Non-frozen preservation protocols for mature mouse oocytes dramatically extend their developmental competence by reducing oxidative stress

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Abstract: The objective of this study was to test whether aging induces oxidative stress (OS) during oocyte preservation at different temperatures and whether the oocyte competence can be extended by antioxidant supplementation. The increase in activation susceptibility was efficiently prevented when oocytes were preserved at 37°C for 9 h in HCZB medium with 10.27 mM pyruvate and 10 μM α-tocopherol, at 25°C for 30 h with 20.27 mM pyruvate, and at 15°C for 96 h and at 5°C for 48 h with 10.27 mM pyruvate. Satisfactory blastocyst development was achieved after oocyte preservation at 37°C for 9 h, at 25°C for 30 h, at 15°C for 48 h and at 5°C for 24 h using the above protocols but with cysteamine/cystine supplementation. Transfer of blastocysts obtained from the above protocols showed no difference in pregnancy outcome between newly ovulated and preserved oocytes. Because oocytes preserved at 15°C for 48 h were fertilized after a 6-h recovery culture, aging of ovulated mouse oocytes has been successfully prevented for 54 h. Assays for ROS and glutathione indicated that in vitro preservation caused marked OS in oocytes. In conclusion, marked OS was observed following in vitro preservation of mature oocytes at different temperatures. Whereas any protocol that reduced OS could inhibit activation susceptibility, only those protocols that decreased OS while increasing glutathione synthesis could sustain oocyte competence.

Key words: aging / antioxidant / low temperature / oocyte preservation / oxidative stress

Introduction

Mammalian oocytes are arrested at the meiotic metaphase II (MII) stage following ovulation. If not fertilized in time, the ovulated oocytes undergo a time-dependent process of aging (Yanagimachi and Chang, 1961; Whittingham and Siracusa, 1978). In vitro culture of matured oocytes also leads to oocyte aging (Longo, 1980; Webb et al., 1986; Tarin et al., 1998; Miao et al., 2005). The post-ovulatory oocyte aging has marked detrimental effects on embryo development (Juetten and Bavister, 1983; Tesarik, 1993; Winston et al., 1993; Tarin et al., 1998) and offspring (Tarin et al., 1999, 2002). The use of aged oocytes resulted in significant decrease in embryonic development following in vitro fertilization, intracytoplasmic sperm injection (ICSI) (Lacham-Kaplan and Trounson, 2008) or nuclear transfer (Cervera and García-Ximénez, 2003; Iwamoto et al., 2005; Wu et al., 2007). Furthermore, methods for short-term preservation of mature oocytes would also be useful for oocyte transportation and the synchronization of treatments or the embryo stage to the surrogate mother. For example, oocytes participating in assisted reproduction technologies are often unavoidably subjected to extended periods of in vitro culture prior to fertilization. One example in which this may occur is when `rescue ICSI’ is performed on the failed-to-fertilize oocytes. Thus, studies on the mechanisms and control of oocyte aging are important for both normal and assisted reproduction.

In addition, although long-term storage of oocytes by vitrification is possible (O’Neil et al., 1997), it is not suitable for oocyte temporary storage pending manipulation/insemination because of the complicated
pretreatment, freezing and warming procedures. Oocyte developmental potential is often compromised after vitrification, and the cryoprotectants used may be toxic to oocytes (Cha et al., 2011; Yan et al., 2011). Furthermore, it was reported that the survival, fertilization rate and embryonic development of mouse oocytes were adversely affected by the cryo-storage duration in liquid nitrogen (Yan et al., 2011) and that oocyte aging should be avoided as much as possible following vitrification (Tan et al., 2009). Thus, studies on the mechanisms and control of oocyte aging are also important for cryopreservation of mature oocytes.

One of the earliest manifestations in aged oocytes is an increase in the susceptibility to activating stimuli (STAS; Kubiak, 1989; Lan et al., 2004) due to a decrease in the maturation-promoting factor (MPF) activity (Wu et al., 1997; Xu et al., 1997), and oocyte aging culminates in apoptosis (Gordo et al., 2000, 2002; Ma et al., 2005; Liu et al., 2009; Takahashi et al., 2009). Cells generate reactive oxygen species (ROS) as by-products through normal metabolic activities (Fridovich, 1986; Yu, 1994). ROS-induced apoptosis was observed in mammalian oocytes (Tatemoto et al., 2000; Chaube et al., 2005). Pyruvate was found to prevent ROS-induced apoptosis in various types of somatic cells (Ramakrishnan et al., 1998; Kang et al., 2001; Mongan et al., 2002; Jagtap et al., 2003; Lee et al., 2003), and it inhibited oocyte aging by maintaining both energy supply and redox potential (Liu et al., 2009; Li et al., 2011). Furthermore, it is well known that hypoxia down-regulates cell metabolism (Chip et al., 2011) and thus reduces ROS production (Kil et al., 1996; Shao et al., 2010). Based on these observations, our recent study showed that oocyte aging could be prevented for longer time by reducing culture temperature while supplementing more pyruvate to culture medium (Li et al., 2012). However, although the results implied oxidative stress (OS) in oocytes cultured for extended durations at various temperatures, no attempt was made in that study to extend oocyte developmental potential by preventing the OS during preservation.

Thus, the objective of the present study was to verify the aging-induced OS during preservation of ovulated oocytes at various temperatures and to test the possibility of further extending their developmental competence by antioxidants supplementation. Results showed that in vitro preservation of mature oocytes caused marked OS and supplementation with antioxidants overcame the deleterious effect of the aging-induced OS. Our revised protocols with thiol and/or α-tocopherol supplementation dramatically extend the developmental competence of mature mouse oocytes preserved at body (37°C), room (25°C), sub-room (15°C) and refrigerator (5°C) temperatures to 9, 30, 54 and 30 h, respectively.

Materials and Methods

The chemicals and reagents used in this study were purchased from Sigma Chemical Co. unless otherwise specified.

Oocytes recovery

Mice of the Kunming breed were kept in a room with 14L:10D cycles, with the dark period starting from 20:00. The animals were handled according to the rules stipulated by the Animal Care and Use Committee of Shandong Agricultural University. Female mice, 8–10 weeks after birth, were induced to superovulate with 10 IU intraperitoneally (i.p.) equine chorionic gonadotrophin (eCG) followed by 10 IU i.p. human chorionic gonadotrophin (hCG) 48 h later. Both eCG and hCG used in this study were from Ningbo Hormone Product Co., Ltd. The superovulated mice were killed 13 h after hCG injection, and the oviductal ampullae were broken in M2 medium (94.66 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl2·H2O, 1.19 mM KH2PO4, 1.19 mM MgSO4·7H2O, 4.15 mM NaHCO3, 20.85 mM HEPES, 23.28 mM sodium lactate, 0.33 mM sodium pyruvate (Na-pyruvate), 5.56 mM glucose, 1 g/l polyvinyl alcohol (PVA), 100 IU/ml penicillin, 50 IU/ml streptomycin, 0.01 g/l phenol red, pH = 7.4) to release newly ovulated oocytes.

In vitro preservation of oocytes

For in vitro preservation, cumulus-intact oocytes were cultured for different times in the HCZB medium [81.62 mM NaCl, 4.83 mM KCl, 1.18 mM KH2PO4, 1.18 mM MgSO4, 5 mM NaHCO3, 1.7 mM CaCl2·H2O, 31.31 mM sodium lactate, 0.27 mM Na-pyruvate, 20 mM HEPES, 1 mM glutamine, 5 g/l bovine serum albumin (BSA), pH = 7.4] supplemented with different concentrations of Na-pyruvate, α-tocopherol (VE) and/or cysteamine/cystine (C/C). α-Tocopherol, cysteamine and cystine were dissolved in DMSO, H2O and 1 N HCl, respectively, to prepare a 100-mM stock solution, and the stock solution was diluted to final concentration with culture medium immediately before use. The osmotic pressure of the medium was adjusted by decreasing the amount of NaCl to 71.62 mM and 61.62 mM, respectively, when 10 and 20 mM Na-pyruvate was added. The culture was conducted in wells (20–25 oocytes per well) of a 96-well culture plate containing 200 μl of medium at different temperatures in humidified air.

Oocytes activation

Our routine work shows that whereas ethanol treatment is a weak stimulus that activates only the aged oocytes and decreases MPF only to some extent, the Sr2+ treatment activated both aged and freshly ovulated oocytes effectively. Furthermore, parthenogenotes developed better after Sr2+ than after ethanol activation. Thus, ethanol stimulus was used to evaluate oocytes’ STAS, and the Sr2+ stimulus was used to assess oocytes’ potential of development. Prior to activation treatment, oocytes were denuded of cumulus cells by pipetting in M2 medium containing 0.1% hyaluronidase. For ethanol activation, the cumulus-free oocytes were first treated with 5% (v/v) ethanol in M2 medium for 5 min at room temperature, then washed three times, and cultured in regular CZB (with 0.27 mM pyruvate, without glucose) containing 2 mM 6-dimethylaminopurine for 6 h at 37°C in a humidified atmosphere of 5% CO2 in air. At the end of the culture, oocytes were observed under a microscope for activation. Only those oocytes that had one or two pronuclei, or two cells each having a nucleus, were considered activated. The activating medium used for Sr2+ activation was Ca2+-free CZB medium supplemented with 10 mM SrCl2 and 5 μg/ml cytochalasin B. After being washed twice in M2 medium and once in the activating medium, the cumulus-free oocytes were incubated in activating medium for 6 h. At the end of incubation, the oocytes were examined for activation and were considered activated when each contained one or two well-developed pronuclei.

In vitro fertilization

Masses of dense sperm were collected from the cauda epididymis of fertile male mice and were placed at the bottom of a test tube containing T6 medium (114 mM NaCl, 3.2 mM KCl, 0.5 mM MgCl2·6H2O, 2 mM CaCl2·H2O, 0.4 mM NaH2PO4·2H2O, 25 mM NaHCO3, 10 mM HEPES, 10 mM lactate, 0.5 mM pyruvate, 5.56 mM glucose, 0.01 g/l phenol red, pH = 7.8) supplemented with 10 mg/ml BSA. After 3–5 min, the supernatant containing highly motile spermatozoa was removed and capacitated in the same medium under mineral oil at 37°C for 1.5 h. After being washed in the fertilization medium (T6 containing 20 mg/ml BSA), the cumulus-intact oocytes were placed in fertilization drops (around 30 oocytes per 100 μl drop). Capacitated sperm were added to the fertilization
drops to give a final sperm concentration of $\sim 1.5 \times 10^7$/ml. Oocytes were observed under a stereomicroscope for fertilization at 6 h after insemination. Oocytes showing two pronuclei and two polar bodies were considered fertilized.

**Embryo culture**

Activated and fertilized oocytes were cultured for 4 days in regular CZB (20–30 oocytes per 100 µl drop) at 37°C under humidified atmosphere with 5% CO$_2$ in air. Glucose (5.5 mM) was added to CZB when the embryos were cultured beyond 4-cell stages.

**Embryo transfer**

Female mice of 8–10-week-old (28–35 g of body weight) were paired with vasectomized males to allow mating. The females were checked for vaginal plugs the next morning, and those showing a vaginal plug were used for pseudo-pregnant recipients. Embryo transfer was done on Day 2.5 postcoitus, and 15 blastocysts were transferred to each recipient, 7 or 8 per uterine horn. The average volume of the medium, including the embryos transferred, was 0.2 µl per recipient. After the embryo transfer, the recipients were housed singly in cages until parturition.

**Assay for intraooocyte ROS**

In order to quantify the ROS in individual oocytes, intraooocyte H$_2$O$_2$ levels were measured using 2,7′-dichlorodihydrofluorescein diacetate (DCHFDA) as described by Nasr-Esfahani et al. (1990). DCHFDA was dissolved in dimethyl sulfoxide at 1 mM as stock solution and stored in dark at −20°C. The stock solution was diluted to 10 mM in M2 immediately before use. Cumulus-free oocytes were stained for 10 min in DCHFDA solution at 37°C. After being washed thoroughly to remove the traces of the dye, at least 10 oocytes were placed on a slide, covered with coverslip and observed under Leica laser scanning confocal microscope. The fluorescence was obtained by excitation at 488 nm. Owing to a rapid increment in the fluorescence intensity soon after staining, photographs were not taken until the fluorescence intensity reached a maximum and stable level ($\sim 3–5$ min after staining). Photographs were taken with fixed microscopic parameters, and the fluorescence intensity from each oocyte was analyzed using Leica software.

**Results**

**Combined effects of preservation temperature and antioxidants supplementation on STAS of mouse oocytes**

The STAS of oocytes was evaluated by observing their ethanol activation rates. Freshly ovulated oocytes were preserved in HCZB medium containing various concentrations of antioxidants at various temperatures for various times before assessment for ethanol activation. Because our previous study had shown that an increase in STAS was efficiently prevented and oocyte competence successfully sustained by oocyte preservation at 37°C for 6 h, 25°C for 24 h, 15°C for 36 h and 5°C for 24 h (Li et al., 2012), this study began oocyte preservation for 9 h at 37°C, 30 h at 25°C, 48 h at 15°C and 24 h at 5°C. When oocytes were preserved at 37°C, the increase in STAS was best inhibited for 9–12 h with 10.27 mM pyruvate plus 10 µM α-tocopherol with or without cysteamine/cystine (C/C) supplementation (Fig. 1). When oocytes were preserved at 25°C, the increase in STAS was best prevented for 30–36 h with 20.27 mM pyruvate alone, and supplementation with 10.27 mM pyruvate alone or with C/C and α-tocopherol showed little effects. The increase in STAS was successfully inhibited for 48–96 h with 10.27 mM pyruvate alone when preserved at 15°C. When oocytes were preserved at 5°C, the increase in STAS was suppressed for up to 48 h with 10.27 mM pyruvate alone, and C/C and/or α-tocopherol showed no effect. Taken together, the results suggested that α-tocopherol, 20.27 mM pyruvate and 10.27 mM pyruvate played the major role in preventing oocyte increase of STAS at 37, 25 and 15°C/5°C, respectively, and C/C showed little effects on the increase of STAS.

**Embryo development in vitro after Sr$^{2+}$ activation of mouse oocytes preserved with different protocols**

The above experiment had selected the culture conditions that could efficiently inhibit the increase in STAS. The objective of the following experiments was to test whether these conditions could sustain oocyte developmental potential as well. In this experiment, freshly ovulated oocytes were preserved with protocols that had been shown to efficiently prevent the increase of STAS at different temperatures, and after preservation, the oocytes were treated with SrCl$_2$ for activation. Because oocytes were not activated by Sr$^{2+}$ treatment immediately following preservation at 15 or 5°C, oocytes in these two groups were Sr$^{2+}$...
treated after a 6-h recovery culture at 37°C in regular CZB containing 0.27 mM pyruvate. Activation rates after Sr²⁺ treatment were high (86 to 93%) and did not differ between different preservation regimens. Blastocyst rates of ≈60% were achieved after oocyte preservation at 37°C for 9 h with 10.27 mM pyruvate, 10 μM α-tocopherol and C/C, at 25°C for 30 h with 20.27 mM pyruvate and C/C, at 15°C for 48 h with 10.27 mM pyruvate and C/C, and at 5°C for 24 h with 10.27 mM pyruvate alone or plus C/C (Fig. 2). With other conditions equal, blastocyst formation was compromised after oocyte preservation without C/C supplementation except for preservation at 5°C. This suggested that C/C was important in sustaining oocyte developmental potential but it was not fully utilized during preservation at 5°C.

### Table

<table>
<thead>
<tr>
<th>Preservation</th>
<th>Pyr (mM)</th>
<th>VE (μM)</th>
<th>C/C (μM)</th>
<th>% Activated oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C 9 h</td>
<td>10.27</td>
<td>0</td>
<td>0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>10</td>
<td>0</td>
<td>c</td>
</tr>
<tr>
<td>12°C 10 h</td>
<td>10.27</td>
<td>0</td>
<td>0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>10</td>
<td>100/200</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>100</td>
<td>0</td>
<td>e</td>
</tr>
<tr>
<td>18°C 10 h</td>
<td>10.27</td>
<td>10</td>
<td>0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>50</td>
<td>100/200</td>
<td>b</td>
</tr>
<tr>
<td>25°C 30 h</td>
<td>10.27</td>
<td>10</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>d</td>
</tr>
<tr>
<td>36°C 20 h</td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>e</td>
</tr>
<tr>
<td>42°C 20 h</td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>a</td>
</tr>
<tr>
<td>5°C 24 h</td>
<td>0.27</td>
<td>0</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>b</td>
</tr>
<tr>
<td>15°C 48 h</td>
<td>0.27</td>
<td>0</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>c</td>
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</tbody>
</table>

**Figure 1** Ethanol activation after newly ovulated mouse oocytes were preserved at various temperatures for various times in HCZB medium containing different concentrations of pyruvate (Pyr), cysteamine/cystine (C/C) and/or α-tocopherol (VE). For controls, FC oocytes were treated with ethanol without preservation. Each treatment was repeated three to four times and each replicate contained ~30 oocytes. (a–e) Values without a common letter in their bars differ (P < 0.05) within temperature groups.
Embryo development in vitro and in vivo after fertilization in vitro of mouse oocytes preserved with different protocols

Freshly ovulated oocytes were preserved with protocols that had produced satisfactory blastocyst rates in the above experiment, and the preserved oocytes were inseminated in vitro for embryo development. Oocytes preserved at 15 and 5 °C were cultured at 37 °C for 6 h for recovery in regular CZB before insemination. For controls, freshly collected (FC) oocytes were inseminated without preservation. Fertilization rates ranging from 86% to 91% did not differ among preservation protocols. Acceptable rates of blastocysts (≏80%) were obtained after oocyte preservation at 37 °C for 9 h with 10.27 mM pyruvate, 10 μM α-tocopherol and C/C, at 25 °C for 30 h with 20.27 mM pyruvate and C/C and at 15 °C for 48 h with 10.27 mM pyruvate alone or plus C/C (Table I).

To test the in vivo developmental potential of the preserved oocytes, embryo transfer was conducted. Blastocysts derived from in vitro fertilization of oocytes preserved with different protocols were transferred to the uteri of pseudo-pregnant recipients. Blastocysts derived from in vitro fertilization of FC oocytes without preservation were also transferred for controls. No significant difference (P > 0.05) was observed between control and preserved oocytes in either pregnancy rate, live young per pregnant recipient, percentage live young/transfered embryos (Table I) or birthweight of the young (2.21 ± 0.02 to 2.27 ± 0.03 g).

Levels of ROS, GSX and GSH/GSSG ratio after preservation of newly ovulated mouse oocytes with different protocols

Freshly ovulated oocytes were preserved with various protocols before assays for ROS and glutathione levels. The ROS level in oocytes was determined according to their fluorescence intensity value. The ROS level in FC oocytes was very low, but it increased dramatically after preservation with any of the protocols (Figs 3 and 4). When oocytes were preserved with 10.27 mM (15 °C or 5 °C) or 20.27 mM (25 °C) pyruvate alone, the ROS level increased with increasing preservation durations (Fig. 4). Supplementation with C/C and/or α-tocopherol decreased the ROS levels significantly at 37 and 25 °C, but the effect became less significant when oocytes were preserved at 15 or 5 °C. When oocytes were preserved at 15 °C for 48 h or 5 °C for 24 h, the ROS level was significantly lower with 10.27 mM pyruvate than with 0 mM or 0.27 mM pyruvate. The ROS level was also significantly lower with 20.27 mM pyruvate than with 10.27 mM pyruvate after oocyte preservation at 25 °C for 30 h.

When oocytes were preserved at 37, 25 or 15 °C without pyruvate or with pyruvate alone, both GSX contents and the GSH/GSSG ratio decreased significantly (Table II). Supplementation with C/C increased the GSX significantly. Supplementation with either α-tocopherol or C/C increased the GSH/GSSG ratio significantly, and supplementation with both increased the ratio further to the level observed in freshly ovulated oocytes. When oocytes were preserved with 10.27 mM (37, 15 or 5 °C) or 20.27 mM (25 °C) pyruvate alone, the GSH/GSSG ratio decreased significantly with increasing preservation durations. The
### Table 1  In vitro development and pregnancy and birth of live young after embryo transfer following in vitro fertilization of mouse oocytes that had been preserved with different protocols.

<table>
<thead>
<tr>
<th>Preservation</th>
<th>Pyr (mM)</th>
<th>VE (μM)</th>
<th>C/C (μM)</th>
<th>% Blastocysts/fertilized oocytes</th>
<th>% Term pregnancy</th>
<th>Live young per pregnant recipient</th>
<th>% Live young/embryos transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>85.7 ± 3.1</td>
<td>80.0 (8/10)</td>
<td>5.1 ± 0.3 (41/8)</td>
<td>27.3 (41/150)</td>
</tr>
<tr>
<td>37°C 9</td>
<td>10.27</td>
<td>10</td>
<td>100/200</td>
<td>83.7 ± 1.4</td>
<td>90.9 (10/11)</td>
<td>5.1 ± 0.2 (51/10)</td>
<td>30.9 (51/165)</td>
</tr>
<tr>
<td>25°C 30</td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>78.9 ± 1.2</td>
<td>81.8 (9/11)</td>
<td>4.8 ± 0.2 (43/9)</td>
<td>26.1 (43/165)</td>
</tr>
<tr>
<td>15°C 48</td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>79.4 ± 2.6</td>
<td>80.0 (8/10)</td>
<td>4.9 ± 0.2 (39/8)</td>
<td>26.0 (39/150)</td>
</tr>
<tr>
<td>5°C 24</td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>81.4 ± 1.9</td>
<td>81.8 (9/11)</td>
<td>4.8 ± 0.3 (43/9)</td>
<td>26.1 (43/165)</td>
</tr>
<tr>
<td>5°C 24</td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>78.7 ± 2.4</td>
<td>81.8 (9/11)</td>
<td>5.0 ± 0.2 (45/9)</td>
<td>27.3 (45/165)</td>
</tr>
</tbody>
</table>

For in vitro fertilization, each replicate (fertilization drop) contained ~30 oocytes. Whereas each treatment was repeated three to four times in the in vitro development experiment, each treatment was repeated 10–11 times (recipients) in the embryo transfer experiment. In the embryo transfer experiment, 15 blastocysts in good shape were selected from around 20 produced from each fertilization drop and transferred to one recipient. Independent samples t-tests were conducted using SPSS to compare the in vivo development between control and preserved oocytes. No difference (P > 0.05) was observed between control and treatments in either rates of fertilization, % blastocysts or % term pregnancy, live young per pregnant recipient, percentage live young/transferred embryos or birthweight of young.

FC, freshly collected oocytes.

*Oocytes were cultured for recovery at 37°C in regular CZB containing 0.27 mM pyruvate.

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**Figure 3** Laser confocal images showing the ROS level (fluorescence intensity) of mouse oocytes following in vitro preservation with different protocols. Oocytes were observed under a laser confocal microscope following DCHFDA staining. As controls, image (A) and (A') show oocytes before preservation observed under bright-field and fluorescence, respectively. Images (B)–(D) show oocytes preserved for 30 h at 25°C in HCZB with 10.27 mM pyruvate, with 20.27 mM pyruvate and with 20.27 mM pyruvate plus C/C, respectively. The fluorescent intensities under these conditions were ~60, 45 and 30, respectively, see Fig. 4.
GSX contents and the GSH/GSSG ratio also decreased significantly after oocyte preservation at 5°C for 24 h with 0.27 mM pyruvate or for 30 h with 10.27 mM pyruvate. Supplementation with 10.27 mM pyruvate significantly increased GSX and the GSG/GSSG ratio in oocytes preserved at 5°C for 24 h, but C/C supplementation had little effect. Taken together, the results suggested that (i) oocyte preservation at different temperatures caused marked OS that intensified with increasing preservation duration; (ii) whereas α-tocopherol could only alleviate OS, C/C not only decreased OS but also promoted glutathione synthesis; (iii) pyruvate counteracted OS in a temperature-related concentration dependent manner and (iv) any protocol of preservation that reduced OS could inhibit STAS, but only those protocols that decreased OS while increasing glutathione synthesis could support embryonic development.

**Discussion**

Aged oocytes show increased STAS due to a decrease in the MPF activity that occurs as early as 4 h after ovulation in mice (Wu et al., 1997; Xu et al., 1997). It is known that a premature activation impairs the subsequent developmental potential of aging oocytes. For example, somatic cell nuclei introduced into enucleated rat oocytes were not properly reprogrammed (Hirabayashi et al., 2003; Ito et al., 2005) due to oocyte spontaneous activation that occurred soon after collection from the oviduct (Keefer and Schuetz, 1982; Chebotareva et al., 2011; Cui et al., 2012). Mouse oocytes electrically pulsed at 3 h after ovulation showed significantly reduced MPF activities and blastocyst rates after nuclear transfer and chemical activation compared with oocytes pulsed immediately after ovulation (Wu et al., 2007). Thus, the inhibition of STAS increase is a prerequisite for the efficient protection of developmental potential in aging oocytes. In our previous study (Li et al., 2012), an increase in STAS was efficiently prevented and satisfactory blastocyst development was achieved after mouse oocytes were preserved with 10.27 mM pyruvate at 37°C for 6 h, 25°C for 24 h, 15°C for 36 h and 5°C for 24 h. By C/C and/or α-tocopherol supplementation, this study has successfully extended these durations to 9 h at 37°C, 30 h at 25°C and 48 h at 15°C. When oocytes were preserved at 5°C, however, C/C and/or α-tocopherol supplementation was unnecessary for inhibiting the increase in STAS and did not protect oocyte developmental potential. Transfer of blastocysts obtained from the above protocols showed no difference between newly ovulated control oocytes and the preserved oocytes in either term pregnancy, live young per pregnant recipient, live young/transferred embryos or birthweight of young. Because oocytes preserved at 15°C for 48 h after a 6-h recovery culture, aging of ovulated mouse oocytes has been successfully prevented for 54 h in this study.

The OS in an oocyte is usually determined by measuring the levels of ROS (Nasr-Esfahani et al., 1990; Zhou et al., 2012) and glutathione (Boerjan and de Boer, 1990; Zhou et al., 2012) because it is known...
that cells generate ROS as by-products through normal metabolic activities (Fridovich, 1986; Yu, 1994) and that as the main redox buffer, the GSH/GSSG ratio is a very important indicator of the redox status of the cell (Wu et al., 2004; Ojha and Srivastava, 2012). By measuring the level of ROS and the ratio of GSH/GSSG, the present study showed that in vitro preservation at different temperatures caused marked OS in oocytes and the OS level increased with preservation duration when other conditions were equal. Thus, following oocyte preservation with 10.27 mM (37, 15 or 5°C) or 20.27 mM (25°C) pyruvate alone, whereas the ROS levels increased, the GSH/GSSG ratio decreased and the change was significant when the duration interval extended to 6 h or longer. In addition, the present results that supplementation with antioxidants α-tocopherol and/or C/C improved the redox status and developmental potential of preserved oocytes further confirmed OS in oocytes preserved at different temperatures. Taken together, our data suggest that at different temperatures, the aged oocyte experiences a state of OS due to the progressive increase in ROS production and the concomitant depletion of antioxidant protection. Although it has been reported by several groups that ROS accumulate in oocytes with increasing post-ovulatory time both in vivo and in vitro at body temperature (Takahashi et al., 2003; Tatone et al., 2011; Lord et al., 2013), reports on OS during oocyte aging under lower temperatures and on depletion of the antioxidant glutathione during oocyte aging are limited.

This study showed that during oocyte preservation at 37°C, supplementation with α-tocopherol alleviated OS but did not promote glutathione synthesis. Supplementation with C/C, however, not only decreased OS but also increased glutathione synthesis. At different preservation temperatures, any protocol that reduced OS could inhibit STAS, but only those protocols that decreased OS while increasing glutathione synthesis could support good embryonic development. Taken together, the present results suggest that glutathione sustained oocyte competence not only by acting as an antioxidant but also by other long-term actions. Glutathione is defined as an index of ooplasmic maturation (Funahashi et al., 1994; de Matos et al., 1997; Abeydeera et al., 1998; Furnus et al., 1998; de Matos and Furnus, 2000); it participates in sperm decondensation and male pronucleus formation (Perreault et al., 1988; Yoshida et al., 1993; Sutovsky and Schatten, 1997). The

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**Table II** GSX contents and GSH/GSSG ratio after newly ovulated mouse oocytes were preserved at various temperatures for various times in HCZB medium containing different concentrations of pyruvate (Pyr), cysteamine/cystine (C/C) and/or α-tocopherol (VE).

<table>
<thead>
<tr>
<th>Preservation</th>
<th>Pyr (mM)</th>
<th>VE (µM)</th>
<th>C/C (µM)</th>
<th>GSX (pmol/oocyte)</th>
<th>GSH/ GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>9</td>
<td>10.27</td>
<td>0/0</td>
<td>5.51 ± 0.08a</td>
<td>2.11 ± 0.04a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.27</td>
<td>100/200</td>
<td>5.36 ± 0.06b</td>
<td>1.50 ± 0.03b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>5.06 ± 0.05b</td>
<td>1.87 ± 0.04b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>100/200</td>
<td>10</td>
<td>5.48 ± 0.01a</td>
<td>2.02 ± 0.04a</td>
</tr>
<tr>
<td>12</td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>4.31 ± 0.12c</td>
<td>1.00 ± 0.01a</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>100/200</td>
<td>0</td>
<td>4.81 ± 0.10b</td>
<td>1.51 ± 0.04b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>4.46 ± 0.13c</td>
<td>1.56 ± 0.03c</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>100/200</td>
<td>10</td>
<td>4.95 ± 0.11b</td>
<td>1.67 ± 0.03c</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
<td>10.27</td>
<td>0</td>
<td>4.16 ± 0.02bc</td>
<td>1.33 ± 0.08b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.27</td>
<td>0</td>
<td>4.32 ± 0.09b</td>
<td>1.60 ± 0.03c</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>5.50 ± 0.15a</td>
<td>2.06 ± 0.09a</td>
</tr>
<tr>
<td></td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>5.09 ± 0.08d</td>
<td>1.73 ± 0.05d</td>
</tr>
<tr>
<td>36</td>
<td>20.27</td>
<td>0</td>
<td>0/0</td>
<td>3.89 ± 0.11c</td>
<td>1.37 ± 0.06b</td>
</tr>
<tr>
<td></td>
<td>20.27</td>
<td>0</td>
<td>100/200</td>
<td>3.96 ± 0.03b</td>
<td>1.25 ± 0.04b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>4.31 ± 0.04c</td>
<td>1.58 ± 0.02c</td>
</tr>
<tr>
<td>15</td>
<td>48</td>
<td>0</td>
<td>0/0</td>
<td>5.20 ± 0.10d</td>
<td>2.07 ± 0.05a</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>3.40 ± 0.12a</td>
<td>0.76 ± 0.07d</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>0.27</td>
<td>0</td>
<td>3.80 ± 0.08b</td>
<td>1.13 ± 0.11b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>5.21 ± 0.06b</td>
<td>1.67 ± 0.04bc</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>5.52 ± 0.06a</td>
<td>2.12 ± 0.04a</td>
</tr>
<tr>
<td>30</td>
<td>10.27</td>
<td>0</td>
<td>0/0</td>
<td>5.03 ± 0.03c</td>
<td>1.62 ± 0.05b</td>
</tr>
<tr>
<td></td>
<td>10.27</td>
<td>0</td>
<td>100/200</td>
<td>5.15 ± 0.04bc</td>
<td>1.79 ± 0.04b</td>
</tr>
</tbody>
</table>

* a In the same column, values without a common letter in their superscripts differ (P < 0.05) within temperature groups. For controls, FC oocytes were assayed without preservation. Each treatment was repeated three to four times and each replicate contained ~35–40 oocytes. The bold values highlighted the optimal protocols that had been selected and tested for oocyte developmental potential in Table I.
intraoocyte glutathione was also important for the development of parthenogenetic embryos (Zhou et al., 2008, 2010). Furthermore, it has been reported that cleavage stage embryos have a limited capacity to synthesize glutathione, susceptible to adverse effects of toxicants or conditions that deplete glutathione, but they have the capacity to reduce the existing GSSG (Gardiner and Reed, 1995a, b), emphasizing the importance for the maintenance of glutathione accumulation during oocyte maturation and aging.

The present results indicated that pyruvate overcame OS and the increase of STAS and protected oocyte competence in a temperature-related concentration dependent manner. For example, to inhibit the increase in STAS, whereas the optimal concentration of pyruvate was 10.27 mM at 37°C (Li et al., 2012), 15 and 5°C (Fig. 1), it increased to 20.27 mM at 25°C. The mechanism for this temperature-associated difference in the efficient concentration of pyruvate is not known but it might be related to changes in the Michaelis constant ($K_m$) because we all know that temperature affects the value of $K_m$ for an enzyme. Thus, the fact that at 37°C, 10.27 mM pyruvate was better than 0.27 mM (Li et al., 2012) or 20.27 mM (Fig. 1) suggested that 10.27 mM was the maximum (saturating) pyruvate concentration to inhibit oocyte aging at this temperature. When temperature decreased to 25°C, $V_{max}$ decreased as the $K_m$ increased, leading to an increased requirement for higher concentrations of pyruvate to counteract oocyte aging. When temperature further declined to 15 or 5°C, however, 20.27 mM pyruvate was found no better than 10.27 mM (Fig. 1). This suggested that at low temperatures, other mechanisms played a more important role than energy metabolism in slowing down oocyte aging, but energy was still essential because the present results showed that 20.27 mM pyruvate was more efficient than 0 or 0.27 mM in preventing the increase of STAS at 15 and 5°C, respectively.

Our previous study (Li et al., 2012) showed that most of the oocytes preserved at 25, 15 or 5°C had spindles disintegrated or disappeared, but after recovery culture, most of the oocytes regained normal morphology of spindles. Sun et al. (2004) also observed that exposure of mature mouse oocytes to 25°C induced spindle disassembly within 10–20 min, and most oocytes reassembled spindles after warming at 37°C. It was reported that defects in spindle assembly or spindle kinetochore attachment, or artificial depolymerization of microtubules, activated the spindle assembly checkpoint (SAC) proteins, which arrested cells prior to the metaphase–anaphase transition with stable cyclin B and elevated MPF activities (Chen et al., 1996; Li and Benezra, 1996).

Further studies confirmed that a complex between anaphase promoting complex (APC), Cdc20 and SAC proteins renders APC inactive and thus activates MPF by preventing cyclin B proteolysis (Li et al., 1997; Ling et al., 1998; Huang et al., 2000; Homer et al., 2005). Thus, cooling might have slowed down the increase of STAS by depolymerizing microtubules, which activated MPF via activating SAC proteins. Furthermore, our previous study showed that whereas most of the oocytes preserved at 25°C had disintegrated spindles, most of those preserved at 15 or 5°C showed no spindles (Li et al., 2012). This might help to explain why the increase in STAS was better inhibited during preservation at 15 and 5°C than at 25°C, because spindle disappearance might represent a more severe damage to microtubules and activate more MPF molecules than spindle disintegration. However, we do not know why the STAS was higher after preservation at 5°C than at 15°C.

In both the present study and our previous study (Li et al., 2012), high fertilization rates of 80–90% were achieved following oocyte preservation with different protocols. This suggested that the oocytes did not undergo cortical reaction during the extended storage time. Our previous study observed no exocytosis but migration inward and toward the vegetal pole of cortical granules following oocyte preservation with different protocols. The migration of cortical granules was totally reversible after oocyte preservation at 25°C for 24 h, at 15°C for 36 h and at 5°C for 24 h (Li et al., 2012). This may offer some explanation as to the lack of cortical reaction during the extended period of oocyte preservation. However, Wakayama et al. (2004) obtained a fertilization rate of lower than 50% following preservation of mouse oocytes for 24 h at 27°C, suggesting the effect of the cortical reaction. It should be noted that a major difference between our studies and the study reported by Wakayama et al. (2004) was that antioxidants were used in our studies to prevent OS. It is known that intracellular calcium increases trigger cortical granule exocytosis (Yanagimachi, 1994) and that ROS inactivates Na+/Ca2+ exchanger, leading to a rise in cytoplasmic calcium and subsequent cell dysfunction (Galán et al., 2010).

In summary, researches on the mechanisms and control of oocyte aging are important both for healthy reproduction and for assisted reproduction techniques. On the basis of our previous results that oocyte aging could be prevented for longer time by reducing culture temperature while supplementing more pyruvate to medium, this study has verified that in vitro preservation of mature oocytes caused marked OS at different temperatures and has successfully extended oocyte competence by antioxidant supplementation to 9 h at 37°C, 30 h at 25°C, 54 h at 15°C and 30 h at 5°C. Thus, with our protocols, mature oocytes can be preserved at body temperature, room temperature or in a refrigerator, at one’s choice. However, species-specific differences are found in the behaviors of the important organelles such as microtubules and mitochondria following cooling and rewarming of oocytes. For example, whereas most of the mouse oocytes reorganized spindles normally (Magistrini and Szollosi, 1980; Sun et al., 2004; Li et al., 2012), spindles of human (Almeida and Bolton, 1995; Wang et al., 2001), bovine (Aman and Parks, 1994) and sheep oocytes (Moor and Crosby, 1985) exhibited only a limited recovery after cooling-rewarming treatment. Different variations on the pattern of mitochondrial aggregation occurred during oocyte maturation of different species (van Blerkom and Runner, 1984; Stojkovic et al., 2001; Sun et al., 2001; Wilding et al., 2001), which may affect cellular metabolism. These factors must be considered when formulating protocols for long-term non-frozen preservation of mature oocytes in human and other species.

**Authors’ roles**

J.-H.T. designed the experiments and drafted the article. T.-Y.W. and Q.L. performed the experiments and analyzed the data. Q.L., H.L., J.Z., W.C. and G.-Z.J. performed the experiments. All authors revised the manuscript and approved the final version to be published.

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Conflict of interest

None declared.

References


Gordo AC, Wu H, He CL, Fissore RA. Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of [Ca(2+)]i oscillations and oocyte age. Biol Reprod 2000;62:1370–1379.


Kubiak JZ. Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. Dev Biol 1989;136:537–545.


