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Effects of different sucrose concentrations on vitrified porcine preantral follicles: Qualitative and quantitative analysis

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ABSTRACT

The aim of the present study was to perform a qualitative and quantitative analysis of the effect of different sucrose concentrations combined with ethylene glycol in the preservation of vitrified porcine preantral follicles. Fragments of ovarian cortex were vitrified in cryotubes containing 200 μl of the vitrification solution (30% Ethylene Glycol; 20% Fetal Bovine Serum; 0 M–0.25 M – 0.75 M or 1 M sucrose) and stored in liquid nitrogen for a week. Histological analysis showed that after vitrification the number of normal follicles decreased compared to the fresh tissue (control). The percentage of normal primordial follicles was sucrose dose dependent. The percentage of normal primary follicles was similar in 0 M or 0.25 M sucrose, while higher concentrations (0.75 M and 1 M) increased significantly the percentage of abnormal follicles ($p < 0.05$). Morphometric analysis showed a statistically significant reduction in the total area of primordial follicles with 0.75 M sucrose and a significant increase in the cytoplasmic area of primordial follicles with 0 M sucrose ($p < 0.05$). The qualitative and the quantitative analysis appear to be a complementary tool when choosing a vitrification protocol. For our cryopreservation system - vitrification of ovarian cortex slices in cryotubes-the best vitrification medium was TCM 199-Hepes with 30% de ethylene glycol, 20% of Fetal Bovine Serum and 0 or 0.25 M sucrose. The present study shows that the use of high sucrose concentrations in the vitrification solution has a deleterious effect on the preservation of porcine preantral follicles contained in ovarian tissue. Consequently, its use at 0.75 M or 1 M wouldn't be recommended.

1. Introduction

Germplasm banks allow storage of gametes and embryos. They are an essential tool to preserve genetic material from endangered species or individuals with a remarkable genotype [3]. In the last years, there have been great advances in cryopreservation of germinal tissue techniques that have contributed greatly to the establishment of these banks [13].

Cryopreservation of oocytes at different stages of meiotic maturation has random results due to multiple factors [11]. Oocytes size (80–120 microns depending on the species) and the characteristics of their cytoplasmic membrane make difficult the dehydration process and intracellular water replacement with cryoprotectant agents (CPA). This process may result in an increase of intracellular calcium, which has the potential to initiate oocyte activation stimulating the disruption and premature release of cortical granules, leading to the zona pellucida hardening [8,11,12]. Furthermore, the meiotic spindle is temperature sensitive and depolymerizes during cooling. Although this process is reversible during the rewarming of the cell, there is a risk of chromosomal loss and the occurrence of aneuploidy at the first maturation division [4].

Ovarian tissue cryopreservation is an alternative that allows preservation of a lot of oocytes contained in preantral follicles (PAF). Unlike oocytes that have completed their growth, germ cells

Abbreviations: CPA, Cryoprotective Agent; PAF, Preantral Follicles; BS, Base Solution; NR, Nuclear Roundness; OR, Oocyte Roundness; NA, Nuclear Area; CA, Cytoplasmic Area; TA, Total Area.

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contained in PAF are less vulnerable to cryogenic damage. These oocytes are smaller, are surrounded by fewer granulose cells, have a low metabolic rate, lack of zona pellucida and cortical granules, and contain a low amount of intracytoplasmic lipids [21]. At this stage, the cells are arrested in prophase and should theoretically have a lower risk of cytogenetic errors. Moreover, primordial follicles have time to repair sublethal damage in organelles and other structures during their prolonged growth phase. Another advantage of this technique is that obtaining samples is not dependent on age or stage of the estrous cycle and can even be applied to animals that die unexpectedly [3,21].

Knowledge about cryopreservation of PAF and ovarian tissue from sows is still limited. Some authors have reported good results using conventional controlled cooling curves in the presence of different permeable CPA -Me2SO, ethylene glycol and glycerol [3,10]. Others found that vitrification is more efficient [1,14] because it allows rapid cooling and leads to the formation of a glassy vitrified state, representing an easy and inexpensive mean of cryopreserving ovarian tissue that does not require special equipment [1,7,18].

Whether it is gradual cooling or vitrification, CPAs replace the intracytoplasmic water, decreasing the formation of ice crystals within the cell.

Fabbri [6] demonstrated that the addition of non-permeable CPAs and bovine fetal serum preserve the structural and functional integrity of the membrane cell. Sucrose (non-permeable CPA), modifies the osmotic gradient promoting cellular dehydration and inhibiting the formation of intracellular crystals [20]. Some studies have shown that it also stabilizes the phospholipids present in the membrane cell [5], and increases cell survival after thawing [26].

In a previous work [9], we found that ethylene glycol was the less harmful CPA in the vitrification process, compared to Me2SO and a combination of both. The number of normal primordial and primary follicles was higher when using this CPA. The aim of the present study was to analyze qualitatively and quantitatively the effect of ethylene glycol combined with increasing sucrose concentrations as a non-permeable CPA in the preservation of porcine PAF histological structure during the vitrification processes.

2. Materials and methods

2.1. Collection and preparation of porcine ovarian tissue

Ovaries were collected from a local abattoir and transported to the laboratory at 32–35 °C in an adiabatic container. They were washed with a sterile saline solution and processed at room temperature (20 °C) within five hours after collection. A total of ten ovaries were included in this study. From each one of them, ten samples of the ovarian cortex (5 mm × 2 mm × 1 mm) were taken using a microtome blade. Two of them were immediately fixed in Bouin solution for 24 h and then placed in formalin 5% until histological processing, to be used as controls.

2.2. Vitrification and warming procedures

The remaining samples were exposed to an equilibrium solution (Base solution (BS): TCM-199, 25 Mm HEPES supplemented with 50 μg/ml penicillin, 100 μg/ml streptomycin; 15% Ethylene Glycol (EG); 20% Fetal Bovine Serum (FBS); 0–0.25 M – 0.75 M or 1 M sucrose) for five minutes, and then placed in the vitrification solution (BS; 30% EG; 20% FSB; 0–0.25 M – 0.75 M or 1 M sucrose) for one minute, as previously described [9]. All the procedures were carried out at room temperature. Cryotubes (1.8 ml Thermo Fisher Scientific, Denmark) containing ovarian tissue immersed in 200 μL of the vitrification solution were exposed to liquid nitrogen and stored at −196 °C for a week.

For warming, cryotubes were removed from the liquid nitrogen, air-warmed for 30 s and then immersed in a 37 °C water bath for 2 min. The samples were then transferred to a wash solution composed of BS with 20% FSB supplemented with 0–0.25 M – 0.75 M or 1 M sucrose according to concentrations of the vitrification solution, for five minutes at room temperature. Finally, they were fixed with the same protocol as the controls.

After being fixed, all samples were dehydrated, clarified, embedded in paraffin and serially sectioned at 5 μm. Histological evaluation of each section was performed by haematoxylin-eosin staining and observed at light microscope (×400 magnification).

2.3. Qualitative analysis

PAF contained in the ovarian tissue were classified into two categories according to the epithelium morphology: those with a single layer of flattened granulosa cells were classified as primordial while those presenting one layer of cuboidal granulosa cells were classified as the primary [23]. PAF surrounded by both, flattened and one or more cuboidal granulosa cells, were classified as primary [25].

Both follicle types were then classified as normal or abnormal taking into account follicular damage according to Rodrigues criteria [17]. Follicles without alterations in the oocyte or in the granulosa cells were considered normal. On the other hand, those with degenerative changes such as injured nuclear membrane, shrinkage of the nucleus, cytoplasm vacuolization or eosinophilia, granulose cells with high pyknosis or disruption of the epithelium were classified as abnormal.

2.4. Quantitative analysis

Digital photographs of PAF contained in each sample were taken using a trinocular microscope LEICA DM4000B LED® and LEICA DCC-380X® camera with the digital support of the LAS LEICA Inc ® capture program. Finally, nuclear (NR) and oocyte roundness (OR), nuclear (NA), cytoplasmic (CA) and total area (TA) of each follicle were obtained using the Leica Qwin Plus ® software, version 3.1 which generates, edits and quantifies binaries. The NA/CA ratio was also calculated.

2.5. Statistical analysis

Statistical analysis was performed using the non-parametric Friedman test of variance ($p < 0.05$), because it was a completely randomized block design.

The appearance of follicular lesions at different levels, oocyte and granulosa cells, was analyzed by descriptive statistical tables and frequency graphs.

3. Results

3.1. Qualitative analysis

Most of the PAF contained in the fresh ovarian tissue, (primordial 75.00%; primary 76.67% over a total of 781 primordial follicles and 714 primary follicles analyzed from 10 ovaries) presented no morphological abnormalities in their oocytes cytoplasm and nuclei. Granulosa cells retained regular spatial disposition and their nuclei chromatin had slight or no signs of pyknosis. Vitrification in presence of increasing concentrations of sucrose significantly reduced the percentage of normal follicles. Follicles with normal and abnormal morphological characteristics are showed in Figs. 1 and 2.