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Combined inhibitory effects of low temperature and *N*-acetyl-L-cysteine on the postovulatory aging of mouse oocytes

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Summary

The postovulatory aging of oocytes eventually affects the development of oocytes and embryos. Oxidative stress is known to accelerate the onset of apoptosis in oocytes and influence their capacity for fertilisation. This study aimed to reveal the roles of temperature and the antioxidant *N*-acetyl-L-cysteine in preventing the aging of postovulatory mouse oocytes. First, newly ovulated mouse oocytes were cultured at various temperature and time combinations in HCZB medium with varying concentrations of *N*-acetyl-L-cysteine to assess signs of aging and developmental potential. When cultured in HCZB with 300 μM *N*-acetyl-L-cysteine at different temperature and incubation time combinations (namely 25°C for 12 h, 15°C for 24 h and 5°C for 12 h), the increase in the susceptibility of oocytes to activating stimuli was efficiently prevented, and the developmental potential was maintained following Sr^{2+} activation or *in vitro* fertilisation. After incubation at either 15°C for 36 h or 5°C for 24 h, oocytes that had decreased blastocyst rates displayed unrecoverable abnormal cortical granule distribution together with decreased BCL2 levels, total glutathione concentrations and glutathione/glutathione disulphide (GSH/GSSG) ratios. In conclusion, postovulatory oocyte aging could be effectively inhibited by appropriate *N*-acetyl-L-cysteine addition at low temperatures. In addition, a simple method for the temporary culture of mature oocytes was established.

Keywords: Aging, Antioxidant, Low temperature, *N*-acetyl-L-cysteine, Oxidative stress

Introduction

Mature mammalian oocytes are arrested in metaphase of the second meiosis (MII) following ovulation. MII-arrested oocytes undergo aging after a delay in the oviduct or culture medium (Yanagimachi & Chang, 1961; Whittingham & Siracusa, 1978), and the developmental potential of such oocytes is impaired (Tesarik, 1993; Winston *et al.*, 1993; Tarin *et al.*, 1998; Bavister, 1989). The most prominent characteristics of postovulatory aged oocytes are increased susceptibility to activating stimuli (Nagai, 1987; Kubiak, 1989; Ware *et al.*, 1989; Fissore & Robl, 1992; Lan *et al.*, 2004), decreased maturation-promoting factor (MPF) activity (Kikuchi *et al.*, 1995, 2000; Wu *et al.*, 1997; Xu *et al.*, 1997), spindle abnormalities (Wakayama *et al.*, 2004), and abnormal cortical granule (CG) distribution (Szollosi, 1971; Ducibella *et al.*, 1990). Postovulatory aging culminates in apoptosis, resulting from the decreased expression of BCL2 proteins and the activation of caspases (Fujino *et al.*, 1996; Gordo *et al.*, 2000, 2002; Ma *et al.*, 2005; Takai *et al.*, 2007; Liu *et al.*, 2009, Lord & Aitken, 2013; Lord *et al.*, 2013). In assisted reproduction technique (ART) practices, the oocytes employed are often unavoidably subjected to extended periods of culture prior to fertilisation (Lord & Aitken, 2013). Although oocytes can be maintained for long periods of time by vitrification (O'Neil *et al.*, 1997), the developmental competence of oocytes is damaged after vitrification, indicating that the cryoprotectant used may be toxic to oocytes during the thawing/warming phase (Cha *et al.*, 2011; Yan *et al.*, 2011). Furthermore, vitrification is complicated and not suitable for the temporary culture of oocytes before manipulation. Thus, non-freezing methods for

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the control of mature oocyte aging *in vitro* are desirable for ART.

It has been reported that after ovulation, either *in vitro* or *in vivo*, reactive oxygen species (ROS) accumulate in oocytes with increased time (Takahashi *et al.*, 2003; Tatone *et al.*, 2011; Lord *et al.*, 2013). Such ROS are by-products of oxidative phosphorylation, and *in vitro* environmental factors such as light exposure and increased oxygen tension also facilitate ROS generation (Marston & Chang, 1964; Maas *et al.*, 1976; Goto *et al.*, 1993; Guerin *et al.*, 2001; Lord & Aitken, 2013). Oxidative stress occurs in postovulatory aged oocytes, in which ROS production is progressively increased and antioxidant protection is concomitantly depleted (Lord & Aitken, 2013). Although intracellular glutathione (GSH) plays an important role in protecting oocytes against oxidative damage, this resource is depleted with postovulatory age (Boerjan & de Boer, 1990; Yoshida *et al.*, 1993). Oxidative stress can generate global damage to the lipid, protein and DNA components of the cell, and such global oxidative damage in aged oocytes is related to poor embryo quality following fertilisation (Fujino *et al.*, 1996; Guerin *et al.*, 2001; Lord & Aitken, 2013; Lord *et al.*, 2013). Oocyte quality could be maintained by the addition of antioxidants to decrease oxidative stress and to reduce ROS-induced damage. There is increasing evidence of the beneficial effects of the antioxidant *N*-acetyl-L-cysteine (NAC) in preventing ROS-induced damage and pathology. In cultured rat ovarian follicles, high concentrations of NAC effectively suppressed apoptosis caused by oxidative stress (Tilly & Tilly, 1995). NAC is less toxic (Atkuri *et al.*, 2007) and may be essential for maintaining the GSH level (Meister & Anderson, 1983). Although the process of mouse oocyte aging *in vivo* can be postponed by appropriate treatment with the antioxidant NAC (Liu *et al.*, 2012), the function of NAC in preventing the postovulatory aging of mouse oocytes *in vitro* remains unknown. Low temperatures decrease cell metabolism and thus reduce ROS generation (Kil *et al.*, 1996; Shao *et al.*, 2010; Chip *et al.*, 2011). However, oocytes of different animals differ in their susceptibility to temperature. Chilling bovine oocytes at both the germinal vesicle (GV) and MII stages to 10°C for 30 min reduced their developmental competence (Martino *et al.*, 1996; Azambuja *et al.*, 1998), In contrast, rabbit and mouse oocytes chilled to 0°C at the MII stage retained normal properties of fertilisation and development (Chang, 1953).

We thus hypothesised that culturing oocytes at a lower temperature with appropriate NAC supplementation would delay postovulatory oocyte aging. Different combinations of culture times and temperatures for newly ovulated mouse oocytes were chosen to understand the mechanisms of oocyte aging and to thus establish a suitable method to inhibit postovulatory oocyte aging. Early developmental competence after *in vitro* fertilisation, GSH content, spindle configuration, CG redistribution, and relative BCL2 levels were evaluated to assess the quality of these oocytes after the inhibition of aging. The postovulatory aging of mouse oocytes could be prevented by culturing the oocytes in HCZB with 300 μM NAC at 25°C for 12 h, 15°C for 24 h or 5°C for 12 h without impairing their quality. To the best of our knowledge, this report is the first to describe the culture of mature mouse oocytes in HCZB with NAC supplementation at low temperatures to arrest aging.

Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich, unless otherwise specified.

Oocyte recovery

Female (6–8 weeks old) and male (8–10 weeks old) Kunming mice (Experimental Animal Centre of Shandong Luye Pharmaceutical Co., China) were housed under a 14 h/10 h (light/dark) cycle regime. Females received an injection of 10 IU human chorionic gonadotrophin (hCG) after 48 h of priming with 10 IU equine chorionic gonadotrophin (eCG). At 13 h after hCG injection, the mice were killed, and oocytes were collected into M2 medium. Both eCG and hCG were purchased from Ningbo Hormone Product Co., Ltd, China. The animals were handled following the rules stipulated by the Animal Care and Use Committee of Yantai University.

In vitro aging of oocytes

To test *in vitro* aging, oocytes were cultured in 96-well plates (25–35 oocytes per well) in HCZB medium (Kimura & Yanagimachi, 1995) containing different concentrations of NAC, covered with mineral oil and maintained in humidified air at various temperatures for various periods of time.

Oocyte activation

Cumulus cells were transferred by pipetting into M2 medium containing 0.1% hyaluronidase, and oocytes were then activated by the addition of Sr^{2+} or ethanol and 6-dimethylaminopurine (6-DMAP). For activation by ethanol with 6-DMAP, oocytes were first soaked with 5% ethanol in M2 medium for 5 min at room temperature. After washing three times, oocytes were cultured in regular CZB (containing 0.27 mM pyruvate, without glucose) with 2 mM 6-DMAP for 6 h at 37°C with 5% CO_2 in air. After

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activation, oocytes containing one or two pronuclei, or two cells each having a nucleus, were considered activated. For activation by Sr^{2+}, oocytes were first washed twice in M2 medium and once in activation medium and then cultivated in activation medium (Ca^{2+}-free CZB medium supplemented with 10 mM $SrCl_2$ and 5 μg/ml cytochalasin B) for 6 h. Oocytes with one or two well developed pronuclei were considered activated. Although the weaker ethanol-incorporation 6-DMAP treatment only activated aged oocytes, Sr^{2+} treatment effectively activated both aged and freshly ovulated oocytes. Parthenotes developed better following Sr^{2+} treatment. Thus, the weaker ethanol-incorporation 6-DMAP treatment was used to assess oocytes' sensitivity to activating stimuli, and Sr^{2+} stimulus was used to evaluate oocytes' developmental potential in this experiment.

In vitro fertilisation

Fresh sperm masses were collected from the cauda epididymis of fertile male mice into 1 ml T6 medium (114 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2{\cdot}6H_2O$, 2 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mM lactate, 0.5 mM pyruvate, 5.56 mM glucose, 0.01 g/l phenol red, pH 7.8) supplied with 10 mg/ml bovine serum albumin (BSA). Then, dispersed sperm cells were capacitated and covered by mineral oil at 37°C for 1.5 h. The oocytes were placed into fertilisation drops (T6 medium containing 20 mg/ml BSA) immediately with the prepared sperms (final sperm 1×10^6/ml) and incubated at 37°C with 5% CO_2 in air for 6 h. Oocytes containing two pronuclei and two polar bodies were considered fertilised.

Embryo culture

Activated or *in vitro* fertilised oocytes were cultured in regular CZB medium covered with mineral oil at 37°C with 5% CO_2 in air. Embryos beyond the 3- or 4-cell stage were placed in CZB medium supplied with 5.5 mM glucose for morula/blastocyst development.

Evaluation of glutathione

Intracellular levels of GSH were measured as previously described (Funahashi *et al.*, 1994) with minor modifications. Briefly, after culture, cumulus-free oocytes were washed three times in phosphate-buffered saline (PBS). Five microlitres of distilled water containing 35–40 oocytes was mixed with 5 μl of 1.25 M phosphoric acid, frozen and thawed three times, and then stored at –80°C until use. Total GSH (GSX) concentrations in the oocytes were determined by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-oxidised GSH reductase-recycling assay (Beyotime). GSX standards (0, 0.5, 1, 2, 5, 10 and 15 μM) were used. The amount of GSX in each sample was divided by the number of oocytes. The GSH values were calculated from the difference between GSX and GSSH for each oocyte.

Immunofluorescence and confocal microscopy

Immunofluorescence was performed as previously described (Li *et al.*, 2012). Briefly, oocytes were exposed to M2 containing 0.5% pronase to remove the zona pellucida and were then fixed with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM ethylene glycol tetraacetic acid, and 4 mM $MgSO_4$, pH 7.0) for 30 min. Fixation was terminated by adding 100 mM glycine, and the samples were incubated at 4°C overnight. Fixed oocytes were stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-α-tubulin antibody for 1 h. To stain CGs, fixed oocytes were incubated with 100 μg/ml FITC-labelled *Lens culinaris* agglutinin in M2 for 30 min in the dark. Chromosomes of the oocytes were stained for 5 min in M2 with 10 μg/ml Hoechst 33342. To stain BCL2, oocytes were incubated with rabbit anti-BCL2 polyclonal antibody (Merck KGaA, Germany) for 1 h and subsequently incubated with a Cy3-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Finally, stained oocytes were mounted onto slides and covered by coverslips. The oocytes were examined with a Leica TCS-SP2 confocal laser-scanning microscope. Blue diode (405 nm), argon (488 nm) and helium/neon (543 nm) lasers were used to excite Hoechst stain, and FITC and Cy3 fluorophores, respectively.

The relative BCL2 concentrations were quantified by measuring the fluorescence intensities with the Image-Pro Plus software (Media Cybernetics Inc.) under fixed thresholds among all slides.

Statistical analysis

Each experiment was repeated at least three times. Data are expressed as the mean ± standard error (SE). The percentage data were subjected to arc-sine transformation and analysed with analysis of variance (ANOVA). Differences were identified with a Duncan multiple comparison test. A P-value < 0.05 was considered statistically significant.

Results

Combination of culture temperature and NAC supplementation on oocyte activation

In previous studies (Li *et al.*, 2012; Wang *et al.*, 2014), we found that oocytes showed different aging

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Table 1 Chemical activation of mouse oocytes after the inhibition of oocyte activation using various culture protocols

Culture		% Oocytes activated at different concentrations of NAC (μM)			
Temperature (°C)	Time (h)	0	300	600	1200
37	6	$56.6 \pm 2.3^{a,B}$	$42.9 \pm 4.2^{b,B}$	$34.7 \pm 2.7^{b,B}$	$32.9 \pm 2.3^{b,C,D}$
37	12	$82.3 \pm 2.3^{a,D}$	$53.0 \pm 2.0^{b,C}$	$44.9 \pm 1.1^{c,C}$	$41.4 \pm 1.7^{c,E}$
25	12	$5.9 \pm 3.8^{a,A}$	$3.2 \pm 1.6^{a,A}$	$4.7 \pm 2.7^{a,A}$	$2.8 \pm 1.4^{a,A}$
25	24	$85.3 \pm 3.1^{a,D}$	$50.6 \pm 2.4^{b,C}$	$42.0 \pm 0.6^{c,C}$	$35.9 \pm 2.6^{c,D,E}$
15	24	$2.5 \pm 1.2^{a,A}$	$2.5 \pm 1.2^{a,A}$	$4.3 \pm 2.6^{a,A}$	$5.4 \pm 1.8^{a,A}$
15	48	$8.4 \pm 2.5^{a,A}$	$3.1 \pm 1.6^{a,A}$	$4.3 \pm 0.4^{a,A}$	$6.8 \pm 2.4^{a,A}$
15	60	$79.8 \pm 2.5^{a,B,C}$	$42.8 \pm 2.4^{b,B}$	$34.5 \pm 2.1^{c,B}$	$26.8 \pm 2.5^{c,B,C}$
5	24	$4.5 \pm 2.8^{a,A}$	$5.5 \pm 2.4^{a,A}$	$6.6 \pm 1.9^{a,A}$	$4.5 \pm 1.0^{a,A}$
5	36	$73.2 \pm 3.8^{a,C}$	$42.2 \pm 2.4^{b,B}$	$32.2 \pm 2.7^{b,c,B}$	$24.6 \pm 3.4^{c,B}$

$^{A–E}$Values with a common letter in their superscripts in the same column do not differ ($P > 0.05$).
$^{a–c}$Values with a common letter in their superscripts in the same line do not differ ($P > 0.05$).
Each treatment was repeated three or four times, and each replicate contained approximately 30 oocytes.

states at the following temperatures: body (37°C), room (25°C), sub-room (15°C) or refrigerator (5°C) temperature, for which preservation protocols have been established (Li *et al.*, 2012; Wang *et al.*, 2014). During our primary experiment, we found that after freshly ovulated oocytes were cultured in HCZB without the addition of NAC, the susceptibility of oocytes to activating stimuli was highly increased at 37°C for 12 h, 25°C for 24 h, 15°C for 60 h, or 5°C for 36 h (data not shown and Table 1), and we chose these temperature and time duration combinations to test whether oocyte susceptibility to activating stimuli could be prevented by NAC. We also examined the effect of NAC on oocyte susceptibility to activating stimuli when the treatment time was shortened at the different temperatures mentioned above.

After freshly ovulated mouse oocytes were cultured in HCZB with different concentrations of NAC for various times at various temperatures, the susceptibility of oocytes to activating stimuli was measured by assessing the activation rates achieved after oocyte activation by ethanol with 6-DMAP. The results (Table 1) showed that oocyte sensitivity to activating stimuli increased with the culture time at a given temperature, and cultured oocytes maintained low activation rates before 12 h at 25°C, 48 h at 15°C, or 24 h at 5°C. Such sensitivity was partially prevented by NAC and was decreased at lower temperatures (from 37°C to 15°C). Surprisingly, for all of the treatments, when cultured at 5°C for only 36 h, the activation rates reached the same levels as when cultured at 15°C for 60 h.

In vitro embryo development after Sr^{2+} activation

Sr^{2+} activation was used to test the developmental potential of oocytes preserved under different conditions that could efficiently prevent oocyte sensitivity to activating stimuli in the above experiment. We found that oocytes preserved at 15°C or 5°C were not immediately activated by Sr^{2+} activation and required recovery culture in regular CZB for 6 h at 37°C before activation. There were no significant differences in the activation rates among all treated oocytes (Fig. 1). Although all oocytes cultured in HCZB containing 300 μM NAC at 25°C for 12 h, 15°C for 24 h and 5°C for 12 h had satisfactory blastocyst rates, oocyte blastulation significantly decreased when the treatment duration was prolonged and the concentration of NAC was increased (Fig. 1).

Effects of different culture protocols on embryo development after *in vitro* fertilisation

The objective of this experiment and the following experiments was to assess the quality of oocytes preserved under the ideal culture conditions that were preliminarily selected from the above two experiments.

After the oocytes were preserved in HCZB containing 300 μM NAC at different temperatures for various times, we further tested the embryo development of oocytes following *in vitro* fertilisation. The fertilisation rates (81.7–87.6%, data not shown) in all treated oocytes did not significantly differ from those of newly ovulated oocytes (control). Although higher blastocyst rates were observed after culturing oocytes at 25°C for 12 h, 15°C for 24 h or 5°C for 12 h, the blastocyst rates significantly decreased when culture time was prolonged to 36 h at 15°C or to 24 h at 5°C (Fig. 2).

Effects of different protocols on intracellular GSH concentration in mouse oocytes

Freshly ovulated oocytes cultured in HCZB containing 300 μM NAC at 25°C for 12 h, 15°C for 24 h or 5°C for 12 h after recovery culture displayed similar GSX

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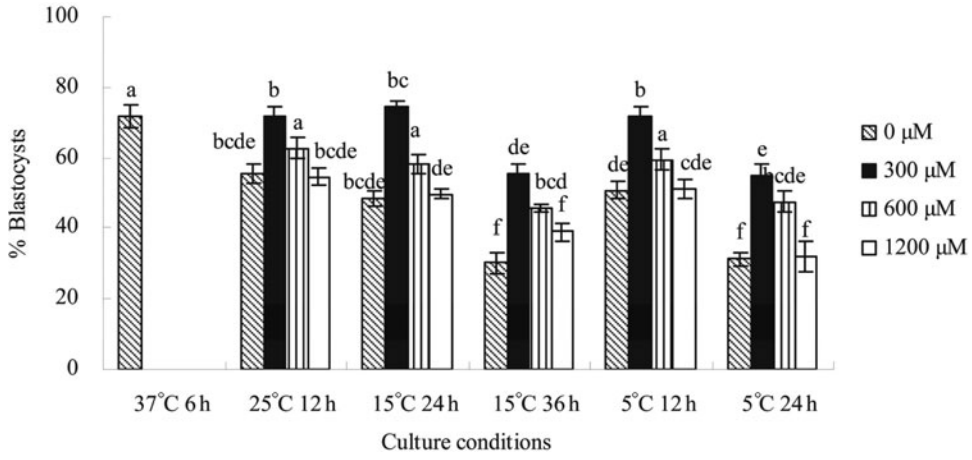

Figure 1 Parthenogenetic development of oocytes after the inhibition of aging by different protocols. ∗Oocytes were incubated for 6 h at 37°C before activation treatment. [a–f]Values with a common letter in their superscripts in the same column did not differ ($P > 0.05$) within oocyte categories. Newly ovulated oocytes that were cultured for 6 h at 37°C were set as control 1. Each treatment was repeated three or four times, and each replicate contained 30–35 oocytes.

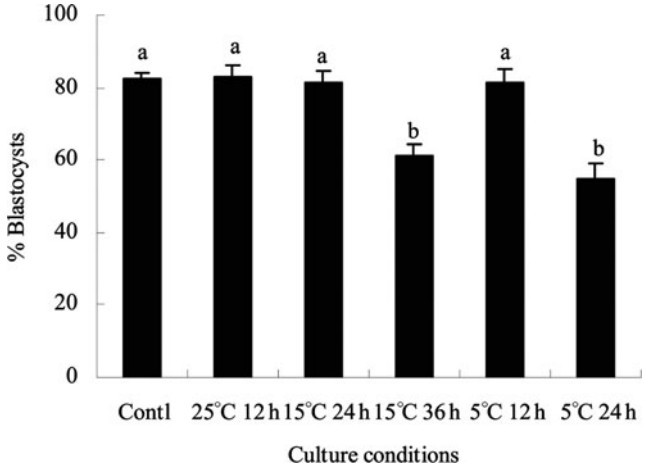

Figure 2 Embryo development after the *in vitro* fertilisation of mouse oocytes that had been cultured for various lengths of time at different temperatures in HCZB containing 300 μM NAC. [a,b]Values with a common letter above their bars do not differ ($P > 0.05$). Freshly ovulated oocytes were set as the controls (Contl). Each treatment was repeated four or five times, and each replicate contained 30–35 oocytes. For recovery culture, oocytes were cultured at 37°C in regular CZB containing 0.27 mM pyruvate.

levels and GSH/GSSG ratios to those in fresh oocytes. However, the GSX concentration and the GSH/GSSG ratio significantly decreased when culture time was extended to 36 h at 15°C or 24 h at 5°C (Fig. 3).

Effects of culture protocols on spindle and chromosome morphology in mouse oocytes

Fresh oocytes cultured in HCZB containing 300 μM NAC at different temperatures for various lengths of time before or after recovery culture displayed spindle and chromosome morphologies that could be divided into five types (Fig. 4): tine-pole spindles with congressed chromosomes, barrel-shaped spindles with congressed chromosomes, disintegrated spindles with scattered chromosomes, disintegrated spindles with congressed chromosomes, and disappeared spindles with congressed chromosomes. Oocytes cultured for 12 h at 25°C before recovery culture displayed scattered chromosomes, whereas the chromosomes of oocytes in all other groups were congressed. Most oocytes cultured at 25°C, 15°C or 5°C for different lengths of time displayed abnormal spindle configurations, whereas almost all of the oocytes showed normal spindle configurations after recovery culture (Table 2).

Effect of culture protocols on the distribution of cortical granules and chromosomes

When mouse oocytes were cultured in HCZB containing 300 μM NAC at different temperatures for various lengths of time before and after recovery culture, CGs were distributed to different degrees. CG distribution was classified into five categories: normal cortical granule distribution with congressed (Fig. 5*A*) or scattered chromosomes (Fig. 5*B*); early (e-) migration of CGs inwards and towards the vegetal pole with congressed (Fig. 5*C*) or scattered chromosomes (Fig. 5*D*); or late (l-) migration of CGs with congressed chromosomes (Fig. 5*E*). Before recovery culture, although about half of the oocytes cultured for 12 h at 25°C or for 24 h at 15°C and one-third of the oocytes cultured for 12 h or 24 h at 5°C underwent early CG migration, whereas oocytes cultured for 36 h at 15°C mainly displayed late

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Table 2 Morphology of meiotic spindles and chromosomes after newly ovulated oocytes were cultured for various lengths of time at different temperatures in HCZB containing 300 μM NAC

Culture°C /h	Tine-pole/ Congressed	Barrel/ Congressed	Disintegrated/ Congressed	Disintegrated/ Scattered	Disappeared/ Congressed
Before recovery					
C/0	100^a	0^a	0^a	0^a	0^a
25/12	3.3 ± 1.9^a	0^a	0^a	96.7 ± 1.9^b	0^a
15/24	2.3 ± 2.3^b	0^a	97.7 ± 2.3^b	0^a	0^a
15/36	1.2 ± 1.2^b	0^a	0^a	0^a	98.8 ± 1.2^b
5/12	5.5 ± 1.2^a	0^a	96.7 ± 1.9^b	0^a	0^a
5/24	3.3 ± 1.9^a	0^a	0^a	0^a	94.5 ± 1.2^b
After recovery					
25/12	96.7 ± 1.8^a	3.3 ± 1.8^a	0^a	0^a	0^a
15/24	97.8 ± 1.1^a	2.2 ± 1.1^a	0^a	0^a	0^a
15/36	97.7 ± 1.2^a	1.1 ± 2.0^a	1.2 ± 1.2^a	0^a	0^a
5/12	96.5 ± 2.1^a	3.5 ± 3.6^a	0^a	0^a	0^a
5/24	95.5 ± 2.2^a	2.3 ± 2.0^a	2.3 ± 1.2^a	0^a	0^a

$^{a, b}$Values with different superscripts are significantly different in each column ($P < 0.05$).
Freshly ovulated oocytes were set as controls (C).
Each treatment was repeated three or four times, and each replicate contained 25–30 oocytes.

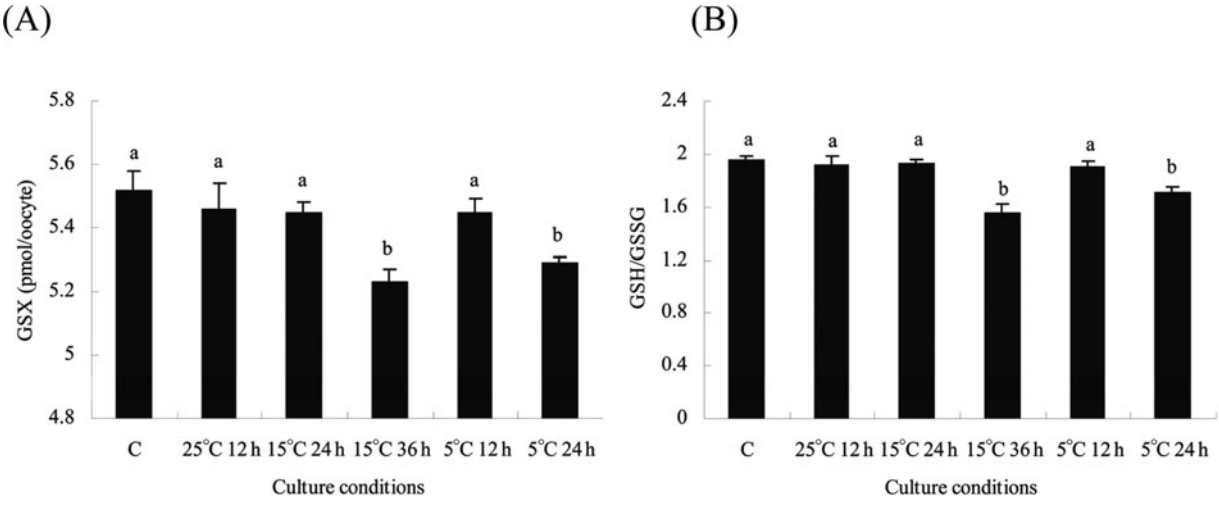

Figure 3 (*A*) Intracellular GSH concentration of mouse oocytes that had been cultured for various lengths of time at different temperatures in HCZB containing 300 μM NAC. (*B*) Ratio of GSH to GSSG. ^{a,b}Values with a common letter above their bars do not differ ($P > 0.05$). Freshly ovulated oocytes were used as controls (C). Each treatment was repeated three times, and each replicate contained approximately 30 oocytes.

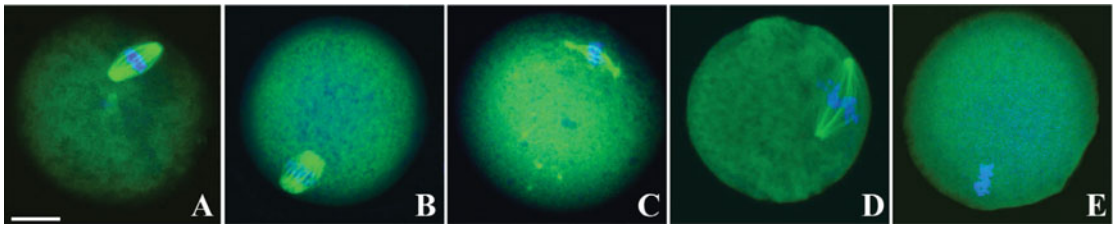

Figure 4 Morphology of meiotic spindles/chromosomes. (*A*) Tine-pole-shaped spindles with congressed chromosomes. (*B*) Barrel-shaped spindles with congressed chromosomes. (*C*) Disintegrated spindles with congressed chromosomes. (*D*) Disintegrated spindles with scattered chromosomes. (*E*) Disappeared spindles with congressed chromosomes. Scale bar is 20 μm. Each treatment was repeated three or four times, and each replicate contained 25–30 oocytes.

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Table 3 Cortical granule/chromosome distribution after newly ovulated oocytes were preserved for various lengths of time at different temperatures in HCZB containing 300 μM NAC

Culture °C/h	Normal/ Congressed	Normal/ Scattered	e-Migration/ Congressed	e-Migration/ Scattered	l-Migration/ Congressed
Before recovery					
C/0	95.3 ± 1.3^{a}	0^{a}	4.7 ± 1.4^{a,b}	0^{a}	0^{a}
25/12	2.4 ± 1.2^{b}	49.8 ± 2.6^{b}	0^{a}	47.8 ± 1.4^{b}	0^{a}
15/24	32.2 ± 3.2^{e}	0^{a}	41.9 ± 4.0^{c}	0^{a}	25.9 ± 3.0^{b}
15/36	9.6 ± 1.4^{c}	0^{a}	5.9 ± 1.0^{a,b}	0^{a}	84.5 ± 1.0^{e}
5/12	34.8 ± 0.8^{e}	0^{a}	33.8 ± 2.4^{e}	0^{a}	31.4 ± 2.1^{c}
5/24	26.3 ± 1.2^{d}	0^{a}	30.7 ± 2.0^{e}	0^{a}	43.0 ± 3.1^{d}
After recovery					
25/12	94.3 ± 1.0^{a}	0^{a}	5.7 ± 1.0^{a,b}	0^{a}	0^{a}
15/24	92.7 ± 0.2^{a}	0^{a}	7.3 ± 0.2^{a}	0^{a}	0^{a}
15/36	85.1 ± 2.7^{f}	0^{a}	14.9 ± 2.3^{d}	0^{a}	0^{a}
5/12	96.3 ± 2.2^{a}	0^{a}	3.7 ± 2.2^{a,b}	0^{a}	0^{a}
5/24	85.7 ± 2.2^{f}	0^{a}	14.3 ± 2.2^{d}	0^{a}	0^{a}

$^{a–f}$In each column, values with different superscripts are significantly different ($P < 0.05$).
Freshly ovulated oocytes were used as controls (C).
Each treatment was repeated three or four times, and each replicate contained 25–30 oocytes.

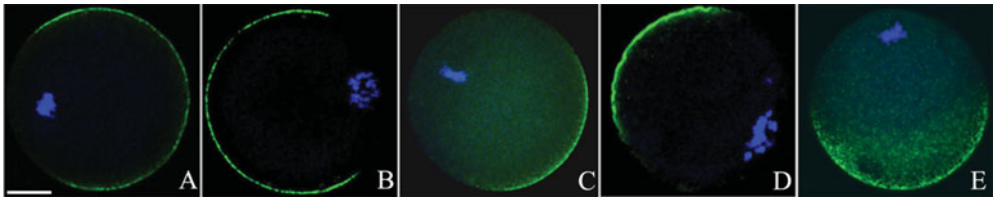

Figure 5 Cortical granule/chromosome distribution. (*A*) Normal cortical granule distribution with congressed chromosomes. (*B*) Normal cortical granule distribution with scattered chromosomes. (*C*) Early (e-) migration of cortical granules inwards and towards the vegetal pole with congressed chromosomes. (*D*) Early (e-) migration of cortical granules inwards and towards the vegetal pole with scattered chromosomes. (*E*) Late (l-) migration of cortical granules with congressed chromosomes. Scale bar is 20 μm.

cortical granule distribution. After recovery culture, most oocytes cultured at 25°C for 12 h, 15°C for 24 h or 5°C for 12 h showed restored normal CG migration, but some oocytes cultured for 36 h at 15°C and 24 h at 5°C remained at early granule migration (Table 3).

Relative BCL2 levels in mouse oocytes following preservation

BCL2 levels were measured in oocytes preserved for 12 h at 25°C, 24 h or 36 h at 15°C or for 12 h or 24 h at 5°C in HCZB containing 300 μM NAC. Different levels of staining were observed among oocytes cultured under different conditions. Freshly ovulated oocytes were heavily stained (Fig. 6*A*); however, after being preserved for 36 h at 15°C, the oocytes were moderately stained (Fig. 6*B*). The relative BCL2 levels were quantified by measuring the fluorescence intensities. The average fluorescence intensity of newly ovulated oocytes was set to 100%, and the other values were normalised to that value. Before recovery culture, there were no significant differences between BCL2 levels in oocytes cultured for 12 h at 25°C and control. After recovery culture, BCL2 levels were similar between the various treatments and the control. However, a low BCL2 level was observed in oocytes cultured for 36 h at 15°C and 24 h at 5°C, either before or after recovery culture.

Discussion

Prevention of the aging of postovulatory oocytes entails maintainance of their meiotic arrest and protection of their developmental potential (Li *et al.*, 2012). The results showed that NAC could partially inhibit an increased sensitivity to activating stimuli in oocytes cultured for different times at various temperatures. Oocytes cultured with the same concentration of NAC displayed entirely inhibited sensitivity to activating stimuli when cultured for 12 h at 25°C, 48 h at 15°C and 24 h at 5°C. This suggests that low temperature itself could inhibit meiotic resumption in oocytes. Moreover,

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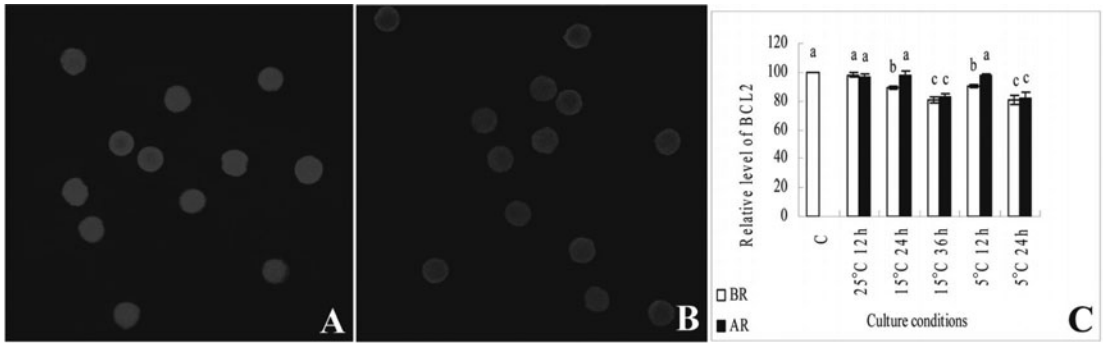

Figure 6 Levels of BCL2 after newly ovulated oocytes were cultured for various lengths of time at different temperatures in HCZB containing 300 μM NAC. (*A*) Confocal images of BCL2 (heavily labelled with anti-BCL2 antibodies) in freshly ovulated oocytes; (*B*) Confocal images of BCL2 (moderately labelled with BCL2 antibodies) in oocytes after preservation for 36 h at 15°C in HCZB containing 300 μM NAC. Photographs in (*A*) and (*B*) were taken using a laser confocal microscope at ×100 magnification. (*C*) Relative levels of BCL2 in mouse oocytes preserved for various lengths of time at different temperatures in HCZB containing 300 μM NAC. BR: Before recovery. AR: After recovery. For recovery culture, oocytes were cultured at 37°C in regular CZB containing 0.27 mM pyruvate. [a–c]Values with a common letter above their bars do not differ ($P > 0.05$). Newly ovulated oocytes were used as controls (C). Each treatment was repeated three times, and each replicate contained approximately 30 oocytes.

low temperatures prevented meiotic resumption in oocytes more efficiently than NAC supplementation. The result of parthenogenetic embryonic development suggested that the developmental potential of oocytes could not be maintained unless an appropriate concentration of NAC was added at a low temperature.

NAC can easily enter cells and becomes the precursor of GSH after being deacetylated. GSH is an important antioxidant involved in various physiological processes (Sies, 1999; Dickinson *et al.*, 2003). It has been reported that GSH and GSX levels and the GSH/GSSG ratio all decrease during the postovulatory aging of oocytes *in vivo* (Boerjan & de Boer, 1990). The GSH/GSSG ratio is a very important indicator of the redox status of a cell; thus, decreases in the GSH content and the GSH/GSSG ratio are equated with oxidative stress (Wu *et al.*, 1997; Ojha & Srivastava, 2012). The present results demonstrated that NAC reduced oxidative stress by increasing the GSH content in oocytes. Low temperature downregulates cellular metabolism and thus reduces ROS production, indicating that low temperature interacted with NAC to inhibit the resumption of second meiosis and to protect the developmental potential of mature oocytes. The present study also showed that the developmental capacity of oocytes decreased under a high concentration of NAC that would produce excessive GSH, likely because too high an intracellular concentration of GSH would upset the redox homeostasis that is essential for normal functions such as gene expression and cell signalling (Arrigo, 1999).

Through these experiments, we optimised a method for the culture of mature oocytes: the aging of postovulatory mouse oocytes could be inhibited for 18 h at 25°C, 30 h at 15°C or 18 h at 5°C without impairing their developmental potential. More specifically, the developmental potential of oocytes could be maintained for a longer time when oocytes were preserved in HCZB containing 300 μM NAC for 12 h at 25°C, 24 h at 15°C or 12 h at 5°C after recovery culture.

Spindle disruption and inward CG migration have been reported in aging oocytes (Szollosi, 1971; Longo, 1974). Although spindle disruption suggests microtubule changes, CG migration indicates microfilament disturbances because microfilaments control peripheral CG migration (Sun & Schatten, 2006). The cytoskeleton was broken at low temperatures. The present study indicated that the degree of spindle damage in oocytes at low temperatures obviously depended on the treatment temperature and time when observed immediately after culture. All abnormal spindle configurations in oocytes cultured in different conditions were restored after recovery culture. In contrast with spindle configurations, CG distributions were more seriously affected by low temperatures and were not restored in oocytes cultured for 36 h at 15°C or 24 h at 5°C after recovery culture.

Oocytes cultured for 36 h at 15°C or 24 h at 5°C displayed decreased normal CG distribution, which was always associated with reduced BCL2 levels. This is likely because NAC alone is insufficient to prevent certain age-related changes such as to the CG distribution and BCL2 levels when the incubation periods were prolonged. With a better understanding of the complex mechanisms controlling oocyte aging, it may be possible to create oocyte culture media that contain a combination of active compounds, including

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an antioxidant agent such as NAC, to allow mature oocytes to be cultured for longer times *in vitro* before IVF/ICSI.

However, species-specific differences were found in the behaviour of spindles following the cooling and rewarming of oocytes. Although a limited effect on the morphology of the second meiotic spindle was observed in mouse oocytes after cooling–rewarming treatment (Magistrini & Szollosi, 1980; Pickering & Johnson, 1987; Sun & Schatten, 2006; Li *et al.*, 2012), only a limited recovery of spindles was observed following the cooling and rewarming of human (Pickering & Johnson, 1987; Almeida & Bolton, 1995; Wang *et al.*, 2001), bovine (Aman & Parks, 1994) and sheep (Moor & Crosby, 1985) oocytes. Therefore, spindle recovery must be considered when formulating methods for the short-term non-freezing culture of mature human oocytes or oocytes from other species *in vitro* before IVF/ICSI.

This study verified that oocyte aging could be delayed by the combination of low temperature and NAC. In our previous reports, the aging of ovulated mouse oocytes could be inhibited for 24 h at 25°C, 42 h at 15°C and 30 h at 5°C after oocyte culture in HCZB with high concentrations of pyruvate, and we further extended the developmental competence of oocytes by antioxidant supplementation at 9 h at 37°C, 30 h at 25°C, 54 h at 15°C and 30 h at 5°C (Li *et al.*, 2012; Wang *et al.*, 2014). The culture time for mature oocytes in this paper was slightly shorter than in our previous reports, suggesting that at low temperatures, the *in vitro* aging of cumulus-enclosed MII oocytes may be divided into at least two periods: early and late. In the early period, the aging of oocytes results mainly from energy insufficiency. Once energy becomes insufficient, oxidative stress is generated and acts as a 'trigger' for aging in postovulatory oocytes (Lord & Aitken, 2013). Thus, antioxidant supplementation would delay the production of oxidative stress. However, oxidative stress plays a more important role than energy insufficiency in the late period of oocyte aging. Cysteamine and cystine must work together to increase GSH synthesis in mouse oocytes, and cystine is reduced to cysteine by cysteamine (Zhou *et al.*, 2010). NAC can easily enter cells, where it is converted to cysteine, which is the precursor of GSH. Thus, when the concentration of pyruvate is increased within HCZB medium, oocyte aging would be delayed by at least the same length of time as by NAC and/or α-tocopherol supplementation, as well as by cysteamine/cystine and/or α-tocopherol supplementation, at low temperatures. This possibility is worthy of further investigation.

In conclusion, our research has demonstrated for the first time that by preventing oxidative stress using appropriate NAC supplementation at low temperatures, aspects of postovulatory aging of oocytes can be delayed. Thus, we have developed a simple method for the short-term culture of mature oocytes *in vitro* without freezing.

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Conflict of interest

The author declares that there is no conflict of interest.

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