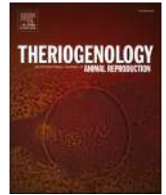
Theriogenology 224 (2024) 163–173

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com



Original Research Article

Vitrification of feline ovarian tissue: Comparison of protocols based on equilibration time and temperature

Isa Mohammed Alkali [a,b], Martina Colombo [a,*], Teresina De Iorio [a,c], Aleksandra Piotrowska [d], Olga Rodak [d], Michał Jerzy Kulus [e], Wojciech Niżański [f], Piotr Dziegiel [d], Gaia Cecilia Luvoni [a]

[a] Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, via dell'Università, 6, 26900, Lodi, Italy
[b] Department of Theriogenology, University of Maiduguri, Maiduguri, Nigeria
[c] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Research Center "Zootechny and Aquaculture", Via Salaria, 31, 00015, Monterotondo, RM, Italy
[d] Department of Histology and Embryology, Wrocław Medical University, ul. Chalubinskiego 6a, 50-368, Wrocław, Poland
[e] Division of Ultrastructural Research, Wroclaw Medical University, 50-368, Wrocław, Poland
[f] Department of Reproduction and Clinic for Farm Animals, Wrocław University of Environmental and Life Sciences, Grunwaldzki Square 49, 50-366, Wrocław, Poland

ARTICLE INFO

Keywords:
Conservation of biodiversity
Field conditions
Cryoprotectant
Ultrastructure
Ovarian fragments
Room temperature

ABSTRACT

Global contraction of biodiversity pushed most members of Felidae into threatened or endangered list except the domestic cat (*Felis catus*) thence preferred as the best model for conservation studies. One of the emerging conservation strategies is vitrification of ovarian tissue which is field-friendly but not yet standardized. Thus, our main goal was to establish a suitable vitrification protocol for feline ovarian tissue in field condition. Feline ovarian tissue fragments were punched with biopsy punch (1.5 mm diameter) and divided into 4 groups. Group 1 was fresh control (Fr), while the other three were exposed to 3 vitrification protocols (VIT_CT, VIT_RT1 and VIT_RT2). VIT_CT involved two step equilibrations in solutions containing dimethyl sulfoxide (DMSO) and ethylene glycol (EG) for 10 min each at 4 °C. VIT_RT1 involved three step equilibration in solutions containing DMSO, EG, polyvinylpyrrolidone and sucrose for 14 min in total at room temperature, while in VIT_RT2 all conditions remained the same as in VIT_RT1 except equilibration timing which was reduced by half. After vitrification and warming, fragments were morphologically evaluated and then cultured for six days. Subsequently, follicular morphology, cellular proliferation (expression of Ki-67, MCM-7) and apoptosis (expression of caspase-3) were evaluated, and data obtained were analysed using generalised linear mixed model and chi square tests. Proportions of intact follicles were higher in Fr ($P = 0.0001$) and VIT_RT2 ($P = 0.0383$) in comparison to the other protocols both post warming and after the six-day culture. Generally, most follicles remained at primordial state which was confirmed by the low expression of Ki-67, MCM-7 markers. In conclusion, VIT_RT2 protocol, which has lower equilibration time at room temperature has proven superior thus recommended for vitrification of feline ovarian tissue.

1. Introduction

Widespread loss or contraction of biodiversity is a threat to stability of the ecosystem [1], particularly in endangered species [2]. Thus, genetic diversity conservation became an indispensable tool that can be achieved through gamete banking in adult animals [3] or gonadal tissue banking in prepubertal ones [4]. Ovarian tissue banking through a combination of cryopreservation and culture and/or transplantation provides a promising and yet experimental technique for genetic diversity conservation. It enables the utilization of ovarian reserve of animals long time after their existence [5]. Similarly, viable ovarian tissue recovered from precious animals that are found dead due to illness, accident or an act of poaching could be saved and preserved for subsequent assisted reproductive technologies especially when the technique has been suitably optimized to field conditions [5]. This is imperative considering the status of the wild feline population in the IUCN

* Corresponding author. Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, via dell'Università, 6, 26900, Lodi, Italy.
E-mail addresses: isa.mohammed@unimi.it (I.M. Alkali), martina.colombo@unimi.it (M. Colombo), teresina.deiorio@unimi.it (T. De Iorio), aleksandra.piotrowska@umw.edu.pl (A. Piotrowska), olga.rodak@student.umw.edu.pl (O. Rodak), mkulus@gmail.com (M.J. Kulus), wojciech.nizanski@upwr.edu.pl (W. Niżański), piotr.dziegiel@umw.edu.pl (P. Dziegiel), cecilia.luvoni@unimi.it (G.C. Luvoni).

https://doi.org/10.1016/j.theriogenology.2024.05.023
Received 23 January 2024; Received in revised form 17 May 2024; Accepted 17 May 2024
Available online 18 May 2024
0093-691X/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

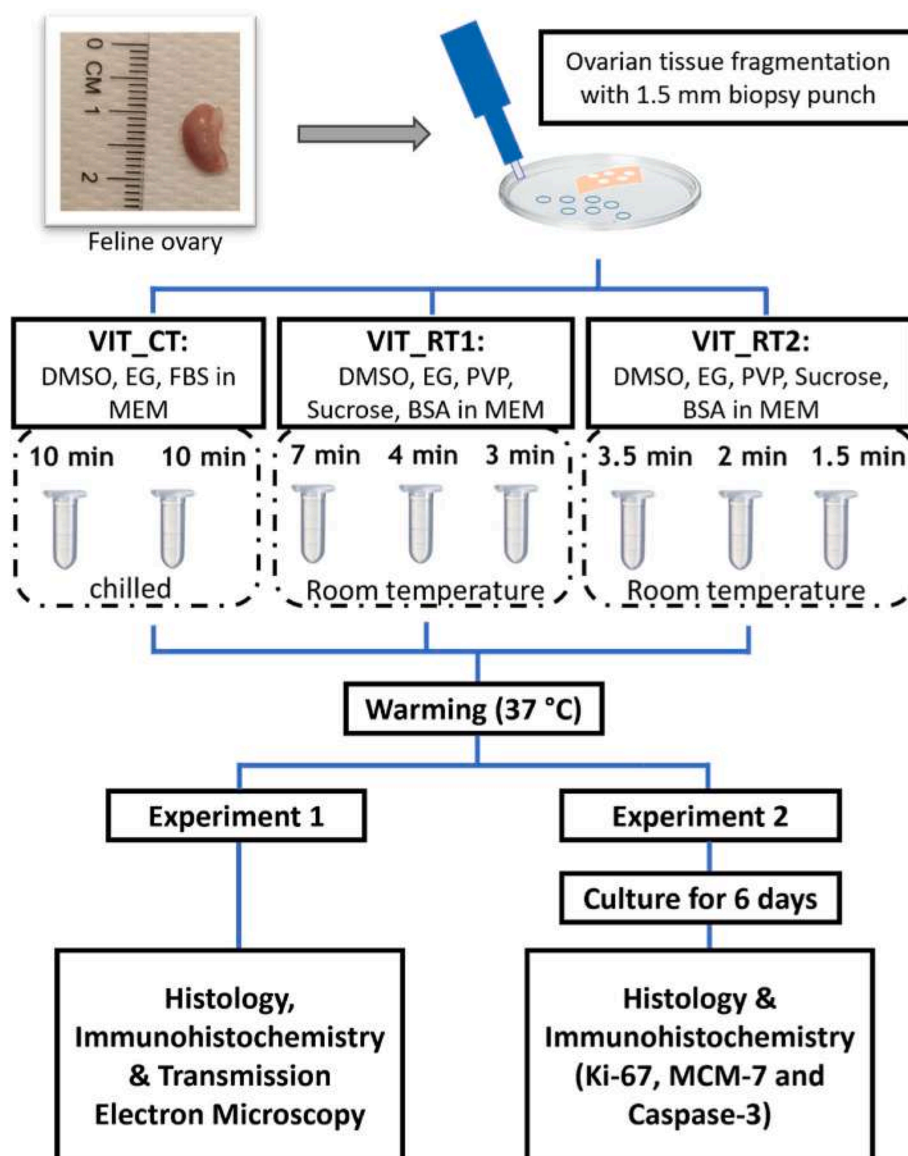


Fig. 1. Experimental design for evaluation of suitable feline ovarian tissue vitrification technique that fits well with field conditions. VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2. DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; FBS: Fetal bovine serum; MEM: Minimum essential medium; PVP: polyvinylpyrrolidone; BSA: Bovine serum albumin; MCM-7: Minichromosome maintenance protein complex component-7; min: Minutes.

(International Union for the Conservation of Nature) red list although certain *in situ* conservation strategies have been successful in some feline species like the Iberian lynx [5,6].

The domestic cat is considerably similar to other members of the Felidae with regards to ovarian structure and ultrastructure [7]. In addition, it is one of the species of least concern in the IUCN red list. Its ovaries can be easily obtained from veterinary clinics or animal shelters as a byproduct of routine spaying which preclude ethical barriers [8]. This makes it a suitable model for reproductive studies aiming at eventual translation into the other members of the family not withstanding some species specific variations [7]. In this regard, the captive population could be the initial focus for gene banking [9].

Ovarian tissue which could be collected postmortem or from a biopsy is laden with different stages of follicles most of which are the less metabolically active primordial follicles [7,10,11]. Known for their resistance to exogenous stress factors, primordial follicles exhibit the potential of withstanding cryopreservation techniques due to their simple morphology [12]. This makes the ovarian cortex an excellent candidate for cryopreservation techniques.

Vitrification has been regarded by many investigators as superior cryopreservation technique for a more complex heterogenous structure such as the ovarian tissue due to two main reasons: 1. Its simplicity and suitability to be carried out in the field since it does not require sophisticated laboratory equipment or an expert technician. The technique may also preclude the need to transport ovaries over a long period of time. 2. Better ovarian tissue quality has been recovered from previous studies comparing vitrification and freezing [13–15]. However, deleterious effects of cryoprotectants (CPAs) are the limitations of the technology because it requires high intracellular concentration of CPAs to produce the necessary viscosity for a glass transition [16]. To achieve such concentration, a number of factors must be considered including rate of tissue penetration [17,18], which is also dependant on the temperature of exposure [19] and the size of ovarian fragment because the later affects both CPA permeation and cooling rate during vitrification [20]. The goal of this study was to determine a field suitable protocol for the vitrification of feline ovarian tissue. Thus, the objectives were to

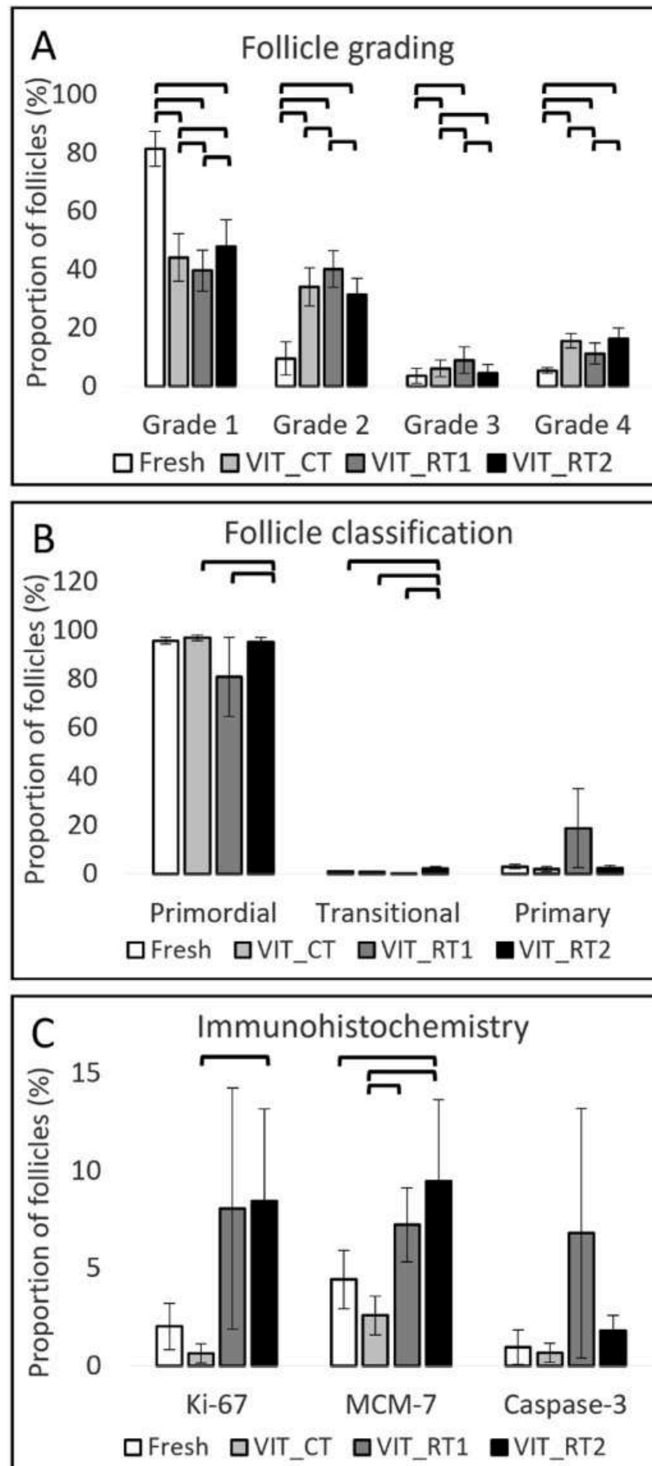


Fig. 2. Graphical representation of proportions of follicles counted from vitrified warmed and fresh feline ovarian tissue (experiment 1). A. Grading of follicles according to morphological intactness (total of 7245 follicles). B. Classification of follicles based on developmental stage (total of 7245 follicles). C. Immunohistochemical staining for proliferative and apoptotic markers (Ki-67, MCM-7 and caspase-3) a total of 9003 follicles were counted. Horizontal back-facing parenthesis on the top of the bars represent statistical significance ($p < 0.05$). Error bars represent the standard error of the mean ($n = 5$). MCM-7: Minichromosome maintenance protein complex component-7. VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2.

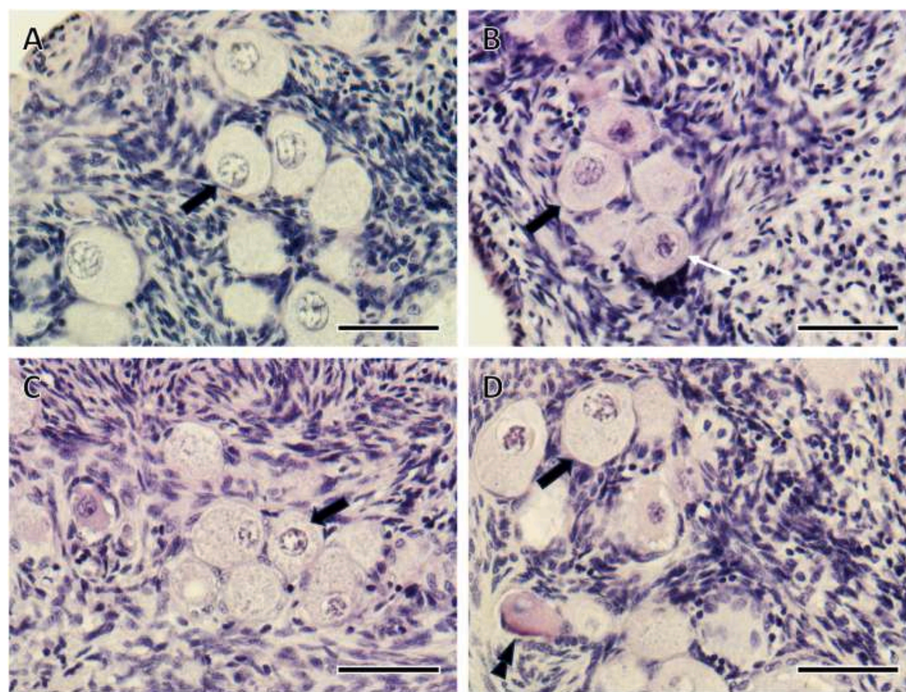


Fig. 3. Representative images from hematoxylin and eosin stained sections of vitrified warmed and fresh feline ovarian tissue (experiment 1). A. Fresh ovarian tissue showing grade one follicles (Black solid arrow), B. VIT_CT showing both grade 1 and grade 2 (white line arrow) follicles, C. VIT_RT1 showing some grade 1 and 2 follicles and D. VIT_RT2 showing grade 1 and grade 4 (double arrow head) follicles. VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2. Scale bar = 50 μm.

evaluate morphological integrity of feline ovarian follicles in different vitrification protocols and to functionally confirm the viability of feline ovarian follicles in an *in vitro* culture system.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) except where specified otherwise.

2.2. Ovarian tissue collection and preparation

Feline ovaries (total of 18 from 9 cats) were collected from animal shelters and veterinary clinics as a byproduct of ovariectomy and immediately transported to the laboratory in washing solution containing phosphate buffered saline supplemented with 0.1 % penicillin and streptomycin (penstrep) combination (Gibco, Bleiswijk, Netherlands). Ovaries were washed three times in the washing solution and then twice in dissection solution composed of 1 % fetal bovine serum (FBS), 1 % penicillin and streptomycin in Leibovitz's (L-15) medium. The ovaries were dissected sagittaly and made to approximately 1 mm thick by removing the medulla before fragmentation using 1.5 mm diameter biopsy punches (Kai medical, Oyana, Japan). The fragments were made from both ovaries such that every group contained at least a fragment from each ovary and every group in each replicate experiment contained at least four fragments.

2.3. Experimental design

The entire experimental design is presented in a simplified flow chart (Fig. 1). The experiment was segmented into two aspects. In experiment 1 (six independent replicates), ovarian tissue fragments were divided into four groups, one group (Fresh) was fixed overnight in form-acetic [21,22] while the remaining three groups were placed on 30G needles (4 fragment per needle) and then exposed to three different vitrification protocols (VIT_CT, VIT_RT1 and VIT_RT2). Vitrified ovarian tissue fragments were warmed and processed for histology, immunohistochemistry, and transmission electron microscopy. In experiment 2 (five independent replicates), the same groups were maintained but cultured post warming for 6 days. Four ovarian fragments from each group were retrieved and fixed at day two, four and six of culture and eventually processed for histology and immunohistochemistry. Six sections (each containing 4 ovarian fragments) were evaluated for each group in every replicate experiment.

2.3.1. VIT_CT

This protocol was based on previous report by Mouttham and Comizzoli [19], which involved equilibrating ovarian tissue fragments in vitrification media (VM)1 containing 7.5 % DMSO (Dimethyl sulfoxide), 7.5 % EG (Ethylene glycol), and 20 % FBS (Fetal bovine serum) for 10 min, and then transferring to VM2 composed of 15 % DMSO, 15 % EG, and 20 % FBS for 10 min, all at 4 °C. Excess VM was dabbed prior to plunging in liquid nitrogen.

2.3.2. VIT_RT1 and 2

Vitrification in VIT_RT1 and 2 were based on previously reported protocol tested on baboon [23,24]. The vitrification solution (VS) is composed of minimum essential medium (MEM) as the base medium supplemented with 10 % DMSO, 26 % EG, 2.5 % polyvinylpyrrolidone (PVP, MW 10 000) and 1 M sucrose in MEM (Gibco, Bleiswijk, Netherlands) + 20 mg/ml bovine serum albumin (BSA). Since there were three equilibration steps, the VS has been serially diluted with MEM +20 mg/ml BSA to form VS1 (25 % VS), VS2 (50 % VS) and VS3 (100 % VS). In the case of VIT_RT1, all equilibrations were carried out at room temperature (RT) for 7 min in VS1, 4 min in VS2 and 3 min in VS3. However, with regards to VIT_RT2, all conditions remained the same except equilibration timing which was reduced by half (3.5 min in VS1, 2 min in VS2 and 1.5 min in VS3). Subsequently, ovarian fragments (on the 30G needles) were removed and dabbed with sterile gauze before

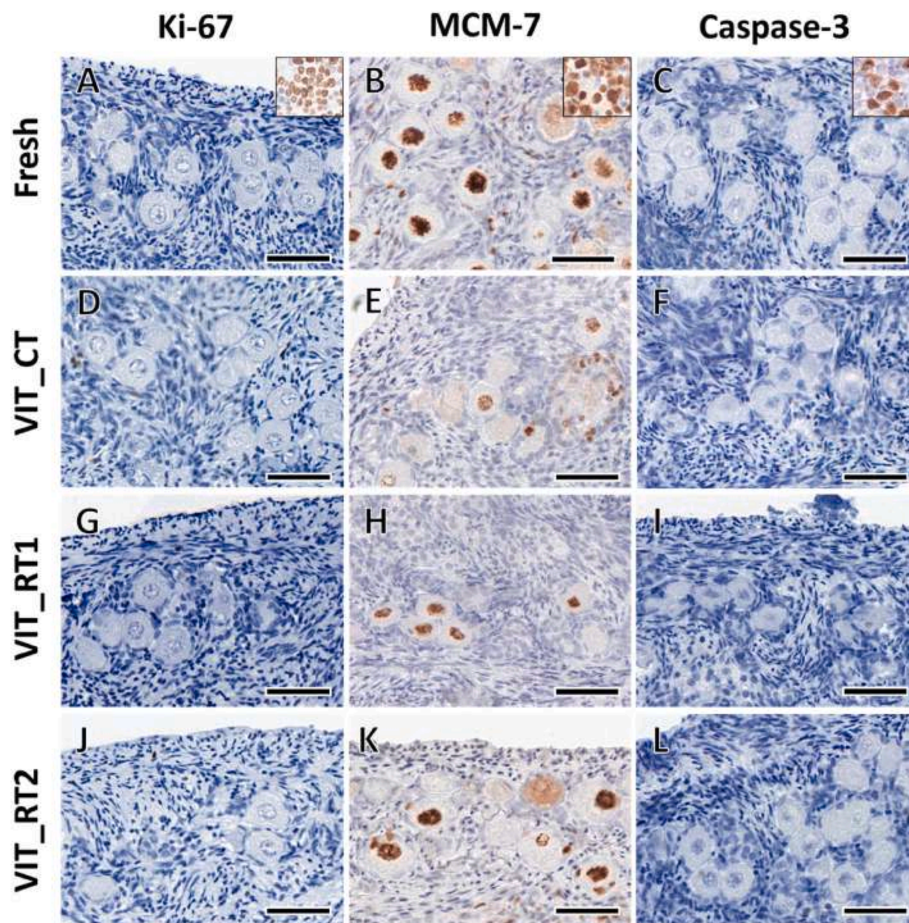


Fig. 4. Representative images from immunolabelled sections of fresh and vitrified warmed feline ovarian tissue (experiment 1). Small squares in panels A, B and C represent the positive controls. Panels A, B and C represent the fresh control, D, E and F represent VIT_CT (vitrification after equilibration at chilling temperature), G, H and I represent VIT_RT1 (vitrification after equilibration at room temperature 1) while J, K and L represent VIT_RT2 (vitrification after equilibration at room temperature 2) immunostained with Ki-67, MCM-7 and caspase-3 respectively. Scale bar = 50 μm.

plunging directly into liquid nitrogen for vitrification.

2.3.3. Warming

Warming for all the protocols involved four washing steps with four serially diluted warming solutions (WS1-4) containing MEM supplemented with 20 mg/ml BSA as the base medium and decreasing concentration (1 M, 0.5 M, 0.25 M and 0 M) of sucrose. The solutions were maintained at 37 °C during warming and tissue fragments were retained in the first warming solution (WS1) for 15 s while subsequent washings were for 5 min each.

2.3.4. Culture

The culture system was adopted from (Fujihara 2012) briefly described as follows: MEM was the base medium supplemented with 50 μM ascorbic acid, 0.3 % Polyvinyl alcohol, 10 μg/ml follicle stimulating hormone, 1 % insulin, transferrin, and selenium mixture (ITS+1) and 1 % penstrep. Ovarian fragments were cultured on agarose gel inserts placed into the wells of 24 well culture plate. The culture medium (400 μl) was added to the inserts so that fragments were not covered with the medium. Distilled water (500 μl) was added in all dish wells without inserts to maintain adequate humidity. Agarose gels were made by heating and dissolving 1.5 g of agarose in distilled water (1.5 % [w/v]) and then poured into sterile 10 cm Petri dish and allowed to set under biological hood. Hexahedrons (~10 × 10 × 5 mm) were dissected with sterile scalpel blade and a 1 cm checkered paper underneath the dish and then culture medium was added and allowed to soak for at least 24 h.

2.3.5. Histology

Ovarian tissue fragments were dehydrated in ethanol and impregnated with paraffin after treatment with Neo-clear. Subsequently, ovarian tissue fragments were embedded into paraffin blocks and sectioned (5 μm thickness). Every sixth section was fixed to glass slide and dried overnight at 37 °C. Slides were stained and counterstained with hematoxylin and eosin following deparaffinization and rehydration of the sections. Afterwards, slides were cover slipped and scanned. Follicles were counted and graded as described previously [22,25] into 4 grades; Grade 1 which is the morphologically intact follicle is when follicle is spherical in shape with evenly distributed follicular cells, intact stroma, spherical oocyte, intact nucleus and nucleolus with homogenous cytoplasm. The second grade (grade 2), is when follicle is spherical in shape with evenly distributed follicular cells, intact stroma and spherical oocyte, misshapen nucleus and/or not homogenous cytoplasm. The third grade (grade 3), is where follicular cells are pulled away from the stroma but the oocyte is spherical, while grade 4 is when the follicular cells are pulled away from the stroma and/or the oocyte is misshapen, vacuolated, with pyknotic nucleus and/or disorganized granulosa cells. Furthermore, the follicles were classified based on developmental stage as primordial, where oocyte is surrounded by flattened follicular cells; transitional, where some of the flattened follicular cells have been converted to cuboidal cells; or primary, where oocyte is surrounded by cuboidal follicular cells.

2.3.6. Immunohistochemistry

Expression of proliferating markers namely Ki-67 and MCM-7 and an

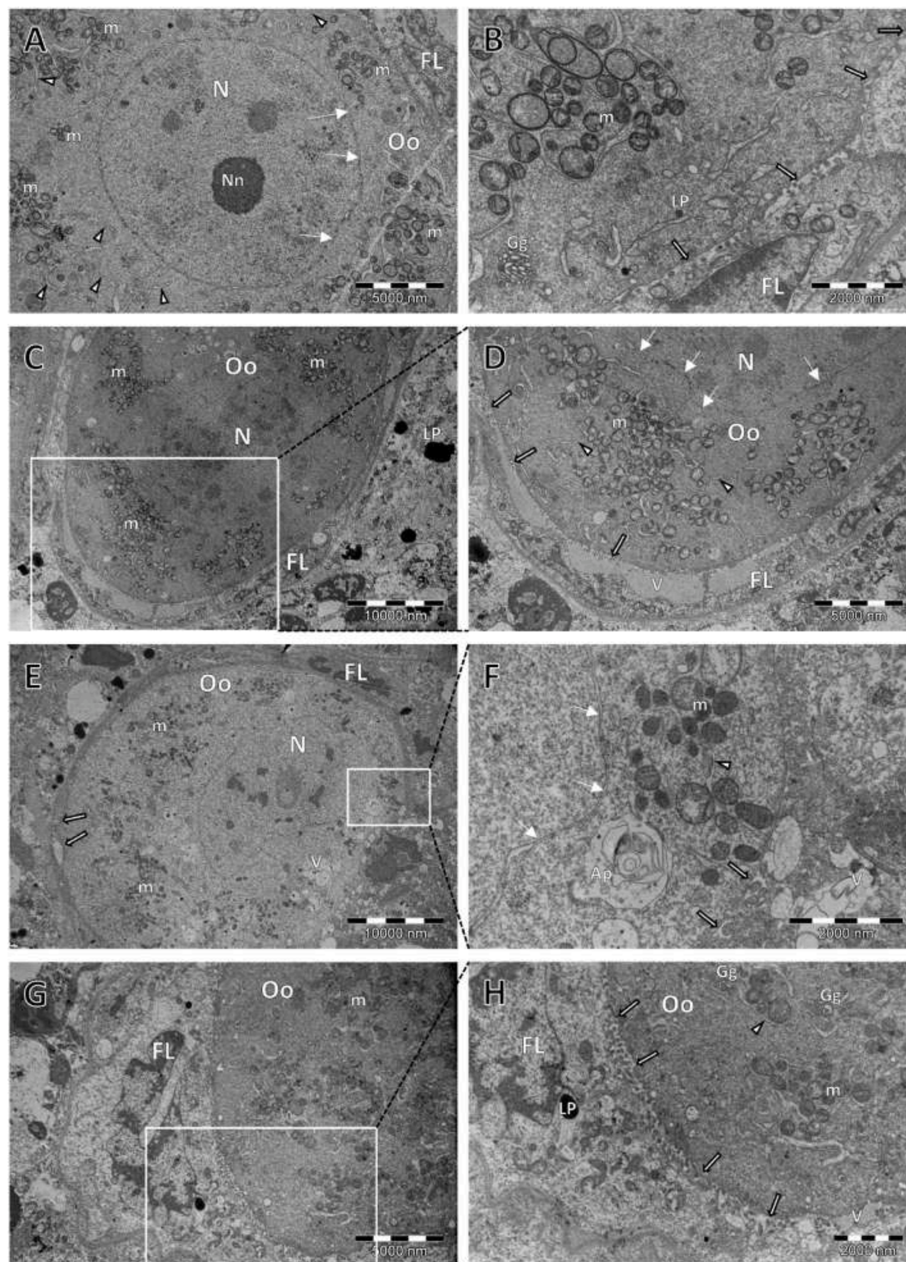


Fig. 5. Representative images from transmission electron microscopy sections of fresh and vitrified warmed feline ovarian tissue (experiment 1). A. Oocyte (Oo) of a primordial follicle from fresh ovarian tissue showing aggregates of mitochondria (m) spherical oocyte nucleus (N) with a distinct nucleolus (Nn), distinct nuclear envelop (white line arrow) and endoplasmic reticulum (arrowhead). B. At higher magnification, some lipid droplets (LP) were observed and there is irregular disposition of the microvilli (solid arrows) within a slightly widen space between oolemma and follicular cells (FL). C. Primordial follicle from VIT_CT (vitrification after equilibration at chilling temperature) showing mitochondrial clusters (m) and a misshapen nucleus (N). D. At higher magnification, shortening and irregular arrangement of microvilli (solid arrows) can be seen and a distinct nuclear (N) envelop folding (white line arrows), very large vacuoles (V) are also present in the follicular cells (FL). E. Primordial follicle from VIT_RT1 (vitrification after equilibration at room temperature 1) also presents similar pattern with microvilli disorganisation (solid arrows) and nuclear (N) envelop folding. F. At higher magnification, the distinct nuclear envelop folding is overt (white line arrows); mitochondrial swelling and presence of autophagosomes (Ap) in both oocyte and follicular cells, close association between mitochondria (m) and endoplasmic reticulum (arrowhead) is also evident. Vacuoles (V) are seen in follicular cells. G. Section from VIT_RT2 (vitrification after equilibration at room temperature 2) showing mitochondrial cluster (m) in oocyte (Oo) and a follicular cell (FL). H. At higher magnification, abundant microvilli (solid arrows) are evident with some disorganisation, several vacuoles (V) including lipid droplets (LP) are seen in the follicular cell (FL). Two normal Golgi apparatuses (Gg) are also evident.

apoptotic marker (activated caspase-3) were evaluated on serially prepared slides using immunohistochemical techniques as described previously [25]. Briefly, paraffin embedded ovarian tissue containing blocks were sliced into sections of five microns and then fixed to immunohistochemistry slides (Dako, California, USA) and dried overnight. Paraffin was removed with xylene and sections were rehydrated prior to antigen retrieval at 97 °C for 20 min in high pH retrieval solution (pH 9.0; Dako EnVision Flex, Glostrup, Denmark) for MCM-7 and activated caspase-3, while a low pH citrate buffer solution (pH 6.0) was used for Ki-67. Subsequently, slides were incubated with primary antibodies: anti-Ki-67 (clone MiB-1; Dako EnVision Flex), anti-MCM-7 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-activated caspase-3 (1:400; Cell signaling, Massachusetts USA). At this point, an automatic immunohistochemistry machine (DAKO Link48, California, USA) was

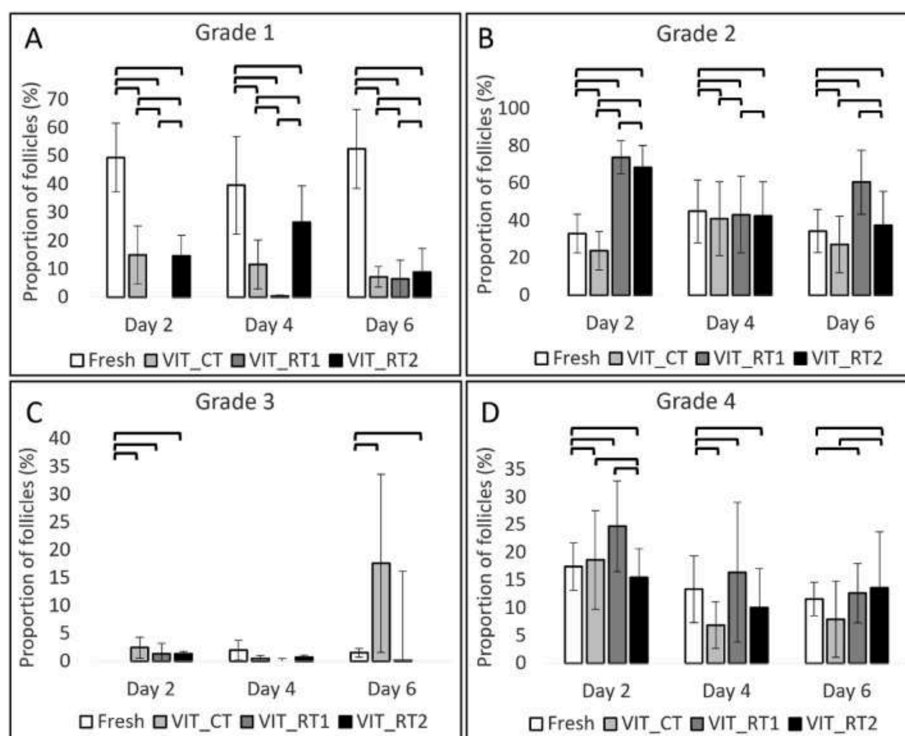


Fig. 6. Graphical representation of proportions of follicles counted (total of 3870) from fresh and vitrified warmed feline ovarian tissue cultured for six days (experiment 2). A. Proportions of grade 1 follicles B. Proportions of grade 2 follicles. C. Proportions of grade 3 follicles. D. Proportions of grade 4 follicles. Horizontal back-facing parenthesis on the top of the bars represent statistical significance ($p < 0.05$). Error bars represent the standard error of the mean (n = 5). VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2.

used for the detection of the three tested markers while adhering to the manufacturer's instructions including counterstaining with hematoxylin (Dako EnVision Flex, Glostrup, Denmark). Feline tonsil was included as a positive control for all the markers due to their high to moderate expression in lymphoid tissues (https://www.proteinatlas.org). Finally, slides were cover slipped, digitally scanned, and evaluated with a case viewer software (3DHistech version 2.3.2) by counting and classifying follicles as positive when at least one follicular cell was immunostained however, in the case of activated caspase-3, follicles were also considered positive when the oocyte was immunostained. Data were presented as proportion of positive follicles against the total number of follicles counted.

2.3.7. Transmission electron microscopy (TEM)

Ovarian tissue fragments were fixed in 2.5 % glutaraldehyde (Heidelberg, Germany) for 24 h at 4 °C and then washed four times at 4 °C in 0.1 M cacodylate buffer (Chempur, Piekary Śląskie, Poland). Fragments were dehydrated in increasing concentrations of ethanol (30–96 %) and acetone, then incubated with epoxy resin and acetone solution (1:1) overnight at room temperature before embedding in epoxy resin (Serva Electrophoresis, Heidelberg, Germany). Epoxy blocks were made by keeping at 60 °C for one week. Semithin sections (600 nm) were sectioned and stained with toluidine blue solution (Serva Electrophoresis, Heidelberg, Germany) on the heating plate (100 °C) until it dried. This was to predetermine the spots with follicles. Subsequently, ultrathin sections (60 nm) were obtained and placed on rhodium copper grids (Maxta form, 200 mesh, Ted Pella, Redding, CA, USA) and contrasted with lanthanides RTU solution (Uranyless; Delta Microscopies, France) and lead citrate RTU solution (Delta Microscopies) before evaluation with transmission Electron Microscope (JEM-1011; Jeol, Tokyo, Japan), at 80 kV and images were captured with Morada Camera (Olympus, Münster, Germany).

2.4. Statistical analysis

Discrete data obtained from the experiment 1 were analysed using Chi square test while data from experiment 2 were analysed with generalised linear mixed model (GLMM) to evaluate the effect of the treatment and culture period, and then Chi square test was applied to compare between the treatments. Confidence interval of 95 % was considered for all analyses. Data was presented as mean percentages of the total follicle counts ± standard error of the mean.

3. Results

3.1. Experiment 1

This study segment was carried out to evaluate the effect of the different vitrification protocols post warming, especially on the morphological integrity of feline ovarian tissue fragments.

3.1.1. Histology

Overall, a total of 7245 follicles were counted. Based on follicular grading, proportion of grade 1 follicles was higher ($p = 0.0287$) in VIT_RT2 than the other tested protocols. However, the general negative effect of vitrification has been delineated by the higher ($p = 0.0392$) proportion of grade 1 follicles in the fresh group when compared with all the vitrified groups wherein only up to 40 % of the follicles were morphologically intact (Fig. 2). Regarding the stages of follicle development, most follicles (more than 80 %) were at the primordial stage (Fig. 2B) and all the groups were statistically the same (Supplementary Table 1). Representative micrographs from hematoxylin and eosin stained sections are presented in Fig. 3.

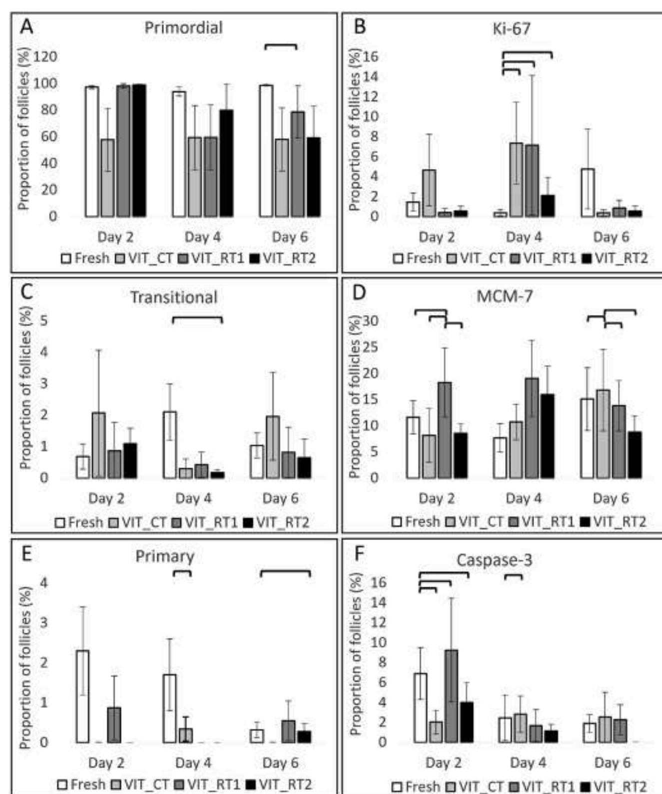


Fig. 7. Graphical representation of proportions of follicles counted from fresh and vitrified warmed feline ovarian tissue cultured for six days (experiment 2). A, C and E presents the distribution of follicles based on their developmental stage (total of 3870 follicles). Chart B, D and F represent the immunohistochemical data (total of 7057 follicles). Horizontal back-facing parenthesis on the top of the bars represent statistical significance ($p < 0.05$). Error bars represent the standard error of the mean ($n = 5$). VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2.

3.1.2. Immunohistochemistry

Generally, there is low expression of all the three markers that were evaluated (Fig. 2C). As per the proliferative markers, they confirm the general quiescent stage of the follicles (total of 9003 follicles) since the ovarian tissue fragments were mainly from the cortex (Fig. 4).

3.1.3. TEM

Being a more advanced morphological parameter, the TEM images (Fig. 5) helped us to digest the morphological defects observed in the conventional hematoxylin and eosin images, although data was mainly observational, and few samples were analysed. We observed some of the major defects at ultrastructural level within sections of all the protocols tested. These defects included but were not limited to nuclear envelop folding, vacuolation of both oocyte and follicular cells, shortening and collapse of microvilli between the oocyte follicular cells. Mitochondrial defects including swelling and presence of autophagosomes where evident both in fresh and vitrified groups and in both oocytes and follicular cells. On the other hand, indicators of good quality oocytes such as close association between mitochondria and endoplasmic reticulum [26] has been spotted in oocytes.

3.2. Experiment 2

To functionally evaluate the integrity of ovarian fragments and to determine potential amelioration of vitrification defects, vitrified warmed ovarian fragments were cultured for six consecutive days *in vitro*. A total of 10 927 follicles were counted.

3.2.1. Histology

Generally, there was a statistical significance among the treatments ($p = 0.0001$) while the culture period (day 2, 4 and 6) was found to have no effect ($p = 0.988$) in all groups. Fig. 6 contains the summary of the different follicle grades wherein grade 1 was higher ($p = 0.0383$) in VIT_RT2 than in the other protocols excluding the fresh control on day six of culture. However, the fresh control is significantly the highest indicating a general negative effects of vitrification procedure. Most of the follicles in the three protocols appear in the grade 2 category (Fig. 6B) with a lot of variations among the groups ($p = 0.004$). Surprisingly, after six days of culture, most follicles remained at the primordial stages evident with over 60 % of primordial follicles in all groups with very little variation among them and a corresponding low proportion of transitional and primary follicles (Fig. 7 and Supplementary Table 3). Representative images of all the groups captured from hematoxylin and eosin stained slides can be found in Fig. 8.

3.2.2. Immunohistochemistry

The general low expression of both proliferative markers (<30 % of follicles) confirms the global lack of initiation of follicular development even after six days of culture (Fig. 7). Similarly, there was low activated caspase-3 activity without any difference within the groups ($p > 0.05$) on the sixth day of culture. Details could be found in Supplementary Table 4 while representative photomicrographs of immunostained slides are presented in Fig. 9.

4. Discussion

In our quest for a suitable vitrification protocol that fits well in the field, we investigated three protocols based on their cryoprotectant composition, temperature of equilibration as well as the equilibration timings. thus, we found that VIT_RT2 presented better result than the other treatments. This may not be unconnected to the composition of the VS which contained in addition to EG and DMSO, sucrose and polyvnyl pyrrolidone as high molecular weight non-permeable CPAs known for maintaining osmotic balance [16]. Albeit, VIT_RT2 was derived from VIT_RT1 which has been tested on baboon ovarian tissue with success post transplantation [23,24], we refined it by choosing smaller sized ovarian tissue fragments (suitable for culture) and concomitant reduction of the timing for equilibration by half. More so, smaller sized tissues tend to equilibrate faster in vitrification solutions and have faster cooling rate during vitrification. Thus, ovarian tissue size has a profound effect on the success of vitrification with smaller size having better results [27,28]. Challenges with larger sized tissues or whole organ vitrification have been associated with fractures that often occur post vitrification or during warming [29,30].

Furthermore, VIT_RT2 is better suited for field condition because all steps are carried out at room temperature. In this way transporting the whole ovary over a long distance from the field thereby risking initiating autolysis can be avoided [15]. Similarly, the equilibration of ovarian fragments at room temperature enhances rate of tissue permeation thereby reducing the equilibration period. Some investigators argued that equilibration at chilling temperature may be more suitable since cellular metabolism is at its lowest phase although this would prolong the equilibration period because the permeation rate will be considerably reduced [31]. In addition, chilling injury which normally take place at sub-physiological temperature may compound the cellular damage [32]. In the current investigation, the results were inconsistent with report from Mouttham & Comizzoli [19], who indicated that exposure of cat ovarian cortex at room temperature had a significant effect on RNA synthesis but not quite profound on the viability of the cortex. This is not surprising because we used a distinct protocol that has been formulated to be carried at room temperature with success [23,24]. A similar protocol for vitrification of cat ovarian tissue has been reported where a

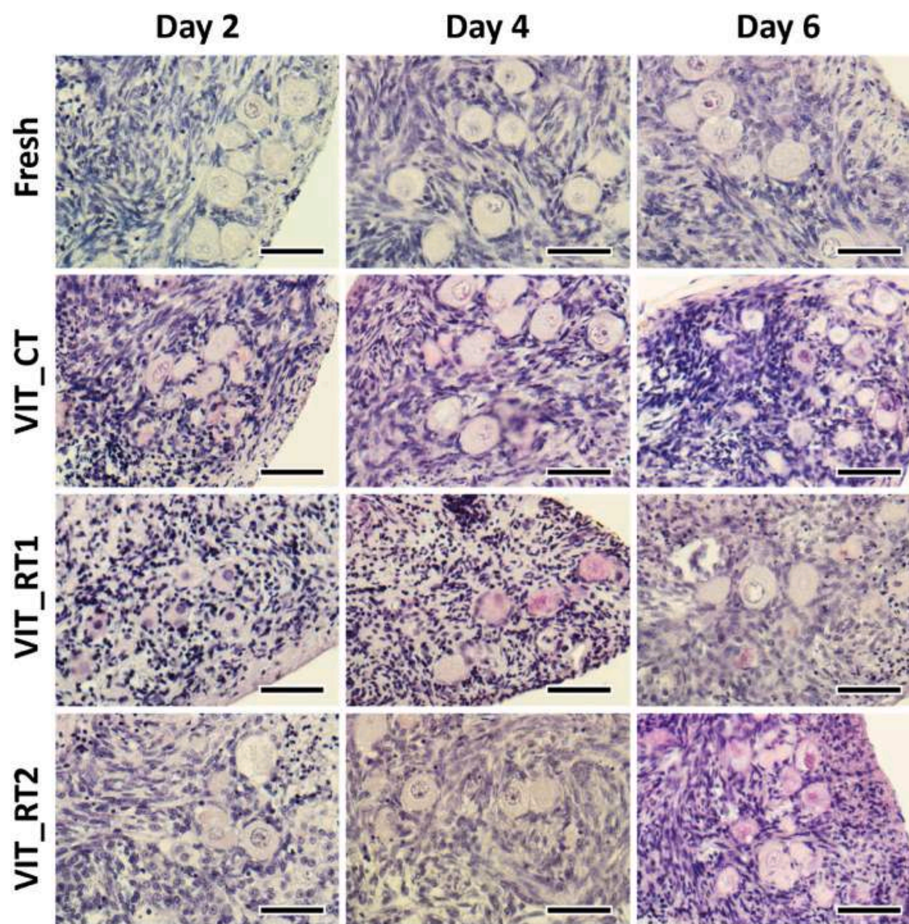


Fig. 8. Representative images from hematoxylin and eosin stained sections of fresh and vitrified warmed feline ovarian tissue cultured for six days (experiment 2). VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2. Scale bar = 50 μm.

three-step equilibration was carried out (5 min in each step) and the first two steps were at room temperature while the final step was chilled [15].

Moreover, the success recorded in VIT_RT2 may not be unconnected to the use of non-permeable CPAs which contributed to attaining the necessary viscosity for a successful vitrification [33]. We reported that VIT_RT2 which has a cumulative equilibration time of 7 min is the best protocol for feline ovarian tissue vitrification. This is conceivable because it has been reported that, prolonged contact of tissues with CPA leads to chemical toxicity [20]. Such toxicity could be associated with osmotic imbalance, cytoskeletal damage or nucleic acid fragmentation [16]. In this regard, DMSO has been incriminated because it exhibits a permissive permeability on cells [34]. Several other degenerative changes were attributed to DMSO toxicity including mitochondrial alterations and vacuolations and extracellular matrix swelling [35]. In the current report, some cellular degenerative changes in the ovarian tissue ultrastructure in almost all the protocols were observed.

After vitrification and warming of ovarian tissue, initial evaluation is often morphological, where structural integrity of the ovarian parenchyma is assessed usually through histology. However, morphology alone is not enough to guaranty tissue viability [35]. The is a need for a functional evaluation of the tissue fragments either through an *in vivo* transplantation on a subject or through an *in vitro* tissue culture system [25,36]. The former has been extensively practiced in humans where warmed ovarian fragments were autotransplanted since ovarian tissue preservation is no longer experimental in humans, otherwise the fragments could be grafted in a secondary subject (xenotransplantation) with the aim of retrieving matured oocytes [37,38]. While in animals, both auto and xenotransplantation can be carried since ovarian tissue cryopreservation is still experimental [36,39]. However, *in vitro* culture may be more preferred because of the complexity and negative consequences of grafting, including being invasive, expensive, cumbersome, and therein lies the possibility of follicular depletion from anoxia or "burn out" [15,40–42]. In the current report, we recorded up to 40 % grade 1 follicles in the treatments post warming (Fig. 2) however, this was followed by a considerable reduction of grade 1 follicles after *in vitro* culture (Fig. 6) which clearly affirms our earlier argument on the necessity to ascertain the ovarian tissue viability functionally.

During the six-day culture, we report low activation of primordial follicles despite maintaining follicle integrity with low follicle apoptosis in all the treatments which may be associated with the culture system employed [25], although regarding the low caspase-3 immunolabeling, we must be cautious not to conclude on overall viability since necrosis and other forms of cell degeneration and death were not evaluated. These findings, however, differ from earlier reports on human ovarian tissue wherein culturing primordial follicles *in situ* helps in their activation and subsequent follicle development until the secondary stage. On the other hand, this trait may be peculiar to the feline species because our findings are in line with Bosch et al. [39], who xenotransplanted thawed cat ovarian fragments on mice and found that more than 90 % of the follicles that survived the technique remained at their primordial stage. Similarly, more than 80 % of follicles remained in their primordial state when cat ovarian tissue was transplanted on chorioallantoic membrane and cultured for five days [43]. In addition, when feline primordial follicles were isolated (*ex situ*) and cultured, their survival was for a short period [7]. In this regard, we can consider

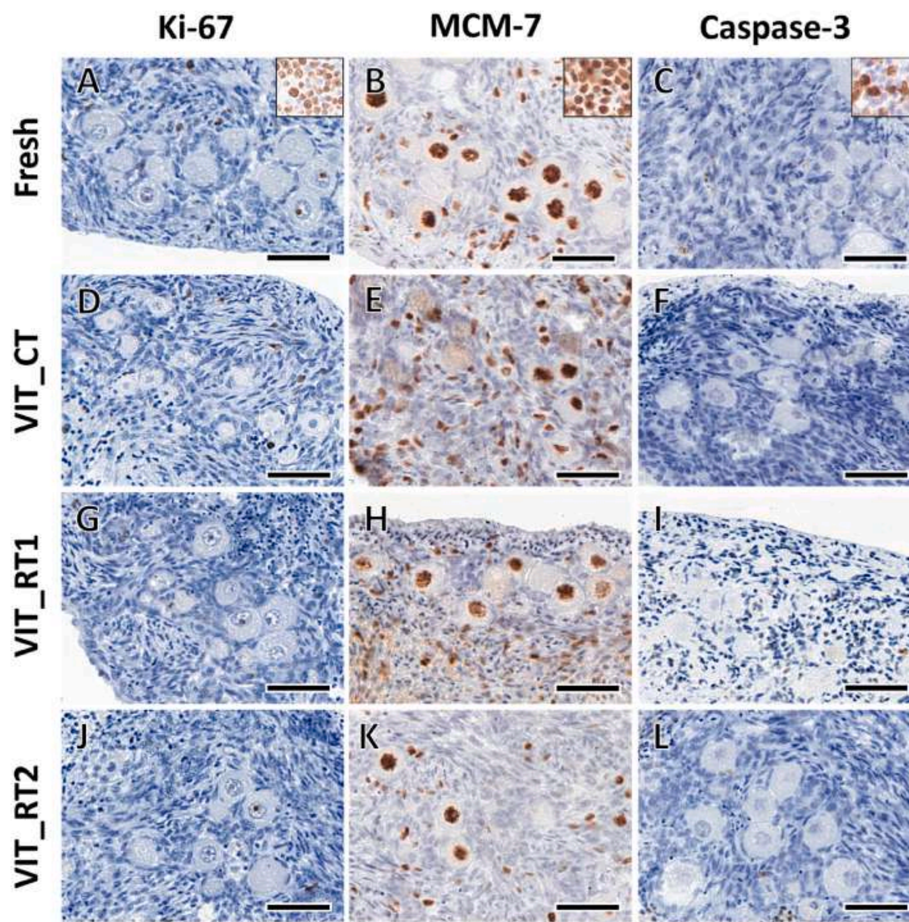


Fig. 9. Representative images from immunostained sections of fresh and vitrified warmed feline ovarian tissue cultured for six days (experiment 2). Small squares in panels A, B and C represent the positive controls. VIT_CT: vitrification after equilibration at chilling temperature. VIT_RT1: vitrification after equilibration at room temperature 1. VIT_RT2: vitrification after equilibration at room temperature 2. Scale bar = 50 μm.

supplementation of the culture medium with activation factors to improve primordial follicle activation in future [44].

In conclusion, we presented a vitrification protocol (VIT_RT2) which has lower equilibration time at room temperature suitable for the field conditions which can be used for vitrification of cat ovarian tissue and perhaps attempts could be carried out on wild felids especially where urgent genetic rescue is required. On a broader note, since our protocol was derived from a protocol that has already been successfully used in humans, baboons, and bovine, it may serve as a stepping stone to a unified ovarian tissue vitrification protocol in several species perhaps with varying equilibration timings.

CRediT authorship contribution statement

Isa Mohammed Alkali: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martina Colombo:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Teresina De Iorio:** Writing – review & editing, Methodology, Investigation. **Aleksandra Piotrowska:** Writing – review & editing, Methodology, Investigation. **Olga Rodak:** Writing – review & editing, Methodology, Investigation. **Michał Jerzy Kulus:** Writing – review & editing, Methodology, Investigation. **Wojciech Niżański:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Piotr Dziegiel:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Gaia Cecilia Luvoni:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Acknowledgements

This work was supported by Regione Lombardia PSR INNOVA n. 201801061529, PSR R-INNOVA n. 202102146691, Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001, and Università degli Studi di Milano "Piano di Sostegno alla Ricerca 2021 (Linea 2 Azione A)". This study was also carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.05.023.

References

[1] Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P, et al. Biodiversity loss and its impact on humanity. Nature 2012;486:59–67. https://doi.org/10.1038/nature11148.

[2] Azizan A, Anile S, Nielsen CK, Paradis E, Devillard S. Population density and genetic diversity are positively correlated in wild felids globally. Global Ecol Biogeogr 2023. https://doi.org/10.1111/geb.13727. n/a.

[3] Huijsmans TERG, Hassan HA, Smits K, Van Soom A. Postmortem collection of gametes for the conservation of endangered mammals: a review of the current state-of-the-art. Animals 2023;13:1360.

[4] Silva AR, Pereira AF, Comizzoli P. Biobanking and use of gonadal tissues - a promising strategy for conserving wildlife from the Caatinga biome. Anim Reprod 2022:19.

[5] Jewgenow K, Zahmel J. Preservation of female genetic resources in feline species. Theriogenology 2020;156:124–9. https://doi.org/10.1016/j.theriogenology.2020.06.040.

[6] Sarmento P, Carrapato C, Eira C, Silva JP. Spatial organization and social relations in a reintroduced population of Endangered Iberian lynx Lynx pardinus. Oryx 2019;53:344–55.

[7] Jewgenow K, Stolte M. Isolation of preantral follicles from nondomestic cats—viability and ultrastructural investigations. Anim Reprod Sci 1996;44: 183–93. https://doi.org/10.1016/0378-4320(96)01549-7.

[8] Colombo M, Alkali IM, Prochowska S, Luvoni GC. Fighting like cats and dogs: challenges in domestic carnivore oocyte development and promises of innovative culture systems. Animals 2021;11:2135.

[9] Henry P, Miquelle D, Sugimoto T, McCullough DR, Caccone A, Russello MA. In situ population structure and ex situ representation of the endangered Amur tiger. Mol Ecol 2009;18:3173–84. https://doi.org/10.1111/j.1365-294X.2009.04266.x.

[10] Luvoni G, Tessaro I, Apparicio M, Ruggeri E, Luciano A, Modina S. Effect of vitrification of feline ovarian cortex on follicular and oocyte quality and competence. Reprod Domest Anim 2012;47:385–91. https://doi.org/10.1111/j.1439-0531.2011.01885.x.

[11] Tenley SC, Gomes RS, Rosasco SL, Northrop EJ, Rich JJJ, McNeel AK, et al. Maternal age influences the number of primordial follicles in the ovaries of yearling Angus heifers. Anim Reprod Sci 2019;200:105–12. https://doi.org/10.1016/j.anireprosci.2018.12.004.

[12] Langbeen A, De porte HFM, Bartholomeus E, Leroy JLMR, Bols PEJ. Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies. Theriogenology 2015;84:477–89. https://doi.org/10.1016/j.theriogenology.2015.04.009.

[13] Kometas M, Christman GM, Kramer J, Rhoton-Vlasak A. Methods of ovarian tissue cryopreservation: is vitrification superior to slow freezing?-ovarian tissue freezing methods. Reprod Sci 2021;28:3291–302. https://doi.org/10.1007/s43032-021-00591-6.

[14] Shi Q, Xie Y, Wang Y, Li S. Vitrification versus slow freezing for human ovarian tissue cryopreservation: a systematic review and meta-anlaysis. Sci Rep 2017;7: 8538. https://doi.org/10.1038/s41598-017-09005-7.

[15] Martins JLA, Lopes MD, de Souza FF, Possebon FS, Wibbelt G, Jewgenow K. Cat preantral follicle survival after prolonged cooled storage followed by vitrification. Cryobiology 2018;81:94–100. https://doi.org/10.1016/j.cryobiol.2018.02.004.

[16] Elliott GD, Wang S, Fuller BJ. Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 2017;76:74–91. https://doi.org/10.1016/j.cryobiol.2017.04.004.

[17] Lotz Jr, Iã§li S, Liu D, Caliskan Sk, Sieme H, Wolkers WF, et al. Transport processes in equine oocytes and ovarian tissue during loading with cryoprotective solutions. Biochim Biophys Acta Gen Subj 2020;1865:129797. https://doi.org/10.1016/j.bbagen.2020.129797.

[18] Lotz J, Icli S, Liu D, Caliskan S, Sieme H, Wolkers WF, et al. Transport processes in equine oocytes and ovarian tissue during loading with cryoprotective solutions. Biochim Biophys Acta Gen Subj 2020;1865:129797. https://doi.org/10.1016/j.bbagen.2020.129797.

[19] Mouttham L, Comizzoli P. The preservation of vital functions in cat ovarian tissues during vitrification depends more on the temperature of the cryoprotectant exposure than on the sucrose supplementation. Cryobiology 2016;73:187–95. https://doi.org/10.1016/j.cryobiol.2016.07.013.

[20] Amorim CA, Curaba M, Van Langendonckt A, Dolmans M-M, Donnez J. Vitrification as an alternative means of cryopreserving ovarian tissue. Reprod Biomed Online 2011;23:160–86. https://doi.org/10.1016/j.rbmo.2011.04.005.

[21] Adeniran BV, Bjarkadottir BD, Appeltant R, Lane S, Williams SA. Improved preservation of ovarian tissue morphology that is compatible with antigen detection using a fixative mixture of formalin and acetic acid. Hum Reprod 2021; 36:1871–90. https://doi.org/10.1093/humrep/deab075.

[22] Alkali IM, Colombo M, Rodak O, Niżański W, Luvoni GC. Effect of fixatives and fixation period on morphology and immunohistochemistry of feline ovarian tissue. Animals 2024;14:825.

[23] Amorim CA, Donnez J, Dehoux J-P, Scalercio SR, Squifflet J, Dolmans M-M. Long-term follow-up of vitrified and autografted baboon (Papio anubis) ovarian tissue. Hum Reprod 2018;34:323–34. https://doi.org/10.1093/humrep/dey355.

[24] Amorim CA, Jacobs S, Devireddy RV, Van Langendonckt A, Vanacker J, Jaeger J, et al. Successful vitrification and autografting of baboon (Papio anubis) ovarian tissue. Hum Reprod 2013;28:2146–56. https://doi.org/10.1093/humrep/det103.

[25] Alkali IM, Colombo M, Luciano AM, Nizanski W, Ali Hassan H, Dziegiel P, et al. Culture of vitrified bovine ovarian tissue on agarose gel inserts maintains follicle integrity. Reproduction 2023. https://doi.org/10.1530/rep-23-0089. REP-23-0089.

[26] Ochota M, Kulus MJ, Młodawska W, Kardasz-Kamocka M, Haczkiewicz-Leśniak K, Podchorska-Okołów M, et al. Ultrastructural changes in feline oocytes during ovary storage for 24-and 48-hours. Theriogenology 2023;197:101–10.

[27] Ali Hassan H, Banchi P, Chayaa R, Pascottini OB, Maniscalco L, Iussich S, et al. Feline ovarian tissue vitrification: the effect of fragment size and base medium on follicular viability and morphology. Theriogenology 2023;198:12–8. https://doi.org/10.1016/j.theriogenology.2022.12.016.

[28] Arav A, Patrizio P. Automation in oocyte and ovarian tissue vitrification. In: Grynberg M, Patrizio P, editors. Female and male fertility preservation. Cham: Springer International Publishing; 2022. p. 387–94.

[29] Arav A. Cryopreservation of oocytes and embryos. Theriogenology 2014;81: 96–102. https://doi.org/10.1016/j.theriogenology.2013.09.011.

[30] Najafi A, Asadi E, Benson JD. Ovarian tissue cryopreservation and transplantation: a review on reactive oxygen species generation and antioxidant therapy. Cell Tissue Res 2023;393:401–23. https://doi.org/10.1007/s00441-023-03794-2.

[31] Yin H, Kim SS, Fisher J, Arnold JR, Gosden RG. Investigation of optimal conditions for equilibrating ovarian tissue with ethylene glycol prior to vitrification. Fertil Steril 2001;76:S101. https://doi.org/10.1016/S0015-0282(01)02298-1.

[32] Guan N, Blomsma SA, Fahy GM, Groothuis GMM, de Graaf IAM. Analysis of gene expression changes to elucidate the mechanism of chilling injury in precision-cut liver slices. Toxicol Vitro 2013;27:890–9. https://doi.org/10.1016/j.tiv.2012.10.009.

[33] Fujihara M, Kaneko T, Inoue-Murayama M. Vitrification of canine ovarian tissues with polyvinylpyrrolidone preserves the survival and developmental capacity of primordial follicles. Sci Rep 2019;9:19–40711.

[34] Gironi B, Kahveci Z, McGill B, Lechner B-D, Pagliara S, Metz J, et al. Effect of DMSO on the mechanical and structural properties of model and biological membranes. Biophys J 2020;119:274–86.

[35] Leonel ECR, Corral A, Risco R, Camboni A, Taboga SR, Kilbride P, et al. Stepped vitrification technique for human ovarian tissue cryopreservation. Sci Rep 2019;9: 20008. https://doi.org/10.1038/s41598-019-56585-7.

[36] Ñaupas LVS, Brito DCC, Souza SS, Brandão FAS, Silva RF, Fernandes CCL, et al. Alpha-lipoic acid in ovarian vitrification solution for in vitro culture or autotransplantation as future strategies for the restoration of ovarian function in sheep. Livest Sci 2022;266:105123. https://doi.org/10.1016/j.livsci.2022.105123.

[37] ASRM. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. Fertil Steril 2019;112:1022–33. https://doi.org/10.1016/j.fertnstert.2019.09.013.

[38] Zhao J, Ge Y, Li J, Zeng Y, Dong G, Zeng L, et al. Experimental study on the vitrification and xenotransplantation of human ovarian tissue. Gynecol Obstet Clin Med 2022;2:38–42. https://doi.org/10.1016/j.gocm.2022.01.006.

[39] Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Lamb SV, et al. Development of antral follicles in cryopreserved cat ovarian tissue transplanted to immunodeficient mice. Theriogenology 2004;61:581–94. https://doi.org/10.1016/S0093-691X(03)00244-9.

[40] Kong HS, Lee J, Youm HW, Kim SK, Lee JR, Suh CS, et al. Effect of treatment with angiopoietin-2 and vascular endothelial growth factor on the quality of xenografted bovine ovarian tissue in mice. PLoS One 2017;12:e0184546.

[41] Gavish Z, Peer G, Hadassa R, Yoram C, Meirow D. Follicle activation and 'burn-out' contribute to post-transplantation follicle loss in ovarian tissue grafts: the effect of graft thickness. Hum Reprod 2014;29:989–96. https://doi.org/10.1093/humrep/deu015.

[42] Anderson RA, Baird DT. The development of ovarian tissue cryopreservation in Edinburgh: Translation from a rodent model through validation in a large mammal and then into clinical practice. Acta Obstet Gynecol Scand 2019;98:545–9. https://doi.org/10.1111/aogs.13560.

[43] Vilela JMV, Leonel ECR, D'Oliveira L, Paiva REG, Miranda-Vilela AL, Amorim CA, et al. Culture of domestic cat ovarian tissue in vitro and in the chick embryo chorioallantoic membrane. Theriogenology 2016;86:1774–81. https://doi.org/10.1016/j.theriogenology.2016.05.038.

[44] Müller MC, Monte APO, Lins TLBG, Macedo TJS, Barros VRP, Ferreira VC, et al. Fibroblast growth factor-2 promotes in vitro activation of cat primordial follicles. Zygote 2022;30:730–4. https://doi.org/10.1017/S0967199421000964.