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Effect of vitrification on meiotic maturation, mitochondrial distribution and glutathione synthesis in immature silver fox cumulus oocyte complexes

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ABSTRACT

The present study was designed to investigate the effects of vitrifying oocytes obtained from silver foxes on nuclear maturation, mitochondrial distribution and glutathione (GSH) synthesis after in vitro culture for 72 h. Immature oocytes were randomly divided into three groups: (1) fresh GV (germinal vesicle) oocytes (Control group), (2) exposure to the equilibration and vitrification solution but without being plunged into liquid nitrogen (exposed group), and (3) vitrification by the cryoloop method (vitrified-warmed group). The number of survival oocytes was not decreased by either being exposed to the cryoprotectant or being vitrified-warmed compared with the control group ($P > 0.05$). After IVM, the percentage of resumption of meiosis for vitrified-warmed oocytes (41.9%) was significantly lower than in the control (81.2%) and exposed (79.1%) groups ($P < 0.05$). However, the proportion of oocytes reaching the metaphase II (MII) stage was similar among the different groups (11.4%, 9.3% and 5.2%, respectively, $P > 0.05$). The translocation of active mitochondria during fox oocyte maturation was revealed using MitoTracker Red staining and confocal laser microscopy. For fresh oocytes at the GV stage, active mitochondria were distributed around the entire cortex with small granulations and various-sized cavities (no MitoTracker signals). After IVM, the mitochondria formed large granulations and clumps throughout the cytoplasm. Vitrification significantly decreased the proportion of MII oocytes with normal mitochondrial distribution compared with the control and exposed groups (35.4%, 71.9% and 59.2%, respectively, $P < 0.05$). Similarly, the GSH content was significantly lower in vitrified-warmed oocytes compared with the control and exposed oocytes after IVM (3.4, 5.7 and 4.7 pM/oocyte, respectively, $P < 0.05$). However, no significant difference was observed between the cryoprotectant exposed and control groups with regard to the normal mitochondrial distribution or GSH content ($P > 0.05$). These results indicate that vitrification of fox immature oocytes using a cryoloop allows them to resume meiosis and develop to the MII stage. The damage to mitochondria and the GSH synthesis deficiency may be associated with the reduced developmental competence of cryopreserved oocytes.

1. Introduction

The silver fox, the domesticated form of the wild red fox, is a seasonally monoestrous breeder belonging to the canine family. Genetic improvements and the preservation of fertility in this species mainly rely on semen cryopreservation and artificial insemination. Compared to the extensive studies in laboratory and livestock species, the limited research in canids has focused on embryo biotechnologies, such as in vitro maturation (IVM), embryo culture and cryopreservation of oocytes. However, there has been even less progress with foxes, although it is critically important to increase their populations, to establish gene banks and to conserve endangered fox species.

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There are few reports regarding cryopreservation of canine oocytes. Abe et al. (2010) [1] vitrified dog GV stage oocytes in DAP213 (2 M dimethyl sulfoxide, 1 M acetamide and 3 M propylene glycol) solution using the cryotop method, and more than 65% of the vitrified oocytes displayed a normal morphology. This result was in agreement with the report by Srirungruang et al. (2012) [2], who vitrified dog GV stage oocytes with a solid surface vitrification (SSV) method. Boutelle et al. (2011) [3] vitrified Mexican gray wolf follicular oocytes using the cryotop method and reported that 61% of the post-thaw intact oocytes were survival. Although a high proportion of vitrified-warmed cumulus-oocyte complexes (COCs) from canine species showed survival, data regarding subsequent in vitro maturation (IVM) and embryo development during in vitro culture are limited, mostly due to challenges arising from the unique reproductive physiology characteristics of those species.

The female gamete in canine species has peculiar characteristics compared with oocytes from most other domestic mammals. Oocytes are ovulated while still at their immature stage and require 2–5 days post-ovulation to complete the first meiotic division within the oviduct [4,5]. Another unusual characteristic of canine oocytes is that they have a large amount of lipid droplets in the cytoplasm and a highly compact cumulus cell layer remains around the fertilized ova until the embryos reach the morula stage [6–8]. These peculiarities are the main obstacles to oocyte cryopreservation and in vitro embryo production. In vitro maturation of ovarian oocytes collected during anestrus has resulted in poor maturation rates in both the silver and blue foxes [9,10]. Although success has been reported for in vitro fertilization of in vitro matured fox oocytes, they failed to undergo cleavage [11]. The ability of oocytes to support subsequent embryonic development involves both nuclear and cytoplasmic maturation. Nuclear maturation refers to resumption of meiosis and completing the metaphase-I and metaphase-II stages. Cytoplasmic maturation includes cytoskeleton changes and translocation of organelles, the storage and accumulation of mRNA and proteins, and changes in cellular metabolism [12,13]. The developmental capacity of in vitro matured oocytes is diminished compared with in vivo matured oocytes, mainly due to insufficient cytoplasmic maturation [14–16]. However, to our knowledge, no studies have been reported on the cytoplasmic maturation and movement of organelles during meiotic maturation of fox oocytes.

Mitochondria are one of the most important organelles in the cytoplasm. They play important roles in respiration, metabolism and cell apoptosis [17,18]. The distribution and metabolic activity of mitochondria undergo changes during oocyte maturation, and the characteristics of their distribution during maturation are different among species [19–22]. Several studies have reported that the mitochondrial morphology of oocytes was affected by vitrification [23,24], and damage to mitochondria or changes in mitochondrial distribution could have strong impacts on oocyte viability and development [25]. No data have been reported on the localization of mitochondria during oocyte maturation in foxes, and whether oocyte vitrification affects mitochondrial distribution also has not been described.

Glutathione (GSH) is a major intracellular thiol compound with important roles in many biological functions, including cellular proliferation, amino acid transport, protein and DNA synthesis and redox state maintenance [26–29]. In oocytes, synthesis of GSH during oocyte maturation is a prerequisite for sperm chromatin decondensation and male pronuclear formation [30,31]. Decreased GSH content of mature oocytes is associated with poor embryo developmental capacity, as reported in the mouse [32], goat [33], cattle [28], pig [31] and dog [34]. Oocytes suffer different types of severe cryodamage after cryopreservation, such as cytoskeleton damage, mitochondrial damage and dysfunction, ATP depletion and apoptosis [35,36]. However, very few studies have been conducted to explore the relationship between oocyte vitrification and GSH synthesis.

For gamete cryopreservation, vitrification appears to be a credible alternative to the conventional slow cooling/freezing technique. Unfortunately, the concentrations of cryoprotectants required for vitrification are much greater than those used in slow cooling, resulting in a hypertonic environment and abnormal osmotic pressure that are detrimental to cell viability. The objectives of the present study were 1) to evaluate the effects of vitrification of silver fox immature oocytes or exposing them to cryoprotectants on nuclear maturation after culturing in vitro for 72 h; 2) to determine the characteristics of mitochondrial distribution in fox oocytes during IVM; and 3) to determine whether vitrification or exposure to cryoprotectant affects mitochondrial distribution and GSH synthesis during fox oocyte maturation.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louris, MO, USA) unless otherwise stated.

2.1. Animals

Silver fox ovaries were obtained from local abattoir from October to December, and transported within 2 h to the laboratory in sterile saline at 30–35 °C. Before collecting COCs, ovaries were washed three times in fresh modified Dulbecco's phosphate buffered saline (mDPBS, DPBS + 0.1% PVA + 100 IU/ml Penicillin G + 100 μg/ml Streptomycin sulphate), then ovarian tissue was sliced repeatedly in petri dishes containing mDPBS to release COCs. Oocytes that had more than three layers of cumulus cell and with a dark uniform ooplasm, ≥110 μm in diameter were selected for use. The sample collection procedure was under the guidance of the Animal Care and Ethics committee established by Institute of Species Animal and Plant Sciences, Chinese Academy of Agricultural Sciences (CAAS).

2.2. Experimental design

There were three experimental groups: (1) fresh collected oocytes were designed as control (Control); (2) oocytes were exposed to equilibrium and vitrification solution without being plung into the liquid nitrogen (exposed); (3) oocytes were vitrified by cryoloop method, stored in liquid nitrogen for 3 days and warmed (Vitrified-warmed). Oocytes were cultured in TCM-199 at 38.5 °C. Following 72 h culture, they were denuded, stained with specific probes and fixed to observe nuclear maturation and mitochondrial distribution, and then directly used for laser-scanning confocal microscopy. Mature oocytes with polar body were used to determine the GSH content.

2.3. Vitrification and warming of immature oocytes

The COCs were vitrified by cryoloop (Hampton Research company, USA) method as previously described [37]. Brifely, the base medium (BM) of vitrification and warming solutions was MEM-alpha supplemented with 10% FBS. For vitrification, COCs were first transferred into equilibrium solution (BM containing 7.5% (v/v) ethylene glycol (EG), 7.5% dimethylsulfoxide (DMSO), and 0.25 M trehalose) for 3 min. Then COCs were moved into a vitrification solution (BM containing 15% (v/v) EG and 15% (v/v) DMSO). Then, the cryoloop was dipped into the vitrification solution so that a thin film was formed by surface tension. Three to five COCs with as little solution as possible were placed on the film and directly plunged