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Caspase activity and oxidative stress of granulosa cells are associated with the viability and developmental potential of vitrified immature oocytes

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ABSTRACT

Objective: The aim of this study is to determine whether caspase activity and oxidative stress of granulosa cells are associated with the viability and developmental potential of vitrified immature oocytes.
Study design: Oocytes from mice were exposed to genistein or/and Z-VAD-FMK with or without vitrification. Ultrastructural alterations of granulosa cells in vitrified immature oocytes were observed. Moreover, the level of superoxide dismutase (SOD) in granulosa cells, incidence of apoptotic follicles, the viability of vitrified-warmed oocytes and their subsequent developmental competence were measured.
Results: Ultrastructural alterations of granulosa cells vitrified in the presence of genistein or Z-VAD-FMK were slighter than that of granulosa cells vitrified in the absence of genistein or Z-VAD-FMK. The incidence of apoptotic follicles vitrified in the presence of genistein or Z-VAD-FMK was significantly lower than that of immature oocytes vitrified in the absence of genistein or Z-VAD-FMK, whereas, the level of SOD in granulosa cells, the viability and developmental competence of immature oocytes vitrified in the presence of genistein or Z-VAD-FMK were significantly higher than that of immature oocytes vitrified in the absence of genistein or Z-VAD-FMK.
Conclusion: Both antioxidant (genistein) and caspase inhibition (Z-VAD-FMK) improve the viability and developmental competence of vitrified immature oocytes. Genistein is superior to Z-VAD-FMK in improving the efficacy of immature oocyte vitrification.

Introduction

Oocyte cryopreservation can significantly contribute to advances in fertility treatment and reproductive biology [1]. It offers the opportunity to preserve fertility in women who want to postpone childbearing or at risk of loss of ovarian function [2,3]. Moreover, cryopreservation of the female gamete could simplify oocyte donation programs and it is an important option for infertile couples who cannot benefit from embryo cryopreservation because of moral, religious, or legal constrains [4].

So far, many studies have been performed to improve the efficiency of oocyte cryopreservation techniques, focusing mostly on mature oocytes [1,3,4]. Less is known about the efficiency and the consequences of cryopreservation on immature oocytes [5,6]. The development of an effective cryopreservation program for immature oocytes has a major impact on in vitro fertilization clinical practice, especially to preserve the fertility of women with polycystic ovarian syndrome at risk of ovarian hyper-stimulation syndrome [7]. Moreover, for cancer patients, transplantation of cryopreserved ovarian tissue is not always conceivable because of the risk of retransmission of the disease due to the presence of neoplastic cells in tissue [8]. In these cases, combining in vitro maturation (IVM) and vitrification offers a novel approach for fertility preservation [9]. Theoretically, given their microstructure, immature germinal vesicle (GV) oocytes should be more resistant

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to the damage caused by cooling and circumvent the risk of polyploidy and aneuploidies, since the chromatins are diffused and surrounded by a nuclear membrane [10]. However, as shown in previous studies, the cryopreservation procedures have detrimental effects on the maturation capacity [5,11]. Fasano et al. confirmed these findings reporting that oocyte maturation rates were significantly reduced when oocytes were vitrified at immature stage followed by IVM [6]. Therefore, more progress is needed to enhance the developmental potential of vitrified immature oocytes and finally to achieve good clinical results.

Granulosa cells are essential for the maturation of the oocytes because germ cell development, meiotic progression and global transcriptional pattern are regulated by the granulosa cells [12,13]. The maturation process in mammalian oocytes includes important nuclear and cytoplasmic changes that are strictly dependent on a co-ordinate development of granulosa and theca cells. When nuclear and cytoplasmic maturation does not occur properly, the oocyte will fail to fertilize and develop successfully [14,15]. Our previous findings suggest that the oxidative stress is induced by cryopreservation in granulosa cells, and caspase is involved in cryopreservation-induced apoptosis in granulosa cells, whereas, supplement of a global caspase inhibitor (Z-VAD-FMK) in vitrification media could improve the efficacy of ovarian tissue cryopreservation [16]. Genistein is a naturally occurring phytoestrogen found in relatively high concentration in soybeans. It has been shown that genistein is effective in reducing the oxidative damage [17–20]. Therefore, the aim of this study is to determine whether inclusion of an antioxidant (genistein) or (and) Z-VAD-FMK can relieve damage to granulosa cells caused by cryopreservation and hence improve the efficacy of immature oocyte cryopreservation.

Materials and methods

Animals

Female ICR mice (5–6 weeks of age) ($n = 50$) were used for oocyte collection. The mice were housed under temperature-controlled conditions (22 ± 2 °C). Food and water were available at all times under photoperiod of 12 h of light and 12 h of dark. The Animal Care Committee of People's Hospital of Laiwu City approved all the experimental procedures carried out in the study. Chemicals were purchased form Sigma–Aldrich Company (St. Louis, MO, USA), unless otherwise indicated.

Oocyte collection

Female mice were super-ovulated by an intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin and were killed 48 h later by cervical dislocation. Ovaries were collected and cumulus-enclosed oocytes (CEOs) were observed by puncture of the antral follicles.

Oocyte vitrification and thawing

The vitrification and warming procedures were similar to those reported by Huang et al. [21]. The CEOs were randomly divided into fresh groups and vitrification groups ($n = 100$ per group).

Group I: equilibration solution was prepared with 7.5% (v/v) ethylene glycol (EG) + 7.5% (v/v) 1.2-propanediol (PROH) in Dulbecco's phosphate buffered saline (DPBS); vitrification solution was prepared with 15% (v/v) EG + 15% (v/v) PROH in DPBS.

Group II: equilibration solution and vitrification solution used for group II were identical to those for group I, but with 100 μM genistein.

Group III: equilibration solution and vitrification solution used for group III were identical to those for group I, but with 60 μM Z-VAD-FMK.

Group IV: equilibration solution and vitrification solution used for group III were identical to those for group I, but with 100 μM genistein and 60 μM Z-VAD-FMK.

Briefly, the oocytes were suspended in an equilibration solution for 3 min. The oocytes were then transferred to the vitrification solution for 45–60 s. 4–5 oocytes were loaded onto a McGill Cryoleaf, which was then immediately plunged into liquid nitrogen for 1 week. For warming, the McGill Cryoleaf was immersed directly in a warming solution for 1 min, then the warmed oocytes were transferred to 0.5 M and 0.25 M sucrose in 10% FBS-supplemented DPBS for 3 min, respectively, and finally washed twice with 10% FBS-supplemented in DPBS. Oocyte survival was assessed by morphologic appearance. The surviving oocytes were transferred to fertilization medium.

Group I: warming solution contained 1.0 M sucrose in 10% FBS-supplemented DPBS. Group II: warming solution used for group II were identical to that for group I, but with 100 μM genistein. Group III: warming solution used for group II were identical to that for group I, but with 60 μM Z-VAD-FMK. Group IV: warming solution used for group IV were identical to those for group I, but with 100 μM genistein and 60 μM Z-VAD-FMK.

Ultrastructural assessment of vitrified-warmed follicles

The follicles were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were then gradually dehydrated for 10 min twice in each concentration in series of ethanol and treated twice for 10 min with propylene oxide, infiltrated with 1:1 propylene oxide/resin in embedding capsules overnight, and finally embedded in fresh resin. Thin sections (60–80 nm) were cut with an ultramicrotome and collected on copper grids. Thin sections were stained with saturated uranyl acetate in 80% methanol and lead citrate. These sections were observed and photographed with a transmission electron microscope at 80 kV.

Determination of superoxide dismutase (SOD) level in granulosa cells

SOD activities in granulosa cells were determined by a SOD Assay Kit-WST. Granulosa cells were obtained after repetitive beat by pipette and mechanical separation. 5×10^6 granulosa cells/vial were washed twice with DPBS by centrifugation. Cell lysates were tested and a standard curve ranging from 0.156 to 20 U/mL was prepared. The colorimetric assay was performed measuring formazan produced by the reaction between a tetrazolium salt and superoxide anion (O^{2-}); the rate of the reduction with O^{2-} was linearly related to the xanthine oxidase activity and was inhibited by SOD. The absorbance was obtained with a Spectra Shell Microplate Reader reading at 450 nm against 620 nm.

TUNEL assay

Apoptotic status was determined using the TUNEL procedure. Follicles were fixed in 4% paraformaldehyde in PBS, embedded in paraffin by the standard method, cut into 5-μm sections, and mounted on poly-lysine-coated slides. Sections were digested by proteinase K and incubated in 50 μL of TUNEL reaction mixture. Then slides were incubated with converter antifluorescein antibody conjugated with peroxides. After washing with PBS, immunoreactions were detected by incubating with substrate solution. Then the slides were dehydrated, cleared, and covered. Negative control samples were incubated with TUNEL reaction mixture without enzyme. Samples were observed with a Nikon TE 2000 fluorescence microscope. Follicles with positive TUNEL