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Cat preantral follicle survival after prolonged cooled storage followed by vitrification

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ABSTRACT

The aim of this study was to investigate the impact of prolonged storage at 4 °C on survival of cat preantral follicles (PAFs) pre- and post-vitrification. Ovaries were obtained from 12 queens and transported at 4 °C within 2–6 h. Parts of the ovaries were stored for an additional 24 h or 72 h. The ovarian cortex was dissected, analyzed for viability (neutral red - NR) and morphology (histology - HE and ultrastructural analysis by TEM) and vitrified. We used 2 mm biopsy punches to obtain equal size pieces as the experimental units. After NR assessment, each sample was fixed and embedded in paraffin for HE staining to determine the number of morphologically intact follicles. Another 2 mm piece of ovary was subjected to TEM. NR viability assessment and HE results showed a similar tendency with PAF survival postvitrification even after prolonged cooling at 24 h and 72 h. With TEM, integrity of mitochondria, plasma and basal membranes as well as the presence of pre-granulose cells of PAFs were documented postvitrification for the control group and 24 h prolonged storage group, but not after 72 h storage. Our results showed that cat PAFs can survive prolonged storage followed by vitrification. The described set of techniques are applicable towards creating a gamete bank for endangered feline species.

1. Introduction

Most of the 35 feline species are listed on the IUCN Red List (International Union for Conservation Nature, 2015) in high categories with seven species listed as endangered, seven as near threatened and 9 as vulnerable. Fragmented and isolated populations promote genetic depletion and lead to species extinction [1,2]. Today, each live individual on the Red List contributes substantially to the species genetic pool. In females, the ovaries contain a huge and important genetic reservoir], the preantral follicles (PAFs) [3].

Mammalian oocytes develop from a few primordial germ cells that multiply into several million cells during fetal life, and migrate to the immature gonad and remain in a quiescent state until recruitment, maturation, growth and ovulation [4,5]. Most ovarian oocytes remain quiescent throughout the female reproductive lifespan, but become a valuable source of gametes for assisted reproductive techniques when the animal dies or undergo castration and if the ovaries can be obtained for preservation of germ cells within a limited time frame.

In the case of wildlife animals, the challenge is to keep the intraovarian oocytes alive after the animal's death. A method for transportation of gonads during a prolonged time period, as well as an easily usable cryopreservation procedure, are required. In this context, vitrification of the ovarian cortex tissue might be the best technique because it does not need sophisticated equipment and can be done quickly under field conditions. Vitrification means ultrafast freezing, whereby the tissue samples are plunged directly into liquid N_2 [3]. In this way, formation of ice crystals is inhibited and the sample becomes a solid vitreous mass of intra- and extra-cellular medium [6].

The cryopreservation strategy for ovarian cortex tissue was first developed for application in human cancer patients to preserve the fertility of women who were about to be subjected to gonadotoxic chemotherapy or radiological treatment [7,8]. In a recent report on

Abbreviations: IUCN, International Union for Conservation Nature; PAF, preantral follicles; TEM, transmission electron microscopy; CEUA, Ethics Committee on Animal Use; DPBS, Dulbecco's phosphate-buffered saline; NR, neutral red; VS1, vitrification solution 1; VS2, vitrification solution 2; VS3, vitrification solution 3; WS1, warming solution 1; WS2, warming solution 2; WS3, warming solution 3; WS4, warming solution 4; RT, room temperature; PBS, phosphate buffered saline; CFN, intact primordial follicles; N, nucleus; SER, smooth endoplasmic reticulum; ZP, zona pellucida; OO, oocyte; GC, granulosa cells; mv, oocyte microvilli; NM, nuclear membrane

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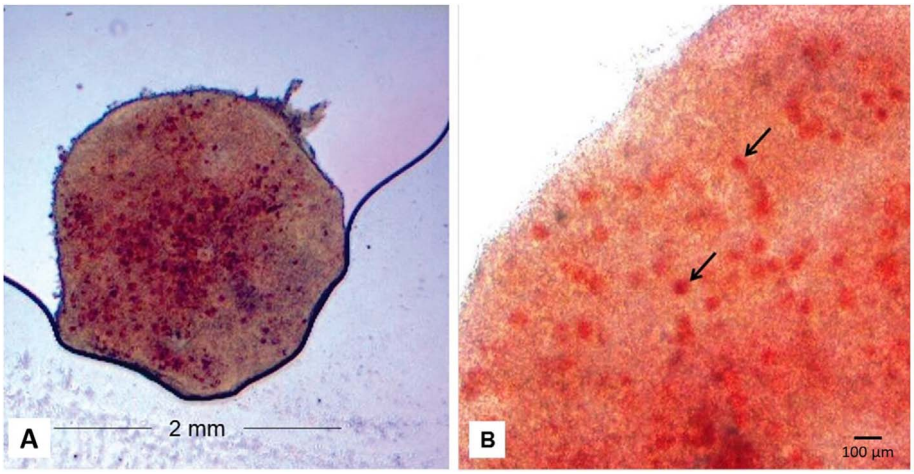

Fig. 1. Photograph of ovarian cortex tissue after vitrification stained with NR. A. Size (2 mm) of ovarian cortex tissue sample. B. Stained PAFs (red points, arrows) with NR (40× magnification). Scale bar, 100 µm (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cryopreservation of human ovaries, better preservation of preantral follicle morphology was observed after cortex vitrification than after a slow-freezing procedure [7]. In particular, the contacts between the oocyte and the granulosa cells were better maintained.

Biotechnology developed for human ovarian tissue might be applied to endangered species for creating gamete banks, if these procedures can be adapted to both the species of interest and the field conditions. In this respect, domestic cats gain relevance because they are excellent experimental models for developing and validating freezing methods to be applied to wild feline species [8–14].

Although vitrification has already been applied for ovarian cortex preservation in bitches [15] and queens [16], the post-warming viability of early oocytes remains unsatisfactory [16–18]. The most challenging aspect is to determine viability of intraovarian oocytes without transferring ovarian tissues back into recipients because it is very expensive and time demanding procedures. Here, better methods that reflect PAF survival will be helpful for evaluating different freezing and warming approaches.

The aim of the present study was to apply a vitrification protocol to feline ovarian tissue that was successfully used in human fertility protection, using domestic cat queens as an experimental model. We investigated the impact of prolonged holding of ovaries at 4 °C for up to 72 h to mimic field and transport conditions. To monitor the survival of feline PAFs post-warming, we used NR staining and morphology assessment by HE staining and TEM.

2. Materials and methods

2.1. Reagents

All chemicals used in this research had the highest possible level of purity and were acquired from Sigma-Aldrich (Taufkirchen, Germany) and Calbiochem (EMD Chemical Inc., San Diego, CA, USA). Any exception to these sources is specified.

2.2. Ethical aspects

This study was performed according to the Ethical Principles of Animal Experimentation and approved by the Ethics Committee on Animal Use (CEUA), Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, SP, Brazil with the Protocol [No./number] 80/2012-CEUA.

2.3. Ovary acquisition and transport

Between November 2013 and February 2014, 12 ovaries were obtained after ovariohysterectomy from the animal clinic of the Berlin shelter (Berlin, Germany). All ovaries were obtained from adult domestic queens who were presented by their owners for castration. The ovariohysterectomies were performed by the clinical personnel. After surgery, the ovaries were placed in plastic 50 mL tubes (Greiner Bio-One, Frickenhausen, Germany) containing transportation medium (Eagle's Minimum Essential Medium, modified with HEPES mM, supplemented with BSA (3 mg/mL); Merck, Darmstadt, Germany) and stored at 4 °C in the refrigerator until transportation to the laboratory. During transportation the ovaries were placed in a Styrofoam box and cooled with ice packs. The time between surgery and arrival at the laboratory could not be exactly determined but it did not exceed 6 h. Upon arrival, ovaries were washed, freed of connective tissues and examined for the presence of corpora lutea, prominent antral follicles or cysts. Only inactive (anestrous) ovaries or those at the early follicular phase (follicles < 0.5 mm) were selected.

2.4. Experimental groups and cold storage

All ovaries were processed to provide samples for both the experimental and control groups. Therefore, each ovary was divided into three parts: a control group and two “prolonged storage” groups that were maintained at 4 °C for 24 h or 72 h to mimic tissue transportation under field conditions. For this purpose, ovary portions allocated for prolonged storage were placed into separate 50 mL tubes with 20 mL Dulbecco's phosphate-buffered saline (DPBS) and returned to the refrigerator. After the prolonged storage, preparation of the ovarian cortex was undertaken as for the control, with samples being collected for morphological and functional analyses and for vitrification (see below).

2.5. Ovarian cortex preparation

A scalpel blade was used to dissect the medullary portion from each ovarian portion until only a thin layer of cortex tissue remained (~200 µm thickness). The procedure was performed within a laminar flow box using ocular magnifiers. The cortex layer was split in half, producing two tissue pieces per ovary portion. One of the pieces was cut into at least three smaller parts (2 mm × 1 mm, Fig. 1A) for vitrification (see below). The other piece was treated with biopsy punches (2 mm, Integra Miltex, York, PA, USA) to obtain same sized samples for viability analysis with NR. After NR staining, the same samples were fixed

for histology. At least one biopsy sample was immediately fixed for TEM (see below).

2.6. Vitrification

Ovarian cortex vitrification was performed as described by Keros et al., 2009 [7]. Briefly, the tissue samples were incubated in vitrification solutions with increasing cryoprotectant concentrations (VS1, VS2, VS3 – see below). The base medium was DPBS supplemented with 10 mg/mL BSA. Dimethylsulfoxide (DMSO), 1,2 -propanediol (PrOH) and ethylene glycol (EG) were used for all three vitrification solutions, polyvinylpyrrolidone (PVP) was added only to vitrification solution 3 (VS3). Vitrification solution 1 (VS1) contained 2.5% of each cryoprotectant with a final cryoprotectant concentration of 7.5%. Vitrification solution 2 (VS2) and vitrification solution 3 (VS3) contained 5% and 10%, respectively, of each of the 3 cryoprotectants. Thus, the final cryoprotectant concentration in VS2 was 15% and in VS3 it was 30%. In addition, VS3 was supplemented with PVP (10% w/v). All vitrification solutions were prepared in advance, aliquoted in 2 mL plastic tubes and stored at −20 °C until use.

The vitrification of ovarian cortex pieces was performed according to the following sequence. Each piece was placed into a tube with 2 mL VS1 for 5 min at RT, thereafter it was gently transferred to VS2 for another 5 min at RT. Finally, the tissue was transferred to 2 mL VS3, which was pre-cooled to 4 °C in an ice bath. After 5 min, the sample was carefully placed into a 0.5 mL open freezing straw and immersed directly into liquid N_2. The straw was prepared with a sloping cut of the tip (see Ref. [7]). All straws from one group were placed together into a properly identified cryocontainer for at least 24 h storage in liquid N_2.

2.7. Warming

The warming procedure included the transfer of tissues through four different solutions that were based on DPBS and BSA (10 mg/mL) and contained decreasing sucrose concentrations. The warming solutions WS1, WS2 and WS3 were supplemented with 0.5 M, 0.25 M or 0.125 M sucrose, respectively. Warming solution (WM4) was sucrose-free. All the warming solutions were prepared in advance and stored in 2 mL aliquots at −20 °C.

For warming, straws were removed from the container within the liquid N_2 and then directly immersed into a 2 mL plastic tube with WS1, which was warmed to 37 °C in a heating block. After 5 min, the tissue was transferred step by step to WS2, WS3 and finally WS4. These steps were performed at RT for 5 min each. Each tissue sample was rewarmed separately.

2.8. Preantral follicle viability

2.8.1. Neutral red staining

Samples of the same size (2 mm), which had been punched out, were stained with NR by placing them into a 4-well dish (Nunc™ 4-well dish, Thermo Scientific, Dreieich, Germany) containing 400 µL washing medium (M199 supplemented with 0.1 mg/mL cysteine, 3 mg/mL BSA, 4 ng/mL sodium lactate, 2.3 mM sodium pyruvate, 6 mM HEPES, 55 µg/mL gentamicin and 3 mM L-glutamine) and 10 µL NR (2.5% v/v). After incubation at 37.5 °C under 5% CO_2 for 16 h, each sample was placed on a slide, compressed with a coverslip and evaluated using an inverted microscope (Axiovert, Carl-Zeiss, Germany) at 40× magnification. All red-stained follicles (Fig. 1B) were counted. The result was expressed as the number of viable PAFs in a 2 mm sample (Table 1). Thereafter, each sample was transferred to Bouin's solution for fixation.

2.8.2. PAF counting in histology samples

Samples were fixed for 24 h in Bouin's solution, then transferred them into PBS. They were kept at 4 °C until processing for standard histology. After dehydration, each sample was positioned vertically in the paraffin block. Serial sections of 3 µm thickness were made and every tenth section was mounted (30 µm distance between mounted sections). The slides were stained with hematoxylin and eosin (HE), (HE Merck, Darmstadt, Germany).

The histological sections were evaluated under a microscope as described previously by Wiedemann et al. [17]. In brief, the number of sections needed for detecting 40 PAFs was noted. In addition, all the counted follicles were characterized as intact or degenerated. Degeneration of PAFs was characterized by follicular, cellular or nuclear membrane ruptures, cellular retraction, altered morphology, vacuolization and other signs. According to Wiedemann et al. [17] the total number of PAFs per piece (number of primordial follicles - CFN) was calculated (CFN = $\pi/4$ [(diameter x n PAFs)/(n x distance)]), and the percentage of follicles was determined. For each ovary and group at least 3 biopsy punch samples were processed.

2.8.3. Transmission electron microscopy

One biopsy punch sample was transferred to Karnowsky solution for fixation and stored for processing by TEM. Although ovarian samples from each group were fixed for TEM, only the best and the worst samples of each group (according to viability by NR) were later analyzed, due to the high work load and great cost.

After washing in PBS, the samples were treated with osmium tetroxide, dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Semi-thin sections of 1 µm were cut and stained with toluidine blue for preselection. Ultrathin sections (70 nm) of the selected tissue part were stained with uranyl acetate, followed by the application of lead citrate. The PAFs present in the selected samples were analyzed and electron micrographs were made at magnifications between 1,250× and 30,000× using a FEI TecnaiSpiritBT device (120 kV; FEI Deutschland GmbH).

Primordial follicles were evaluated for ultrastructure by assessing the integrity of the basal and cytoplasmic membranes, the presence and density of mitochondria, organelles and nucleoli. In addition, granulosa cells were analyzed for morphology. All the primordial follicles present in each sample were evaluated.

2.9. Statistical analysis

Initially, the distribution of variables was determined using graphical analysis (histogram, qq plots) and the Shapiro-Wilk normality test. The number of viable follicles determined by NR was transformed to log scale to achieve normality. A multivariate analysis of variance for repeated measures (PROC MIXED, SAS Institute, 2011) was used to compare the average number of follicles among the treatments and times. A term of interaction between treatment and time was included in all models to test the hypothesis that differences among the treatments were dependent on time. An autoregressive covariance structure was used to model the correlation between the measurements obtained within the same animal. Tukey's test was used to adjust the p values resulting from multiple comparisons.

To study the effect of vitrification on the CFN within each time, a generalized linear model with logarithmic response was adjusted to use a negative binomial binding function and to consider repeated measurements [19,20]. Comparisons were made using the LS Means of the SAS Proc Genmod procedure (Statistical Analysis System, version 9.3). The goodness of fit was checked by the deviation per degree of freedom (scaled deviance). A non-linear regression analysis was used to evaluate the correlation between NR and CFN techniques, when a type 2 polynomial curve was fitted and the R^2 calculated. For all analyses, the level of statistical significance was set at [$p < 0.05$].

Table 1
Mean ± standard deviation of CFN counted in the histological samples stained with HE in cortical tissue of the same size (2 mm) of the domestic cat.

	Before Vitrification (n = 6)			After Vitrification (n = 6)		
Cooled storage time (4 °C)	Control	24 h	72 h	Control	24 h	72 h
PAF number	138 ± 243	107 ± 93	137 ± 194	201 ± 273	68 ± 108	31 ± 34

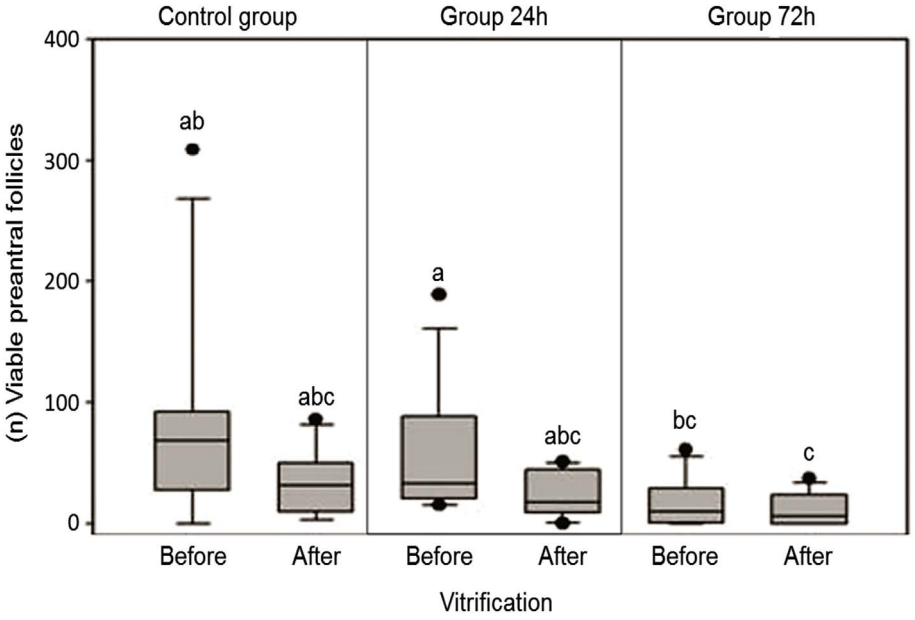

Fig. 2. Box plot of PAFs viability in samples of the same size (2 mm) from the ovarian cortex of the domestic cat before and after vitrification, stained by NR (median, mean and *outliers*). Different letters (a, b and c) indicate significant difference between the groups (n = 12, $p < 0.05$).

3. Results

3.1. PAF viability by neutral red (NR)

The results of neutral staining are presented in Fig. 2. The effect of vitrification on the average number of PAFs varied according to how the data were grouped. An effect of vitrification was not observed when the groups were analyzed separately. Thus, no significant difference was found after 24 h and 72 h storage of control samples according to the average number of viable PAFs. The comparison between groups, however, revealed a significant difference pre- and post-vitrification: pre-control *vs.* 72 h post-control ($p = 0.014$), pre-24 h *vs.* post-72 h ($p = 0.009$) and between the post-24 h *vs.* post-72 h ($p = 0.036$) (Fig. 2).

To assess the impact of vitrification alone, we combined the pre-freezing groups (control + 24 h + 72 h) and the post-freezing groups (control + 24 h + 72 h). In this case, a significant effect of vitrification was determined ($p = 0.038$; mean ± standard deviation, 52 ± 63 *vs.* 23 ± 21 viable follicles per piece, respectively) although a large data variation was observed.

In this scenario of uncertainty about the total number and distribution of PAFs in the ovarian cortex, we considered analyzing the percentile changes after prolonged storage and vitrification, thus the effect of time and/or treatment on follicular viability as determined with NR. First, the control at 0–6 h was set as 100% (control 0–6 h: 83.8 PAFs per piece = 100%) and viability after 24 h and 72 h storage was calculated for the pre-freezing samples (storage for 24 h: 55.8 PAFs per piece = 67.2%, storage for 72 h: 15.9 PAFs per piece = 19.2%), and for the post-warming samples (control: 33.7 PAFs per piece = 40.6%, storage for 24 h: 23 PAFs per piece = 27.7%, storage for 72 h: 10.7 PAFs per piece = 12.9%). For the second analysis, post-warming viability of the control was set at 100% (control: 33.7 PAFs per piece 100%), whereas the survival after 24 h and 72 h prolonged storage pre-vitrification was 68.1% (23 PAFs per piece) and 31.8% (10.7 PAFs per piece) post-warming, respectively.

Finally, the viability determined by NR was compared between the two sample pairs for each storage time (before and after vitrification): for controls 40.6% survived vitrification (83.8 per piece *vs.* 33.7 per piece), for 24 h storage groups 41.2% survived (55.8 per piece *vs.* 23.0 per piece) and for 72 h storage groups 67.5% survived (15.9 per piece *vs.* 10.7).

3.2. Histology

The histological analysis indicated a reduction in the number of intact PAFs after prolonged storage and after vitrification (Table 1); however, this decrease was mitigated by a large variation in the number of PAFs between samples. The total CFN was consistent with the mean number of viable PAFs counted using NR (Table 1), but no significant difference was observed between the CFN groups ($p = 0.49$).

The majority of PAF samples from the control and 24 h groups (Fig. 3), pre- and post-vitrification, showed good morphology, i.e., preservation of their cell structures. Degenerated PAFs with substantially altered morphology were more commonly found in the 72 h group.

The non-linear regression coefficient (R^2) was 0.51, thus indicating a moderate correlation between both techniques in the present study. If NR was used to estimate the CFN, only 51% of the data variability should be contemplated, the remaining 49% of the variability would be attributed to unknown variables not measured by this experiment.

3.3. Transmission electron microscopy

For TEM analysis, samples from the control and 24 h groups post-

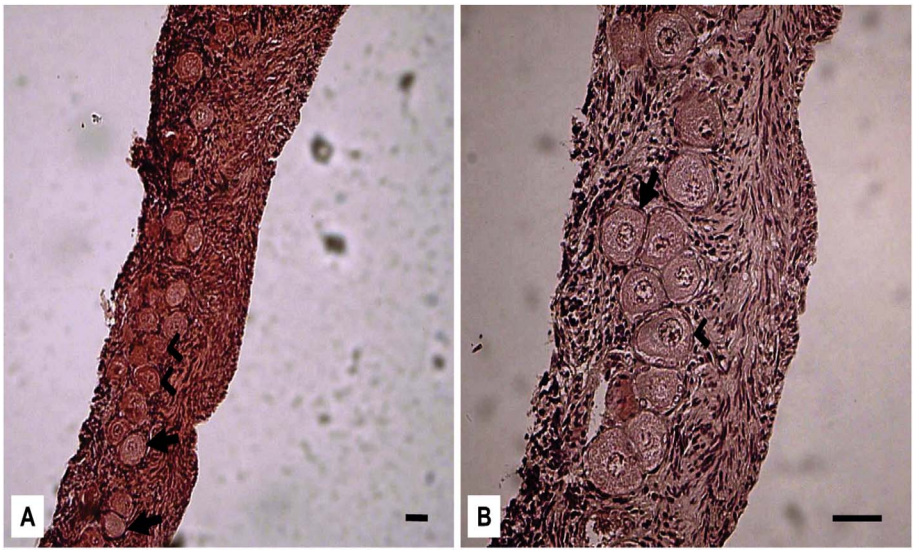

Fig. 3. Microphotography of domestic cat ovarian cortex tissue stained with HE. A. PAFs with normal morphology non-stained (arrow) or stained (arrowhead) with NR (100× magnification). B. Intact primordial follicle (arrow) containing oocyte surrounded by follicular layer of flattened cells and transitional PAF (arrowhead) containing oocyte surrounded by follicular layer of flattened and cuboid cells (200× magnification). Scale bar, 50 µm (A and B).

warming showed good cellular preservation of follicle structures including membranes and mitochondria. A loss of morphological integrity was observed in both 72 h groups, pre- and post-vitrification. Pre-freezing, cellular arrangement was impaired but follicular structure was still preserved. After warming, a total loss of all follicular and cellular arrangements was evident, with basal and cytoplasm membrane rupture and strong cytoplasm disorganization in the oocytes and in the granulosa cells.

The effect of prolonged storage pre- and post-vitrification is described in detail below and in Fig. 4.

3.3.1. *Pre-vitrification*

In the control group, intact follicles with condensed chromatin (Fig. 4A) were observed. One of the samples exhibited endoplasmic reticulum surrounded by mitochondria (Fig. 4A1). Mitochondria were present in lobular and round shapes, with light ridges and a double membrane (Fig. 4A1). Pre-granulosa cells had normal morphology, and the follicles had intact membranes. In the 24 h group, morphological preservation of follicular structures was observed. Oocytes with zona pellucida and microvilli (Fig. 4C) and pre-granulosa cells with normal morphology were present. After storage for 72 h, samples showed

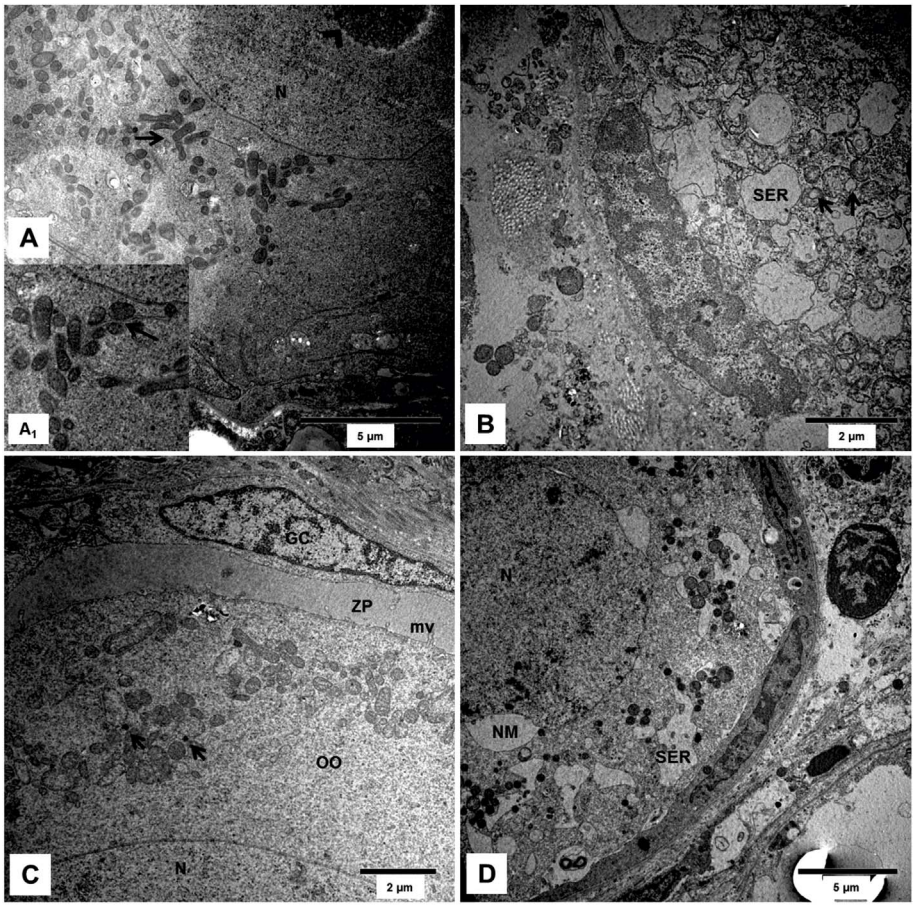

Fig. 4. TEM photomicrographs of the primordial follicles of cats. A) Condensed chromatin (arrowhead) in nucleus (N) and intact mitochondria with intact morphology (arrow) in the control group. A1) Rough endoplasmic reticulum surrounded by mitochondria (arrow) in the control group. B) Smooth endoplasmic reticulum (SER) with enlarged, vacuolated mitochondria (arrows) in the 72 h group. C) Zona pellucida (ZP) between the oocyte (OO) and granulosa cells (GC), oocyte microvilli (mv) and cortical granules (arrows) in the 24 h group. D) Expanded nuclear membrane (NM) in the 24 h group.

disrupted membranes in the oocytes and pre-granulosa cells, with cytoplasmic disorganization. Vacuolization was observed in the mitochondria, and dilations were observed in the smooth endoplasmic reticulum (Fig. 4B).

3.3.2. Post-vitrification

The samples from the control group were characterized by adequate preservation of all cell structures. However, some PAFs were observed with a mild rippling of the basal membrane, a discrete loss of follicular structure, and loss of the arrangements between the oocyte and granulosa cells. The mitochondria showed loss of ridges, and within the smooth endoplasmic gratings dilation of the cisterns were found. The nuclear membranes appeared wavy. Oocytes were better preserved than granulosa cells. Samples from the 24 h group contained follicles with dilation of the perivitelline space and dilatation of smooth endoplasmic reticulum (Fig. 4D). The basement membrane of the follicle retained its integrity, but severe disorganization of the cytoplasm and cell organelles was observed. The 72 h group samples exhibited complete follicular disruption.

4. Discussion

The absence of significant differences between the control and 24 h storage groups post-vitrification is the main finding of the experiment and is valuable because it enables us to transport gonadal material for one day without any significant loss of viable PAFs. To the best of our knowledge, this is the first demonstration of cat PAFs surviving after 24 h of cooled storage at 4 °C followed by vitrification.

Working with ovarian cortex produces a great challenge to assess the viability of cells within a complex matrix of connective tissue. As shown by Matos et al. [21], different methods are applied based either on intactness of follicular morphology or viability of PAFs. Techniques applied include histomorphology [3], fluorescent viability staining [22], immunohistochemical techniques [16] and short-term follicle culture [17]. Here, we applied a set of methods to directly compare between the morphology approach (HE and TEM) and viability staining (NR). In this way, our results are able to demonstrate the advantages and disadvantages of each technique, and provide a comprehensive reflection of follicular survival within the ovarian cortex.

A major difficulty in analyzing viability of intraovarian follicles is related to the unequal number and distribution of follicles in the cortex. It was suggested that the variations are associated with the species, animals' ages, nutritional status, health and estrous cycle stage [23,24]. The continuing process of follicular recruitment and growth opens the ovarian tissue matrix and constantly changes the position and distribution of the PAFs in the cortex layer. We found in the same ovary neighboring tissue samples without any PAFs and samples with > 100 follicles. To minimize PAF number fluctuation, we used a biopsy punch to obtain a standard size piece (2 mm) and subsequently performed NR and HE analysis on the same sample, but still high standard variations were found in all groups and probably masked differences between groups and time points. Also the correlation between NR counts and CFN calculation, although performed on the same samples, revealed only 51% of the data variability should be contemplated. By combining NR viability staining with subsequent histology, we observed red stained follicles in our HE samples indicating the persistence of NR during histological procedures. Other morphologically intact follicles, however, were NR-free. This suggests that cells can have a normal appearing morphology but yet were not necessarily alive at the time of fixation.

The morphological approach requires fixation followed by thin slicing of the tissue. In this way, further use of a particular tissue sample is excluded, and only a small sector can be evaluated. For normal histology, serial sections (CFN) might provide a broader picture but this procedure is time-consuming. In the case of TEM, only a very small piece of tissue can be assessed, and it is often hard to detect whole follicles. Viability stains, like NR, do not change the tissue architecture, and even allow further processing of the same piece. Chambers et al. [24] predicted the density of follicles in slices of ovarian cortex from humans and small ruminant species by NR before culture. They showed that staining or incubation in NR prior to culture did not compromise subsequent follicle survival. The disadvantage of viability stains, however, is the impaired permeability and therefore visibility through dense cellular layers. Cortex slices must be quite thin (~200 µm) to obtain equal staining of all follicles. In addition, we observed that for good NR staining prolonged incubation (16 h) was required. Other viability stains, like ethidium/calcein [25], act more quickly but are less suitable for field conditions, because of the need for fluorescence microscopy.

Our results demonstrate that it is very useful to combine morphological and functional (viability) methods to minimize disadvantages and obtain complementary information. Thus, it was possible to find PAFs with good morphology (CFN) and positive NR staining after 24 h storage at 4° C followed by vitrification. TEM analysis underlined the results obtained by histology and NR, although it represents more of a "snap-shot" result, due to the small number of follicles assessed. However, we confirmed the ultrastructure of PAFs provided by Carrijo et al. [26], especially with respect to the shape and distribution of mitochondria and the appearance of membranes. Also, our TEM data revealed the preservation of subcellular structures after cooling for 24 h prior to vitrification and re-warming.

Until now, the only way to really prove PAF survival after cryopreservation is xenotransplantation [27,28], but while this complex and expensive procedure might be eligible for excluding tumor cells in human ovarian tissue designated for fertility restoration in cancer patients, it cannot be used in routine research. Other researchers suggested brief culture for seven days [27] or 14 days [17] of *culture* after warming to evaluate the impact of cryopreservation on PAF survival. But again, for cell culture sophisticated laboratories are required, thus this approach is not suitable for any field conditions.

Vitrification of cat ovarian cortex was performed in only two studies before. Luvoni et al. [16] used a different protocol. They used DAP 213 solution (2 M DMSO, 1 M acetamide and 3 M propylene glycol) at 0°C with tissue fragments maintained on ice for 5 min. They focused on in vitro maturation of fully grown oocytes obtained from the ovarian tissue after thawing. Differences in aims and experimental design makes comparison difficult between their results and our findings. Recently, Mouttham et al. [29] showed that successful vitrification of cat ovarian cortex tissue depended on the duration and temperature of exposure to cryoprotectant solution (15% EG, 15% Me2SO, 20% FBS in MEM-Hank's) which should contain 0.5 M sucrose. Incubation in the cryoprotectant solution for 10 min at 4°C improved PAF survival post-vitrification. Tissue survival was shown by histological evaluation of follicle morphology after two days of culture. We applied a different cryoprotectant composition, published by Keros et al., which contained DMSO, PROH, ETOH and PVP at higher overall concentration (40% total) but exposure time was only 5 min at 4°C. For post-thaw cryoprotectant dilution we also used sucrose. Future protocols for cat cortex tissue vitrification could combine 0.5 M of sucrose as used in the "Keros vitrification solution" and expose tissue for 10 min at 4°C, as described for Mouttham [29,30] in attempts to improve our results.

Despite the mean loss of 40% in PAF viability, the protocol for ovarian storage at 4 °C prior to cortex preparation and vitrification can be recommended to preserve the feline ovarian matrix. In one sample, even after 72 h of cold storage followed by vitrification, we still found more than 100 viable PAFs with good morphology. Therefore, we suggest that 72 h is the upper time limit for transportation of ovaries in PBS at 4 °C. In 1997, Wood et al. [31] suggested 48 h as a time limit for preserving oocyte viability at 4 °C, but they exclusively applied morphological analysis to draw their conclusions. Anyway, for gamete preservation the fastest transportation at 4 °C has to be achieved, with an ideal maximum of 24 h before cryopreservation.

Overall, the technique set and protocols described here, in particular the simple vitrification procedure and the easily applied viability assessment *via* NR, support the feasibility of preserving germ cells from feline ovaries. Our protocol is very portable and all its important steps can be performed under field conditions without any freezing machine. In an actual global scenario of climate changes we strongly encourage the distribution of methods like these as part of the efforts to prevent extinction in zoological and wildlife feline populations, and for building a base for a future female gamete bank of wild cat neotropical species.

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Conflicts of interest

The authors declare there are no known conflicts of interest associated with this publication.

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