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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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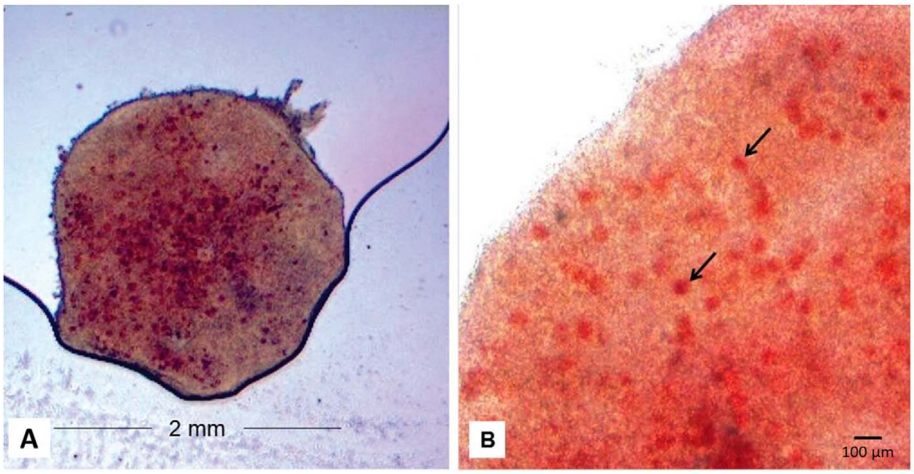

Fig. 1. Photograph of ovarian cortex tissue after vitrification stained with NR. A. Size (2 mm) of ovarian cortex tissue sample. B. Stained PAFs (red points, arrows) with NR (40× magnification). Scale bar, 100 µm (B). (For interpretation of the references to colour in this figure legend, the reader is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