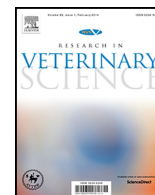
Research in Veterinary Science 97 (2014) 140–147

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Catalase addition to vitrification solutions maintains goat ovarian preantral follicles stability

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ARTICLE INFO

Article history:
Received 2 October 2013
Accepted 16 May 2014

Keywords:
Cryopreservation
Ovary
ROS levels
DNA damage
Apoptosis

ABSTRACT

The aim of this study was to verify whether the addition of catalase (20 IU/mL) at different steps of goat ovarian tissue vitrification affects ROS levels, follicular morphology and viability, stromal cell density, apoptosis and the expression of proteins related to DNA–damage signaling (γH2AX) and repair (53BP1). Goat ovarian tissues were analyzed fresh (control) or after vitrification: without catalase (VS–/WS–), with catalase in vitrification solutions (VS+/WS–), with catalase in washing solutions (VS–/WS+) or with catalase in both solutions (VS+/WS+). The vitrification without catalase had higher ROS levels than the control. The catalase, regardless the step of addition, maintained ROS levels similar to the control. There were no difference between treatments regarding follicular viability, stromal cell density and detection of γH2AX and 53BP1. There was no difference in follicular morphology and DNA fragmentation between groups vitrified. In conclusion, catalase addition to vitrification solutions prevents ROS formation in cryopreserved goat ovarian tissues.

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1. Introduction

Cryopreservation has been largely used for genetic material preservation from males and females (Fernández-Santos et al., 2008; Gupta et al., 2010; Luz et al., 2012). However, the exposure of biological samples to cryoprotectant agents associated with rapid cooling and warming rates generate physical and chemical changes (Fernández-Santos et al., 2008). These changes may lead to an increase in production of reactive oxygen species (ROS; Wang et al., 1997), resulting in oxidative stress (Agarwal et al., 2005). ROS include hydroxyl radical ($OH^{\bullet}$), superoxide anion ($^{\bullet}O_2^{-}$) and hydrogen peroxide (H_2O_2) (Agarwal et al., 2005; Rahimi et al., 2003). H_2O_2 is a nonpolar molecule, which crosses the cell membrane easily (Veal et al., 2007), and can be converted to $OH^{\bullet}$ (Yan et al., 2009), a highly reactive and toxic radical to the cell.

The increase in ROS levels affects the cell microenvironment (Fernández-Santos et al., 2008) resulting in damage to the morphology of ovarian preantral follicles (Luz et al., 2012), change gene expression (Harvey et al., 2002), and possibly in DNA (Imlay and Linn, 1988; Ménézo et al., 2010). ROS can react rapidly with nucleotides (Evans and Cooke, 2004), inducing DNA double-strand break (DSB; Ménézo et al., 2010), which can be evidenced by the phosphorylation of the histone H2AX (γH2AX), one of the proteins responsible for DNA DSB signaling (Burma et al., 2001). After H2AX phosphorylation, other proteins involved in DNA repair, such as the 53BP1 (p53 binding protein 1), are recruited to the sites of DNA damage (Rappold et al., 2001). 53BP1 binds to the central binding domain of p53 and moves to the site of DNA damage in response to DSB (Ward et al., 2003).

Despite the occurrence of the cellular events mentioned above, cells have natural defense mechanisms against oxidative stress comprising the action of antioxidants, which inhibit ROS production or capture and inactivate them (Veal et al., 2007). Among the latter, catalase is responsible for the hydrolysis of H_2O_2 into water and oxygen (Fernández-Santos et al., 2008), removing this important initiator of chain reactions that can lead to formation of other ROS (Aitken et al., 1995).

It has been shown that procedures such as cryopreservation result in increased ROS production (Mazzilli et al., 1995; Wang et al., 1997) with consequent alteration of the cellular redox system. For this reason, addition of antioxidants has been investigated in cryopreservation of sperm (Baumber et al., 2003; Chi et al., 2008),

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http://dx.doi.org/10.1016/j.rvsc.2014.05.006
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oocytes (Dinara et al., 2001; Gupta et al., 2010) and ovarian tissue (Luz et al., 2012; Sanfilippo et al., 2013). Since supplementation of cryopreservation solutions with antioxidants has only recently been investigated, there is a need to better understand the role of these substances in a complex tissue as the ovary.

Higher ROS production can also occur during the resumption of cell metabolism after freezing and thawing (Agarwal et al., 2005). Although previous studies have investigated the effect of catalase addition during the warming procedure (Fernández-Santos et al., 2008; Kim et al., 2004), the ideal period for the inclusion of antioxidants during the cryopreservation process (e.g., vitrification or cryoprotectants removal) has so far not been properly tested. Therefore, the aim of this study was to assess the effect of catalase supplementation of vitrification and washing solutions on ROS production in vitrified–warmed goat ovarian tissues.

2. Materials and methods

This experiment was approved and performed under the guidelines of Ethics Committee for Animal Use of the State University of Ceará. The cryoprotectants (ethylene glycol and dimethyl sulfoxide) were obtained from Dinâmica (Dinâmica Química, Diadema, SP, Brazil) and the other chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA), unless otherwise stated. The pH was adjusted to 7.4 in all solutions used in this study.

2.1. Preparation of ovarian tissue

Ovaries ($n = 10$) were collected from five adult cross-bred goats (*Capra hircus*) at a local slaughterhouse. Immediately postmortem, the ovaries were washed once in 70% ethanol for 10 s and then washed twice in HEPES-buffered minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. Ovaries were then transported to the laboratory in MEM at 20 °C within 1 h after collection. At the laboratory, ovaries were stripped of the surrounding fat and fibrous tissue and the ovarian cortex from each ovarian pair was cut into small fragments ($n = 18$; $3 \times 3 \times 1$ mm) using a scalpel blade under sterile conditions. Part of the fragments ($n = 6$) were used as fresh control samples and the remaining fragments ($n = 12$) were vitrified.

2.2. Experimental design and vitrification procedures

The ovarian fragments were exposed to vitrification solutions (VS) and washing/removal solutions (WS) of cryoprotectant agents, either with or without catalase (from bovine liver; Sigma: C1345), resulting in four different conditions: (1) vitrification *without* catalase followed by washing *without* catalase (VS−/WS−), (2) vitrification *without* catalase followed by washing *with* catalase (VS−/WS+), (3) vitrification *with* catalase followed by washing *without* catalase (VS+/WS−) and (4) vitrification *with* catalase followed by washing *with* catalase (VS+/WS+). The catalase concentration (20 IU/mL) used in this study was based on a previous study from Luz et al. (2012), who observed similar rates of morphologically normal preantral follicles after freezing goat ovarian tissues as compared to fresh ovarian tissues.

The vitrification was performed using the Ovarian Tissue Cryosystem (OTC), a new vitrification protocol developed by our team (Carvalho et al., 2013). Briefly, the fragments were exposed to two VS. The VS1 consisted of MEM supplemented with 10 mg/mL bovine serum albumin (BSA), 0.25 M sucrose, 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO). VS2 had a similar composition of VS1 but with higher concentration of cryoprotectants (20% EG and 20% DMSO). Tissue fragments ($n = 6$) were exposed either to vitrification solutions (VS1 and VS2) without catalase or ($n = 6$) to the same solutions but with catalase. The fragments were initially exposed to VS1 for 4 min followed by VS2 for 1 min. Both exposures were performed using the OTC. The vitrification solution was then removed and the OTC containing the ovarian tissue was closed and immediately immersed vertically into liquid nitrogen. After cryostorage for up to 1 week, OTCs containing the vitrified ovarian fragments were warmed in air at room temperature (RT ~25 °C) for 1 min, followed by immersion in a water bath (37 °C) for 30 s. After warming, the cryoprotectants were removed by a three-step washing solutions (WS; 5 min each) in WS1: MEM + 3 mg/mL BSA + 0.5 M sucrose, WS2: MEM + 3 mg/mL BSA + 0.25 M sucrose and WS3: MEM + 3 mg/mL BSA. The three WS were either supplemented or not with catalase (20 IU/mL).

2.3. ROS levels

The ROS levels were determined by a spectrofluorimetric method, using 2′,7′-dihydrodichlorofluorescein diacetate (DCHF-DA) assay (Loetchutinat et al., 2005). Prior, fresh control and vitrified tissues were homogenized in cold ice 50 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at $3000 \times g$, and the pellet was discarded. The low-speed supernatants (S1) were separated and used for ROS level assays. To estimate the level of ovarian homogenate ROS production an aliquot of S1 (10 µL) was incubated with 10 µL of DCHF-DA (1 mM) and the oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured for the detection of intracellular ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) using a spectrofluorimeter (Shimadzu model RF-5301PC) 30 min after the addition of DCHF-DA to the medium.

2.4. Evaluation of follicular morphology and stromal cell density

Fresh control and vitrified–warmed ovarian fragments were fixed in Carnoy's solution at RT for 4 h, dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax, and serially sectioned into 7 µm thickness. The sections were stained with hematoxylin and eosin for histological analysis and follicular morphology was examined by microscope (magnification ×400). For each treatment, 150 preantral follicles were counted only in sections where the oocyte nucleus was visible. Preantral follicles were morphologically classified as (i) normal if they contained an intact oocyte and intact granulosa cells and (ii) degenerate if they contained a pyknotic oocyte nucleus, shrunken ooplasm, accompanied or not by disorganized granulosa cells (e.g. increase in volume with or without detachment from the basement membrane). The presence of at least one of the aforementioned features was indicative of atresia.

Ovarian stroma cell density was evaluated by calculating the number of stromal cells in an area of 100×100 µm. For each treatment, 10 fields per animal were assessed, resulting a total of 50 fields per treatment, and the mean number of stromal cells per field was calculated.

2.5. Viability analysis by trypan blue and by fluorescent markers

Preantral follicles were isolated from the fresh control and vitrified–warmed ovarian fragments by the mechanical method described by Lucci et al. 1999, with slight modifications. Briefly, with a tissue chopper (The Mickle Laboratory Engineering, Gomshal, Surrey, United Kingdom) adjusted to sectioning interval of 75 µm, samples were cut into small pieces and placed in 2 mL MEM supplemented with 3 mg/mL BSA. Samples were then suspended 100 times with a large Pasteur pipette (inner diameter ~1600 µm), followed by 100 times with a smaller Pasteur pipette (inner diameter ~600 µm) to dissociate preantral follicles from stroma. The

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