh (control)	92	–	84 (91.3)^{a}
Slow frozen	73	69 (94.5)^{a}	53 (76.8)^{b}
Vitrified	96	72 (75)^{b}	53 (73.6)^{b}

^{ab}Values with different superscripts in the same column are significantly different at $p < 0.05$.
*On the total no. of examined oocytes.
**On the no. of oocytes with normal morphology after thawing/warming.

Table 2. The effect of the Syngro bovine vitrification kit® and slow freezing method on resumption of meiosis of cat oocytes after 48 h of IVM

Treatment groups	Oocytes examined No. (%)	GV No. (%)	Meiosis resumption* No. (%)	Degenerated No. (%)
Fresh (control)	92	8 (8.7)^{a}	76 (82.6)^{a}	8 (8.7)^{a}
Slow frozen	69	11 (15.9)^{a}	42 (60.9)^{b}	16 (23.2)^{b}
Vitrified	72	26 (36.1)^{b}	27 (37.5)^{c}	19 (26.4)^{b}

GV, germinal vesicle.
*Meiosis resumption = GVBD-MII.
^{abc}Values with different superscripts within columns are significantly different at $p < 0.05$.

Discussion

The use of vitrification kits to cryopreserve bovine embryos has increased in the past decade, mostly owing to its practical procedure. Commercial products have also the advantage to be standardized, easy to perform and most of them do not contain components of animal origin, which minimizes the risk of contaminating pathogens (Hasler 2010). For these reasons, we decided to test the feasibility of the use of a commercial vitrification kit developed for bovine embryos to cryopreserve cat oocytes for the first time. To make sure that there were no deviations of equilibrium nature or in timing, we executed the procedure following the kit manufacturer's instructions in details, including the amount of diluents and vitrification solution recommended. Our results showed that the percentage of intact morphology after thawing/warming was remarkably lower in vitrified oocytes than in the frozen ones. However, vitrified oocytes were able to resume meiosis following IVM and showed similar degeneration rates after culture than those cryopreserved by the conventional slow freezing method.

During the process of cryopreservation, oocytes suffer considerable morphological and functional damage that are critical to development (Prentice and Anzar 2011). Post-thaw/warm oocytes often exhibit zona pellucida or oolemma fractures and cytoplasm degeneration; evaluation of morphological integrity immediately after thawing/warming has been used by several authors as a non-invasive parameter indicative of cryoinjury, while assessment of morphology after *in vitro* culture as an indicative of resilience. Therefore, assessment of survival has been accomplished based on morphological criteria and developmental potential *in vitro* in several species, including humans (Tsagias et al. 2006; Prentice and Anzar 2011). In the present study, 75% of vitrified oocytes were recovered in morphologically normal form, while 94.5% of the slow frozen ones maintained their integrity after thawing. However, despite the clear morphological evidence that slow frozen oocytes were more resistant to cryodamage than vitrified, similar proportions of oocytes presented normal morphology after *in vitro* maturation (73.6% vs 76.8%), which demonstrated that thawed and warmed oocytes that resist to cryodamage have the same chances to maintain their integrity after 48 h of culture.

The percentage of morphological survival obtained in the current study (74%) after vitrification-warming-culture of GV stage oocytes was higher than those found

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by Murakami et al. (2004) and Merlo et al. (2008) after vitrification of *in vitro* matured cat oocytes in straws (50.7%) and in cryoloop (50.2%), respectively, and also than that reported by Cocchia et al. (2010) after vitrification of immature cat oocytes using the open pulled straws technique (OPS: 45.3%). It is important to point out that in the latter study, the number of surviving oocytes was assessed following cFDA/Trypan blue staining, while in the others and in the present study, morphological criteria were used to judge cell injury, therefore direct comparison between data reported by Cocchia et al. and those obtained in the present study is unreliable. The high percentage of intact oocytes found in the current study compared to that from Murakami et al. (2004) and Merlo et al. (2008) might be attributed to the different meiotic stage of the oocytes before cryopreservation (immature vs mature) and to the minimum period of exposure to vitrification solutions and diluents during warming. Murakami et al. (2004) investigated the effects of incubation period (0.5, 1 or 5 min) in a sucrose solution during dilution of cryoprotectant from vitrified-warmed cat oocytes. According to them, cat oocytes incubated in the sucrose solution for 5 min showed lower rates of survival compared to their counterparts incubated for a shorter period (0.5 min). In the present study, straws were shaken like a clinical thermometer to mix diluents and vitrification solution, and oocytes were immediately recovered and transferred to a petri dish where they were washed two times in maturation medium, with a total period of exposure of less than a minute. We believe that this procedure could have better maintained morphological integrity after vitrification, even though was insufficient for the developmental competence of the oocytes once the ability to mature *in vitro* was severely impaired. Indeed, Luvoni et al. (1997) have reported that morphologically normal oocytes were not able to resume meiosis after ultrarapid freezing–thawing culture, demonstrating that morphology is not predictive of the ability to resume meiosis once that structurally and subtle damage that cause important modifications might not be identified when only morphology is evaluated through light microscope. Taken together, the above-mentioned study and our results reinforces the idea that a morphologically normal oocyte could be incompetent for meiosis and so morphological assessment after thawing is a limiting factor in predicting developmental potential.

To confirm viability of morphological normal oocytes and their cryotolerance after cryopreservation, resumption of meiosis was evaluated; rates of resumption of meiosis occurred in vitrified oocytes, even though at lower rates (37.5%) compared to frozen (60.9%) or to the fresh ones (82.6%). Similar findings were reported by Luciano et al. (2009) with regard to mature (Telophase I-MII stage) oocytes obtained after cryopreservation/thawing/IVM (14.1%, 32.5% and 71.2% for vitrified, slow frozen and fresh oocytes, respectively). In the referred study, the authors demonstrated that the slow freezing method preserved the cytoskeleton organization better than vitrification, even though the integrity and cytoplasmic organization were severely affected by both procedures. Also, they reported a relation between an abnormal organization of cytoskeleton and the lower meiotic capability after IVM, which explain the low competence of cryopreserved cat oocytes, irrespective of the method used. Moreover, we should take into account that feline oocytes are considerably less cryostable compared to others, such as bovine; and oocytes are more sensitive to cryoinjury than embryos because of their large size and consequently low surface to volume ratio (Pereira and Marques 2008). These differences between these two species and the fact that the vitrification kit used in our study was developed for bovine embryos may explain the limited developmental capacity of vitrified cat oocytes after *in vitro* culture. Future studies using other commercial kits and/or different cryodevices for vitrification should be tested for feline species to find an appropriate cryoprotectant mixture that would improve maturation rates and embryo development. Recently, Comizzoli et al. (2009) investigated the compactation of GV chromatin prior to the vitrification procedure and reported that thawed oocytes successfully developed into embryos (>60% advancing to 4–7 or 8–16 cell stage). Cocchia et al. (2010) demonstrated that immature cat oocytes vitrified in OPS were able to develop *in vitro* until the blastocyst stage. The above-mentioned results are at least encouraging and prompt to the need of new investigations that would improve vitrification procedures and reduce cryodamage. In fact, up to date there are no data regarding the ultrastructural changes, besides cytoskeleton organization and cumulus-oocyte gap junction–mediated communication (Luciano et al. 2009), observed in cat oocytes in response to their sensitivity to low temperatures that would certainly help to optimize the vitrification procedure. Moreover, further studies focused on enrichment of culture systems are needed to provide a better environment for thawed/warmed oocytes that have lost their *cumulus* cells during cryostorage, producing higher rates of embryo development.

In conclusion, these results suggest that immature cat oocytes vitrified with a kit for bovine embryos are able to resume meiosis after warming and culture, albeit at lower rates than slow frozen oocytes. Vitrification and slow freezing methods show similar proportions of morphologically normal oocytes after culture, which demonstrate that thawed and warmed oocytes that resist to cryodamage have the same chances to maintain their integrity after 48 h of culture.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Luvoni and Apparicio contributed to design the study, experimental phase, analyse the data and draft the paper. Ruggeri contributed during the experimental phase in the laboratory and with the draft of the paper.

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