The use of a metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation, after different times and temperatures of transport

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Abstract

Purpose Cryopreservation of ovarian tissue is paramount for fertility preservation, with important clinical applications, especially for women suffering from an oncological condition. Several cryopreservation methodologies have been tried in search of better outcomes, especially in terms of primordial and primary follicles integrity post-cryopreservation. Vitrification has successfully been applied to ovarian tissue using different carriers for tissue exposure to the liquid nitrogen (LN₂).

Methods We developed an enclosed metal vessel, which has the advantage of a faster heat transfer, when in contact with LN₂ avoiding at the same time, the direct contact with tissue. Additionally, we assessed the effect of different times and temperatures of transport between the collection of mouse ovaries and the beginning of cryopreservation, on follicular morphology after vitrification.

Capsule Vitrification of ovarian tissue in a metal container results in high rates of morphologically normal primordial follicles up to 4 hrs between tissue collection and cryopreservation.

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Results Our results suggest that 37 °C and R.T. help to maintain normal primordial and primary follicle morphology for up to 4 hrs after collection and beginning of vitrification in a metal container.

Conclusion These data show that the metal container is an appropriate carrier for mouse ovary vitrification. The rate of morphologically normal primordial follicles up to 4 hrs.

Keywords Metal container · Vitrification · Ovaries

Introduction

Ovarian tissue cryopreservation has been gaining growing attention by the infertility and oncologist experts, as it represents an enormous benefit for young women facing a cancer treatment and may be at risk of premature ovarian failure and sterility.

Previous studies have proven that the cryopreservation of human ovarian tissue is possible and may result in successful live births after auto-transplantation [3,6,15,22]. Vitrification, as a choice of cryopreservation, has shown promising outcomes in terms of follicle and stromal preservation in rodents and other species including humans [1,2,4,10,13,16,17,19,21]. Despite these promising results, little attention has been paid to how the tissue should be handled before cryopreservation, to guarantee the best possible post-storage outcome. In a previous report we showed that the size of the ovarian fragment directly affects follicular survival after vitrification and it is an important parameter to be taken into account, when preparing the tissue for cryopreservation [7]. On the other hand, limited information is available on the ideal conditions to which ovarian tissue should be exposed

before cryopreservation, such as the time before cryopreservation and the temperature that the fragments should be maintained. These are important parameters to consider for the effectiveness of the cryopreservation process, as in several situations the Hospital or clinic, where the patient may have the ovarian fragments removed shall not possess cryopreservation facilities and the tissue samples will have to travel to a different institution, which may take hours before the procedure begins.

Another important point to appraise is the growing concerns on the possible risks of contamination due to the direct contact of the biological material with the liquid nitrogen, when vitrification is the choice method for cryopreservation. This issue has impelled scientists to develop systems, in which the biological material is never directly exposed to the LNi during cryopreservation and subsequent cryostorage. In this regard, closed systems were developed for the vitrification of macaque and the human ovarian tissue using cryovials [17,20]. However, both studies employed plastic devices to enclose the ovarian fragments, and attention should be given to alternative materials that present faster heat-conduction, a key factor for successful vitrification. Metals usually conduct heat extremely well and may represent an alternative material for making a closed, clinical grade system to vitrify human ovarian tissue.

Despite the fact that mice ovaries may not be the ideal model for human ovarian tissue cryopreservation, because of their looser structures [8], we chose the mouse model to develop this study, because of the easiness to obtain them from the Biological Facilities of our University, in contrast to the ethical issues and limited access to donated human ovarian specimens.

The present study investigated the possible effects of three different temperatures of the medium in which mouse ovaries were placed before cryopreservation and two different times between collection and the beginning of vitrification. For the vitrification procedure, we developed a simple hand-made aluminum foil container. The morphology of the fragments was studied using light microscopy after re-warming, giving special attention to the structure of the primordial and primary follicles of the ovarian cortex.

Material and methods

Animals

Female Albino mice were maintained under controlled lighting (12L:12D), temperature (20 to 25 °C) and humidity (40–60 %) conditions in a the Animal Facility of our University. The study was approved by our Institutional Ethics Committee.

Vitrification

Whole ovaries were dissected from 18, 2–3 week-old albino female mice. Each experimental group was composed of three ovaries from different females. One ovary of each of the 18 females was placed in TC199 (Sigma-Aldrich, USA) medium supplemented with 20 % fetal calf serum (FCS; Nutricel, Campinas, SP) in one the following experimental groups: 1) at 4 °C on ice, 2) at R.T.(23–25 °C) or 3) in an incubator at 37 °C. The other ovary of each of the same 18 females was fixed in Bouin's solution as control. For each of these three experimental groups another subdivision was made relative to the timing of transportation a) 2 h and b) 4 h. Thus, there were in total 6 experimental groups and one control group.

Cryopreservation protocol followed a modification of the method described by Choi et al. [5] for the vitrification of mice ovaries. Ovaries were carefully removed from the collection medium and stepwise equilibrated by exposure to an equilibrium solution (ES) of TCM199 containing 20 % (v:v) FCS, 7.5 % (v:v), ethylene glycol (EG) and 7.5 % (v:v) dimethyl sulfoxide (DMSO) for 15 min at R.T., then exposed to the vitrification solution (VS) containing 15 % (v:v) EG, 15 % (v:v) DMSO and 0.5 M sucrose. From the VS solution the ovaries were gently transferred with a minimum volume of medium to a handmade aluminum foil caddy, within 90 s of exposure to VS and the loose ends of the caddy were closed with a pair of forceps. The bottom side of this container was placed in contact with the surface of the LNi, where a 1.8 ml Nunc cryotube was immersed with its open side above the surface to avoid LNi coming inside the tube. The closed aluminum caddy was placed inside the cryotube, which was then tightly closed, plunged into the LNi and stored for a period between 2 days and 4 weeks before warming.

Warming and histological analysis

To rewarm, the cryotubes were taken from the LNi, the lids removed and the caddy containing the vitrified ovary was exposed to R.T. for 30–40 s and then to water bath at 37°C till the medium surrounding the tissue has melted. After removal from the aluminum container, the ovaries went through two 5 min steps in warming solutions consisting of TCM199 medium and 20 % FCS, supplemented with 1 M and 0.5 M sucrose, respectively, at R.T. Ovaries were then fixed in Bouin's solution for 2 h. The fixative was replaced with 70 % alcohol and processed for histological analysis. Ovaries were serially sectioned at 5 µm thickness. The sections were mounted on glass slides and stained with hematoxylin and eosin. Every second section of each ovary was analyzed for primordial and primary follicular morphology. Slides were stained with hematoxylin & eosin (HE).

Only follicles with a visible nucleus in the oocyte were considered for counting to avoid counting twice the same oocyte and follicle. We considered primordial follicles as those surrounded by one flat layer of follicular cells and primary follicles as those surrounded by one layer of cuboidal follicular cells.

We did not analyze growing or secondary, antral follicles. We considered intact follicles the ones that did not present any contraction of the oocyte cytoplasm or pyknotic nuclei. The oocyte had to be closely surrounded by the follicular cells. The follicular cells should not be detached from the basement membrane and there should not be any enlarged space between them. Analyses were performed at final magnification of 400 using an Olympus BX41 light microscope. Slides underwent blind analysis, by two independent embryologists.

Statistical analysis

Differences between groups were analyzed using one-way analysis of variance. Analyses were performed on the percentage of normal follicles in each experimental group. A value of $P < 0.05$ was considered statistically significant

Results

The mean percentages of morphologically normal primordial and primary follicles in vitrified and control ovaries submitted to the different temperatures and times before cryopreservation are presented in Table 1. Representative pictures of whole ovary and follicular morphology are shown in Figs. 1 and 2.

Primordial follicles from ovaries that were exposed to 37 °C or R.T. did not present significant changes, when compared

Fig. 1 Representative cross section of whole mouse ovary post-vitrification (experimental group R.T./2 h). Light microscopy (100X-HE)

with controls. However, the groups of ovaries exposed to 4 °C for 2 or 4 h, before vitrification showed a significant lower percentage of normal primordial follicles, when compared with controls. In addition, when each experimental group was independently compared against the others, only 4 °C for 2 and 4 h showed significant differences in the mean percentage of morphologically normal primordial follicles after vitrification. This result suggests that 37 °C and R.T. helps to maintain normal primordial follicle morphology for up to 4 h after collection and beginning of vitrification.

On the other hand, primary follicles showed a pronounced decrease in normal morphology in all experimental groups, when compared with controls. Again, the poorer results in terms of follicular damage were observed when ovaries were exposed for 2 or 4 h to 4 °C.

These observations suggest that primary follicles are more sensitive to cryodamage, than primordial follicles (Fig. 2).

Overall our data suggest that R.T. and 37 °C are more appropriate temperatures to transport mouse ovaries than 4 °C to preserve their primary and primordial follicles for up to 4 h before cryopreservation.

Discussion

The results presented in this study show that we can successfully vitrify mouse ovarian tissue using a metal container that goes inside a plastic cryovial, ensuring a fast cooling rate and avoiding the risk of contamination during vitrification and storage. Most relevant, we show that the normal morphology of primordial follicles do not undergo any significant morphological damage after exposure at 37 °C or R.T. for up to 4 h, using the metal caddy as a vessel for vitrification. Primordial follicles showed a remarkable recovery in terms of normal morphology and they represent the ovarian reserve or the fertility potential of a woman. Thus, their integrity after cryostorage is paramount in a fertility preservation program for women facing a cancer

Table 1 Means and standard deviation of the percentage of primordial and primary follicles showing normal morphology after different times and temperatures before vitrification

Experimental Group	Primordial Follicles (%) Mean (SD)	Primary Follicles (%) Mean (SD)
R.T./2 h	77.30 (24.86) ^{ab}	23.49 (18.27) ^{bc}
R.T./4 h	81.19 (25.02) ^{ab}	26.79 (27.65) ^{bc}
4 °C/2 h	37.98 (28.16) ^c	14.16 (22.42) ^{bc}
4 °C/4 h	54.41 (40.92) ^{bc}	9.97 (18.77) ^c
37 °C/2 h	85.42 (24.62) ^a	37.37 (30.80) ^b
37 °C./4 h	69.15 (30.14) ^{abc}	35.27 (43.24) ^{bc}
Control group	84.78 (28.82) ^a	71.16 (35.87) ^a

Different subscripts within a column indicate significant differences among groups

R.T., room temperature

Fig. 2 Light microscopy of mouse ovarian cortex showing primordial and primary follicles (400X-HE) after vitrification. In **a**) one primordial and one primary follicle not affected after treatment (experimental group R.T./2 h); **b**) primary follicles not affected by treatment (experimental 37 °C/2 h). In **c**) one primary follicle damaged (red arrow) and one not affected (blue arrow) after treatment (experimental group 37 °C/2 h)

treatment or any other condition that may affect their ovarian pool. Previous reports have already demonstrated high rates of primordial follicle survival after different cryopreservation protocols [4,11]. In addition, a more recent study showed that morphology and markers of early follicle growth of primate primordial follicles are well preserved after slow freezing and thawing [12].

Primary follicles seem to be more sensitive to cryopreservation in comparison with the primordial ones, as they presented a higher rate of damage after vitrification using the same metal container. Primordial follicles are smaller

than primary ones, the physical connections between the oocyte and surrounding squamous granulosa cells are not well established, which is a different situation from the primary structure, where cuboidal epithelial cells, tightly attached one to another form a well defined layer around the oocyte. Possibly, the recovery from cryodamage may be less likely, when cell connections have already been established forming a well defined cell layer surrounding the oocyte, than in the case where a looser structure of flattened cells limits the primary oocyte in a primordial follicle.

One study comparing slow-freezing and vitrification of macaque ovarian tissue showed no difference in morphology of primordial and primary follicles in frozen or vitrified tissue compared with those in fresh tissue [20]. However, the authors based their conclusions in morphological observations and photographs, without any statistical analysis or information of numbers of normal follicles in each experimental group.

Interesting to observe that the poorer results in terms of normal follicular morphology, for both primordial and primary follicles, were observed when the ovaries were placed in ice at 4 °C, prior to cryopreservation. It seems a surprising result considering that in human organ transplantation practice, the specimens are transported in a hypothermic state for best organ preservation and survival until it is ready to be transplanted. Accordingly, one recent publication describes the transport of human ovarian tissue from the collection site to the laboratory in flushing medium at 4 °C [17], while another study used medium at R.T. for the transport of the ovarian fragments [13]. In both studies, cryopreservation started within 5 min after collection, in the same Institution, a situation which may not be possible in several clinical situations, when the time and temperature of transport may play a key role on the outcome of the cryopreservation procedure.

On the other hand, it is a common practice among scientists that work with cattle ovaries obtained from abattoirs, to transport them to the laboratory at R.T or at 37 °C. This observation led us to perform this experiment to investigate whether 4 °C would benefit follicular survival and consequently IVM and IVF results, when dealing with assisted reproduction in cattle or humans, or fertility preservation, when concerning oncological patients.

Unfortunately, we did not perform any viability tests on the warmed specimens. The final proof of viability of the ovarian tissue and follicles after cryopreservation using our metal vessel technique for vitrification, would be to produce healthy puppies in oophorectomized mice. However, we believe that the morphology assessment is a good marker of cell and follicular viability as previous studies have already shown [4,11,12]. Also, considering the recent progresses in biomaterial-based systems that promote angiogenesis after transplantation using capsules made of fibrin

embedded with vascular growth factor [18] or the addition of different vascular endothelial growth factor isoforms to the grafts to improve angiogenesis of ovarian tissue xenotransplantation, the well preserved follicles shall have enough blood supply to survive and support natural conception [9,14]. Further work is underway to test this possibility in an improved version of the metal container for vitrification of bovine ovarian tissue.

In conclusion, our results using the mouse model and a metal container for vitrification show that ovarian tissue can be maintained at R.T. or 37 °C for up to 4 h. between collection and cryopreservation to best preserve follicular morphology, specially the primordial follicle pool. The metal container for vitrification can potentially be employed as a clinical grade device for cryopreservation of tissue fragments, as it is not cytotoxic and it avoids any direct contact with the LNi. This will enable us to comply with the quality requirements of Health Authority Bodies that shall create strict rules for the cryopreservation and storage of human tissues before transplantation in our and other countries, as has been the case in Europe with the European tissue directive 2004/23, 2006/17/EG, and 2006/86/EG (www.EU).

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References

- Almodin CG, Minguetti-Câmara VC, Meister H, Ferreira JO, Franco RL, Cavalcante AA, Radaelli MR, Bahls AS, Moron AF, Murta CG. Recovery of fertility after grafting of cryopreserved germinative tissue in female rabbits following radiotherapy. *Hum Reprod.* 2004;19:1287–93.
- Almodin CG, Minguetti-Câmara VC, Meister H, Ceschin AP, Kriger E, Ferreira JO. Recovery of natural fertility after grafting of cryopreserved germinative tissue in ewes subjected to radiotherapy. *Fertil Steril.* 2004;81:160–4.
- Andersen CY, Rosendahl M, Byskov AG, Loft A, Ottosen C, Dueholm M, Schmidt KL, Andersen AN, Ernst E. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod.* 2008;23:2266–72.
- Chen SU, Chien CL, Wu MY, Chen TH, Lai SM, Lin CW, et al. Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. *Hum Reprod.* 2006;21:2794–800.
- Choi JY, Lee J-Y, Lee EY, Yoon B-K, Boe DS, Choi DS. Cryopreservation of the mouse ovary inhibits the onset of primordial follicle development. *Cryobiology.* 2007;54:55–62.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, van Langendonck A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet.* 2004;364:1405–10.
- Ferreira M, Bos-Mikich A, Frantz N, Rodrigues JL, Brunetto AL, Schwartzmann G. The effects of sample size on the outcome of ovarian tissue cryopreservation. *Reprod Dom Anim.* 2009;45:99–102.
- Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA, Ragni G. Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. *Fertil Steril.* 2006;85:1150–6.
- Henry L, Fransolet M, Labied S, Blacher S, Munaut C, Colige A, Kirschvink N, Noël A, Nisolle M, Foidart J-M. Does isoforms 111 and 165 of vascular endothelial growth factor (VEGF111 and 165) improve transplanted ovarian tissue survival? *J Assist Reprod Genet.* 2011;28:1007–24.
- Isachenko V, Lapidus I, Isachenko E, Krivokharchenko A, Kreienberg R, Woriedh M, Bader M, Weiss JM. Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation. *Reproduction.* 2009;138:319–27.
- Jeremias E, Bedaiwy MA, Nelson D, Biscotti CV, Falcone T. Assessment of tissue injury in cryopreserved ovarian tissue. *Fertil Steril.* 2003;79:651–3.
- Jin SY, Lei L, Shea LD, Zelinski MB, Stouffer RL, Woodruff TK. Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation. *Fertil Steril.* 2010;93:2627–32.
- Keros V, Xella S, Hultenby K, Pettersson K, Sheikh M, Volpe A, Hreinsson J, Hovatta O. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. *Hum Reprod.* 2009;24:1670–83.
- Labied S, Delforges Y, Blacher S, Munaut C, Signole N, Colige A, Janssen L, Henry L, d'Hauterive SP, Jouan C, Kirschvink N, Noël A, Nisolle M, Foidart J-M. Isoform 111 of vascular endothelial growth factor (VEGF111) improves angiogenesis of ovarian tissue xenotransplantation. *J Assist Reprod Genet.* 2011;28:1007–24.
- Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, Schiff E, Dor J. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. *N Engl J Med.* 2005;353:318–21.
- Migishima F, Suzuki-Migishima R, Song SY, Kuramochi T, Azuma S, Nishijimi M. Successful cryopreservation of mouse ovaries by vitrification. *Biol Reprod.* 2003;68:881–7.
- Sheikh M, Hultenby K, Niklasson B, Lundqvist M, Hovatta O. Clinical grade vitrification of human ovarian tissue: an ultrastructural analysis of follicles and stroma in vitrified tissue. *Hum Reprod.* 2011;26:594–603.
- Shikanov A, Zhang Z, Xu M, Smith RM, Rajan A, Woodruff TK, Shea LD. Fibrin encapsulation and vascular endothelial growth factor delivery promotes ovarian graft survival in mice. *Tissue Eng Part A.* 2011;17:3095–104.
- Sugimoto M, Maeda S, Manabe N, Miyamoto H. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. *Theriogenology.* 2000;53:1093–103.
- Ting AY, Yeoman RR, Lawson MS, Zelinski MB. In vitro development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow-rate freeze or vitrification. *Hum Reprod.* 2011;26:2461–72.
- Tokieda Y, Ishiwata I, Segino M, Ishikawa H, Sato K. Establishment of a novel method for cryopreservation and thawing of the mouse ovary. *Hum Cell.* 2002;15:230–7.
- von Wolff M, Donnez J, Hovatta O, Keros V, Maltaris T, Montag M, Salle B, Sonmezer M, Andersen CY. Cryopreservation and autotransplantation of human ovarian tissue prior to cytotoxic therapy- a technique in its infancy but already successful in fertility treatment. *Eur J Cancer.* 2009;45:1547–53.