Antioxidant supplementation overcomes the deleterious effects of maternal restraint stress-induced oxidative stress on mouse oocytes

Hua-Yu Lian, Yan Gao, Guang-Zhong Jiao, Ming-Ju Sun, Xiu-Fen Wu, Tian-Yang Wang, Hong Li and Jing-He Tan

College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai-an City 271018, Shandong Province, People's Republic of China

Correspondence should be addressed to J-H Tan; Email: tanjh@sdau.edu.cn

Abstract

In this study, using a mouse model, we tested the hypothesis that restraint stress would impair the developmental potential of oocytes by causing oxidative stress and that antioxidant supplementation could overcome the adverse effect of stress-induced oxidative stress. Female mice were subjected to restraint stress for 24 h starting 24 h after equine chorionic gonadotropin injection. At the end of stress exposure, mice were either killed to recover oocytes for \textit{in vitro} maturation (IVM) or injected with human chorionic gonadotropin and caged with male mice to observe \textit{in vivo} development. The effect of antioxidants was tested \textit{in vitro} by adding them to IVM medium or \textit{in vivo} by maternal injection immediately before restraint stress exposure. Assays carried out to determine total oxidant and antioxidant status, oxidative stress index, and reactive oxygen species (ROS) and glutathione levels indicated that restraint stress increased oxidative stress in mouse serum, ovaries, and oocytes. Whereas the percentage of blastocysts and number of cells per blastocyst decreased significantly in oocytes from restraint-stressed mice, addition of antioxidants to IVM medium significantly improved their blastocyst development. Supplementation of cystine and cysteamine to IVM medium reduced ROS levels and aneuploidy while increasing glutathione synthesis and improving pre- and postimplantation development of oocytes from restraint-stressed mice. Furthermore, injection of the antioxidant epigallocatechin gallate into restraint-stressed mice significantly improved the blastocyst formation and postimplantation development of their oocytes. In conclusion, restraint stress at the oocyte prematuration stage impaired the developmental potential of oocytes by increasing oxidative stress and addition of antioxidants to IVM medium or maternal antioxidant injection overcame the detrimental effect of stress-induced oxidative stress. The data reported herein are helpful when making attempts to increase the chances of a successful outcome in human IVF, because restraint was applied at a stage similar to the FSH stimulation period in a human IVF program.

Reproduction (2013) 146 559–568

Introduction

Studies suggest that psychological stress can exert detrimental effects on reproduction in women (Copper et al. 1996, Csemiczky et al. 2000, Kee et al. 2000, Klonoff-Cohen et al. 2001, Schröder et al. 2004, Neggers et al. 2006). Adverse effects of psychological stress on reproduction have also been reported in sows (Tsuma et al. 1996, Peltoniemi et al. 1999) and ewes (Smith et al. 2008). Restraint of small animals is an experimental procedure developed for studying psychogenic stress (Paré & Glavin 1986, Glavin et al. 1994). Mice and rats exposed to restraint stress during pregnancy exhibit impaired function of corpora lutea and reduced pregnancy rates and litter size (Wiebold et al. 1986, Sugino et al. 1994, Mairesse et al. 2007). According to Hobel & Culhane (2003), stressors present early in pregnancy are most probably present before pregnancy, and, hence, to improve pregnancy outcome, interventions will have to begin before conception. However, evidence for the direct effect of psychological stress on the oocyte is limited (Zhang et al. 2011, Zhou et al. 2012).

In a healthy body, reactive oxygen species (ROS) and antioxidants remain in balance. When the balance is disrupted toward an overabundance of ROS, oxidative stress occurs (Agarwal et al. 2005). Oxidative stress has been identified to play a key role in the pathogenesis of subfertility in both males and females. However, whereas the adverse effects of oxidative stress on sperm quality and functions have been well documented, its effect on oocytes and female reproductive functions remains largely unclear (Agarwal et al. 2012). In somatic cells, physical and psychological stressors cause...
oxidative stress by upsetting the balance between prooxidant and antioxidant status (Ejchel-Cohen et al. 2006, Zaidi et al. 2003, Derin et al. 2006, Sahin et al. 2006, Akpinar et al. 2008). Furthermore, studies using rat models have demonstrated that restraint stress induces oxidative stress in different organs (Zaidi et al. 2003, Derin et al. 2006, Sahin et al. 2006, Akpinar et al. 2008). However, although our recent study has provided preliminary evidence that maternal restraint stress increases oxidative stress in the oocyte (Zhou et al. 2012), reports on whether restraint stress causes oxidative stress in the ovary are limited. Thus, further trials are necessary to confirm the causal link between maternal restraint stress and oocyte oxidative stress and to elucidate the precise mechanisms through which oxidative stress affects the oocyte.

Patients undergoing IVF often suffer from anxiety and concern. For example, unsuccessful cycles and failure to release cumulus–oocyte complexes (COCs) may be associated with significantly increased stress levels (Verhaak et al. 2005, 2007). The costs of treatment and medical aspects of the procedures such as surgery, anesthesia, and pain can also cause concern (Klonoff-Cohen & Natarajan 2004, Dawson et al. 2005, Polinder et al. 2008). A prospective study has indicated that stressful life events may reduce the chances of a successful outcome following IVF, possibly through psychological mechanisms affecting medical end points such as oocyte retrieval outcome (Ebbeisen et al. 2009). However, it is unknown whether IVF-associated psychological stress would impair the developmental potential of oocytes by causing oxidative stress. Furthermore, although antioxidant supplementation has been suggested to control ROS generation and cure reproductive disorders (Agarwal et al. 2012), studies on its use to overcome the detrimental effect of oxidative stress on oocytes are limited.

Our hypothesis was that restraint stress would impair the developmental potential of oocytes by increasing oxidative stress and that antioxidant supplementation to culture media or maternal antioxidant injection would overcome the adverse effect of stress-induced oxidative stress on oocytes. The objective of this study was to test this hypothesis by using a restraint-stressed mouse model.

Materials and methods

Unless otherwise specified, all chemicals and reagents used in this study were purchased from Sigma Chemical Co.

Mice and their treatment

Mice of the Kunming strain were handled according to the rules stipulated by the Animal Care and Use Committee of Shandong Agricultural University. Female mice, 8–10 weeks old, were injected with equine chorionic gonadotropin (eCG). Mice were subjected to restraint stress for 24 h starting 24 h after eCG injection, to mimic the human IVF-associated distress taking place sometime during the follicle-stimulating hormone (FSH) stimulation period in the follicular phase (Macklon et al. 2006). Procedures followed for restraint stress were exactly those reported by Zhang et al. (2011). For antioxidant treatment, mice were injected (i.p.) 1 h before restraint stress exposure with different amounts (mg/kg) of epigallocatechin gallate (EGCG, Zhejiang Yixin Pharmaceutical Co. Ltd., Lanxi, China) in saline.

Oocyte recovery and in vitro maturation

Mice were killed immediately after restraint stress exposure, and large follicles in the ovaries were ruptured in M2 medium to release cumulus–oocyte complexes (COCs). COCs were cultured in groups of about 30 in 100 µl drops of maturation medium. For most of the experiments, the maturation medium used was a modified α-MEM (CaCl₂, 1.8 mM; MgSO₄, 0.81 mM; KCl, 5.3 mM; NaHCO₃, 26.2 mM; NaCl, 117.2 mM; NaH₂PO₄, 1.0 mM; d-glucose, 5.6 mM; phenol red, 0.03 mM; and sodium pyruvate, 1 mM) supplemented with 2 mM glutamine, 4 mg/ml BSA, and 10 IU/ml eCG. Because oocytes matured in α-MEM could not be fertilized properly, oocytes used for IVF were matured in TCM-199 medium (Gibco) supplemented with 10% (v/v) FCS (Gibco), 1 µg/ml 17β-estradiol, 24.2 µg/ml sodium pyruvate, 0.05 IU/ml FSH, 0.05 IU/ml luteinizing hormone, and 10 ng/ml epidermal growth factor. To observe the effect of antioxidant supplementation, antioxidants including cysteamine and cystine, vitamins E and C, and N-acetyl-l-cysteine (NAC) were added to the maturation medium.

Oocyte activation, IVF, and embryo culture

Procedures followed for oocyte activation, IVF, and embryo culture were exactly those reported by Zhou et al. (2010), except that zona drilling was not carried out before insemination in this study. Blastocysts obtained were either stained with Hoechst 33342 for cell counting or transferred into surrogate mothers to observe postimplantation development.

Collection and cell counting of in vivo embryos

Embryo donor mice were injected with human chorionic gonadotropin (hCG) and caged with male mice 48 h after eCG injection. Mice were examined for vaginal plugs the next morning (day 0 p.c.). In the afternoon of day 3 p.c., mice having a vaginal plug were killed to collect embryos. Blastocysts obtained were either transferred into surrogate mothers or processed for cell counting.

Embryo transfer

Female mice aged 8–10 weeks (28–35 g body weight) were used as pseudopregnant recipients. IVF blastocysts and in vivo blastocysts recovered 3.5 day p.c. were transferred into the 2.5-day p.c. pseudopregnant recipients. Fifteen embryos were transferred into each recipient, eight or seven embryos per uterine horn.
Assay for intra-oocyte ROS

Intra-oocyte H$_2$O$_2$ levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) as described by Nasr-Esfahani et al. (1990). Cumulus-free oocytes were stained for 10 min with the 0.01 mM DCHFDA in M2 medium and then observed under a Leica laser scanning confocal microscope. Fluorescence was obtained by excitation with a 488 nm line of laser. Photographs were taken using fixed microscopic parameters, and the fluorescence intensity from each oocyte was analyzed using Leica Software.

Assay for intracellular glutathione (GSH)

Samples (each containing 35–40 cumulus-free oocytes) were frozen and thawed three times. The concentrations of total GSH (GSX) in oocytes were determined by the 5,5'-dithiobis (2-nitrobenzoic acid)-oxidized glutathione (GSSG) reductase-recycling assay (Funahashi et al. 1994). Standards (0.01, 0.02, 0.1, 0.2, and 1.0 mM) of GSX and a sample blank without GSX were also assayed. The amount of GSX in each sample was divided by the number of oocytes to determine the intracellular GSX concentration per oocyte (pmol/oocyte). The reduced GSX (GSH) values were calculated from the difference between GSX and GSSG values for each oocyte.

Oocyte chromosome preparation and analysis

At 17 h of in vitro maturation (IVM), oocytes were separated from cumulus cells and placed in 1% sodium citrate (hypotonic solution) for 1.5 h at 4°C. Oocytes were then transferred with minimal solution onto glass slides and fixed in situ with drops of methanol:acetic acid (3:1). After air drying, the slides were stained with 2% Giemsa, observed for chromosomes, and photographed under a light microscope. All oocytes were anonymized to ensure blind analysis, and the anonymized oocytes were analyzed by a single observer (the first author). The numbers of haploid (20 chromosomes, each with two chromatids), hypohaploid (<20 chromosomes), and hyperhaploid (>20 chromosomes) oocytes were determined. We ignored spreads with missing chromosomes and calculated only the percentage of hyperhaploid oocytes.

Observation of chromosome spindles

At 8.5 h of IVM, oocytes were separated from cumulus cells and zona pellucida. Oocytes were first fixed with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO$_4$, pH 7.0) for at least 30 min, followed by treatment with 1% Triton-X 100 in PHEM for 10–15 min. Then, oocytes were blocked overnight in PHEM containing 1% BSA and 100 mM glycine at 4°C. Finally, oocytes were incubated for 1 h with FITC-conjugated anti-α-tubulin MAB (1:50) in 3% BSA in M2 medium and for 10 min with 10 μg/ml Hoechst 33342 in M2 medium. The spindle and chromosome morphology of oocytes was observed under a fluorescence microscope. All the oocytes were anonymized to ensure blind analysis, and the anonymized oocytes were analyzed by a single observer (the first author).

Measurement of total oxidant status and total antioxidant status in serum and ovaries

Preparation of serum

Mice were killed by decollation, and trunk blood (about 1 ml) was collected into ice-cooled centrifuge tubes. Blood was then allowed to clot for 30 min before centrifugation (1500 g, 10 min, 4°C) to recover serum.

Ovarian homogenisation

Ovaries were weighed and transferred into a homogenizer with 800 μl PBS per 100 mg ovarian tissue. Homogenization was carried out by hand at room temperature. Homogenates were centrifuged (1500 g, 10 min, 4°C), and the supernatant was collected for use.

Total oxidant status determination

Total oxidant status in serum and ovarian homogenates was measured using the method of Erel (2005). Briefly, 225 μl Reagent 1 (150 μM xylene orange, 140 mM NaCl, and 1.35 M glycerol in 25 mM H$_2$SO$_4$ solution, pH 1.75), 35 μl sample, and 11 μl Reagent 2 (5 mM ferrous ion and 10 mM o-dianisidine in 25 mM H$_2$SO$_4$ solution) were used for the reaction. The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in an acidic medium and on the measurement of the colored complex formed by the ferric ion with xylene orange at 560 nm. The first absorbance was determined before the mixing of R1 and R2 (as sample blank), and the last absorbance was determined when the reaction trace drew a plateau line (about 3–4 min after the mixing). The assay was calibrated with H$_2$O$_2$, and the results are expressed in μM H$_2$O$_2$ equivalent (μM H$_2$O$_2$ equiv.).

Total antioxidant status determination

Total antioxidant status was measured using the method of Erel (2004). Briefly, 200 μl Reagent 1, 5 μl sample, and 20 μl Reagent 2 were used for the reaction. While Reagent 1 was a 0.4 M acetate buffer (pH 5.8), Reagent 2 contained 10 mM 2,2'-azino-di-(3-ethylbenz-thiazoline sulfonate) (ABTS) in 30 mM acetate buffer (pH 3.6). This assay relies on the ability of antioxidants in the sample to inhibit the formation of colored ABTS$^*$ from the oxidation of ABTS. This change in color was measured as a change in absorbance at 660 nm. The first absorbance of the assay was taken before the mixing of R1 and R2 (as sample blank) and the last absorbance was taken at the end of the incubation period (5 min after the mixing). The reaction rate was calibrated with Trolox dissolved in phosphate buffer (30 mM, pH 7.4), and the assay results are expressed in mM Trolox equivalent (mM Trolox equiv.).

Oxidative stress index

The total oxidant status to total antioxidant status ratio is considered as the oxidative stress index (Aycicek & Erel 2007). The total antioxidant status unit of mM Trolox equivalent was converted into μM Trolox equivalent, and the oxidative stress index value was calculated as follows: oxidative stress index = (μM H$_2$O$_2$ equiv./μM Trolox equiv.)/100 (Aycicek et al. 2005).
**Statistical analysis**

There were at least three replicates for each treatment unless otherwise stated. Percentage data were arc sine-transformed and analyzed with ANOVA when each measure contained more than two groups or with an independent t-test when each measure had only two groups. A Duncan multiple comparison test was used to find differences during ANOVA. The software used was Statistics Package for Social Sciences (SPSS 11.5, SPSS, Inc.). Data are expressed as means ± S.E.M., and \( P<0.05 \) was considered significant.

**Results**

**Restraint stress caused oxidative stress in mouse serum, ovaries, and oocytes**

Female mice were exposed to restraint stress for 24 h starting 24 h after eCG injection. At the end of restraint stress exposure, mice were killed to collect blood, ovaries, and oocytes. Following restraint stress exposure, total oxidant status and oxidative stress index increased significantly in both serum and ovaries (Fig. 1A). Total antioxidant status decreased in both serum and ovaries, although the difference was not statistically significant in the ovaries. Whereas the intra-oocyte levels of ROS and GSSG increased, the level of GSH decreased significantly after restraint stress exposure, leading to a decreased ratio of GSH:GSSG in oocytes both before and after IVM (Fig. 1B).

**Combined supplementation of cysteamine and cystine \((C+C)\) to IVM medium was the optimal protocol that improved the preimplantation development of oocytes from restraint-stressed mice**

Oocytes from restraint-stressed or unstressed control mice were matured in α-MEM with or without antioxidant supplementation. Mature oocytes were treated with SrCl₂ for activation. Compared with those in oocytes from unstressed control mice, the percentage of four-cell embryos and blastocysts and number of cells per blastocyst decreased significantly in oocytes from restraint-stressed mice (Table 1). Supplementation with C+C increased the percentage of blastocysts to the highest level in oocytes from both unstressed and restraint-stressed mice. Although vitamin E supplementation increased the percentage of blastocysts in oocytes from restraint-stressed mice to the level observed in those from control mice matured without C+C supplementation, the number of cells per blastocyst observed with vitamin E supplementation was significantly lower than that observed in oocytes from control mice. Thus, C+C supplementation was the optimal protocol that increased the developmental potential of oocytes from restraint-stressed mice to the level observed in those from unstressed control mice.

**C+C supplementation to IVM medium improved both pre- and postimplantation development of oocytes from restraint-stressed mice**

Oocytes from restraint-stressed or control mice were matured in TCM-199 medium with or without C+C supplementation. Mature oocytes were inseminated in vitro. Restraint stress decreased the rates of fertilization, percentage of four-cell embryos and blastocysts, and number of cells per blastocyst significantly (Table 2). Supplementation with C+C increased these parameters in oocytes from restraint-stressed mice to levels observed in those from control mice matured without C+C supplementation. Supplementation with C+C also improved the development of oocytes from control mice, but the extent of improvement was not statistically significant. After embryo transfer, the percentage of live young/transferred embryos was significantly lower in oocytes from restraint-stressed mice than in those from control mice (Table 3). The percentage of term pregnancy and live young per pregnant recipient also decreased in oocytes from restraint-stressed mice, although the difference from that in oocytes from control mice did not reach statistical significance.
Supplementation with C+C increased all these parameters in oocytes from restraint-stressed mice to levels observed in those from control mice.

**C+C supplementation to IVM medium alleviated oxidative stress while improving redox potential of oocytes from restraint-stressed mice**

Oocytes from restraint-stressed mice were matured in α-MEM with or without C+C supplementation before being assayed for the levels of ROS and GSH. Supplementation with C+C significantly reduced the level of ROS (Fig. 2). Whereas the levels of GSX and GSSG increased, the levels of GSH and the GSH:GSSG ratio did not change significantly after C+C supplementation. This suggests that C+C supplementation promoted the synthesis of GSX, but some of the synthesized GSH was oxidized to reduce the increased amounts of ROS.

**C+C supplementation to IVM medium reduced MII aneuploidy while improving MI spindle assembly in oocytes from restraint-stressed mice**

Oocytes from restraint-stressed and unstressed control mice were matured in α-MEM with or without C+C supplementation. At 8.5 and 17 h of maturation respectively, oocytes were processed to observe MI spindles and MII chromosomes. Figure 3A and B shows MII oocytes with a normal haploid (n=20) and a hyperhaploid (n=21) complement of chromosomes. According to our reported criteria (Zhou et al. 2012), whereas the barrel-shaped spindles and the chromosomes that were well congressed on the metaphase plate were considered normal, the asymmetric spindles with one pole wider than the other and the chromosomes that were not well congressed on the metaphase plate (often with displaced chromosomes) were considered abnormal. Thus, in this study, the MI oocytes were classified into those with both normal chromosomes and normal spindles (Fig. 4A), those with either normal spindles (Fig. 4B) or normal chromosomes (Fig. 4C), and those with neither normal spindles nor normal chromosomes (Fig. 4D). Restraint stress increased the number of MII oocytes with hyperhaploid chromosomes (Fig. 3C) while decreasing the number of MI oocytes with both normal spindles and normal chromosomes significantly (Fig. 4E). Supplementation with C+C restored both the parameters in oocytes from restraint-stressed mice to levels observed in those from control mice.

### Table 1 Effects of supplementation of different antioxidants to IVM medium (α-MEM) on embryo development in oocytes from control and restraint-stressed mice following Sr²⁺ activation.

<table>
<thead>
<tr>
<th>Mouse treatments</th>
<th>Antioxidants</th>
<th>Concentration (µM)</th>
<th>Percentage of four-cell embryos/activated oocytes</th>
<th>Percentage of blastocysts/four-cell embryos</th>
<th>Number of cells per blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C+C</td>
<td>0</td>
<td>88.1 ± 1.1 b</td>
<td>29.0 ± 1.2 d</td>
<td>39.3 ± 2.0 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 + 200</td>
<td>89.9 ± 1.8 a</td>
<td>44.3 ± 2.9 b</td>
<td>51.0 ± 0.5 d</td>
</tr>
<tr>
<td>Stressed</td>
<td></td>
<td>0</td>
<td>74.2 ± 3.3 b</td>
<td>18.0 ± 3.0 a</td>
<td>29.1 ± 1.1 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 + 200</td>
<td>92.2 ± 2.8 a</td>
<td>41.8 ± 6.2 b</td>
<td>38.2 ± 0.8 b</td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td>0</td>
<td>72.8 ± 4.9 b</td>
<td>16.9 ± 3.3 b</td>
<td>27.6 ± 1.4 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>69.2 ± 6.3 b</td>
<td>38.9 ± 7.5 b</td>
<td>28.6 ± 1.3 a</td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td>0</td>
<td>69.2 ± 1.7 b</td>
<td>16.7 ± 3.0 b</td>
<td>32.6 ± 3.1 a,b,c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>70.4 ± 2.7 b</td>
<td>31.7 ± 4.4 b</td>
<td>31.7 ± 1.6 a,b</td>
</tr>
<tr>
<td>NAC</td>
<td></td>
<td>0</td>
<td>75.7 ± 3.0 b</td>
<td>10.0 ± 1.7 b</td>
<td>29.4 ± 4.2 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>74.6 ± 5.2 b</td>
<td>20.7 ± 2.6 b</td>
<td>33.7 ± 3.9 a,b,c</td>
</tr>
</tbody>
</table>

*a,b,c,d,eValues without a common letter in their superscripts differ (P<0.05, ANOVA) within columns. Each treatment was repeated three to four times, and in each replicate, 30–40 oocytes were observed. Antioxidants were used at the optimal concentrations selected in preliminary experiments (data not shown). Oocyte activation rates ranging from 87 to 96% did not differ among the treatments. C+C, combined supplementation of cysteamine (100 µM) and cystine (200 µM); NAC, N-acetyl-L-cysteine.

### Table 2 In vitro development after IVF of oocytes from unstressed control or restraint-stressed mice that had been matured in TCM-199 medium with (+) or without (−) C+C supplementation.

<table>
<thead>
<tr>
<th>Mouse treatments</th>
<th>C+C supplementation</th>
<th>Percentage of fertilized oocytes/observed oocytes</th>
<th>Percentage of four-cell embryos/fertilized oocytes</th>
<th>Percentage of blastocysts/four-cell embryos</th>
<th>Number of cells per blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>91.6 ± 1.8 a</td>
<td>89.5 ± 2.6 a</td>
<td>80.4 ± 2.2 a</td>
<td>51.9 ± 0.6 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>93.1 ± 1.5 a</td>
<td>91.0 ± 2.1 a</td>
<td>85.7 ± 1.6 a</td>
<td>54.7 ± 0.8 a</td>
</tr>
<tr>
<td>Stressed</td>
<td>−</td>
<td>78.0 ± 1.7 b</td>
<td>75.2 ± 4.2 b</td>
<td>60.2 ± 8.5 b</td>
<td>37.8 ± 1.8 b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>87.0 ± 3.1 a</td>
<td>86.0 ± 4.0 a</td>
<td>80.9 ± 4.5 a</td>
<td>48.8 ± 2.8 a</td>
</tr>
</tbody>
</table>

Values without a common letter in their superscripts differ (P<0.05, ANOVA) within columns. Each treatment was repeated three to four times, and in each replicate, 30–40 oocytes were observed. Oocytes that were recovered from unstressed mice and cultured for IVM without C+C supplementation were used as controls. C+C, combined supplementation of cysteamine (100 µM) and cystine (200 µM).
Control K

important indicator of the redox status (Wu et al. 2012). By measuring total oxidant status, total antioxidant status, and oxidative stress index, the present study showed that oxidative stress increased in both blood and ovaries after restraint stress exposure. Furthermore, both our assays for intra-oocyte ROS and glutathione and C+C supplementation to IVM medium demonstrated that restraint stress increased oxidative stress in oocytes. It has been reported that stresses including restraint stress cause the disruption of homeostasis and an imbalanced antioxidant status in blood and several organs (Sahin et al. 2004, Derin et al. 2006, Sahin & Gumuslu 2007). However, stress-induced oxidative stress has not been observed in ovaries, although our previous study has indicated that restraint stress causes oxidative stress in oocytes (Zhou et al. 2012). Furthermore, measurement of total oxidant status, total antioxidant status, and oxidative stress index in ovaries has not been reported.

The results of the present study indicated that restraint stress impaired the developmental potential of oocytes by increasing oxidative stress. Thus, whereas the percentage of blastocysts and number of cells per blastocyst decreased significantly in oocytes from restraint-stressed mice to levels observed in oocytes from control mice.

Discussion

As the effects of oxidants or antioxidants can be additive and measuring individual ones separately is both time consuming and labor intensive, a measurement of the combined activities of total oxidant status or total antioxidant status is now often used to estimate the overall oxidative or antioxidant status (Erel 2004, 2005). Notably, oxidative stress index is considered to be a more accurate index of oxidative stress, because it represents a comprehensive measurement of total antioxidant status and total oxidant status. Oxidative stress in an oocyte is usually determined by measuring the levels of ROS (Nasr-Esfahani et al. 1990, Zhou et al. 2012) and glutathione (Boerjan & de Boer 1990, Zhou et al. 2012), because it is known that GSH/GSSG is the main redox buffer and the GSH:GSSG ratio is a very important indicator of the redox status (Wu et al. 2004, Ojha & Srivastava 2012). By measuring total oxidant status, total antioxidant status, and oxidative stress index, the present study showed that oxidative stress increased in both blood and ovaries after restraint stress exposure. Furthermore, both our assays for intra-oocyte ROS and glutathione and C+C supplementation to IVM medium demonstrated that restraint stress increased oxidative stress in oocytes. It has been reported that stresses including restraint stress cause the disruption of homeostasis and an imbalanced antioxidant status in blood and several organs (Sahin et al. 2004, Derin et al. 2006, Sahin & Gumuslu 2007). However, stress-induced oxidative stress has not been observed in ovaries, although our previous study has indicated that restraint stress causes oxidative stress in oocytes (Zhou et al. 2012). Furthermore, measurement of total oxidant status, total antioxidant status, and oxidative stress index in ovaries has not been reported.

The results of the present study indicated that restraint stress impaired the developmental potential of oocytes by increasing oxidative stress. Thus, whereas the percentage of blastocysts and number of cells per blastocyst decreased significantly in oocytes from restraint-stressed mice to levels observed in oocytes from control mice.

**Maternal injection of the antioxidant EGCG improved both in vitro and in vivo development of oocytes from restraint-stressed mice**

Mice were injected with EGCG 1 h before restraint stress exposure. At the end of restraint stress exposure, mice were either killed to recover oocytes for IVF in α-MEM or injected with hCG and caged with male mice overnight. The IVM oocytes were activated with SrCl₂ to observe in vitro development. Caged mice showing vaginal plugs were killed on day 3 p.c. to collect in vivo embryos. In vitro (Table 4), the percentage of four-cell embryos and blastocysts and number of cells per blastocyst decreased significantly in restraint-stressed mice that were not injected with hCG compared with those in unstressed control mice, and maternal injection of EGCG (65 mg/kg body weight) eliminated the deleterious effect of restraint stress. In vitro (Table 4), the percentage of four-cell embryos and blastocysts and number of cells per blastocyst decreased significantly in restraint-stressed mice that were not injected with hCG compared with those in unstressed control mice, and maternal injection of EGCG (65 mg/kg body weight) eliminated the deleterious effect of restraint stress. In vivo (Table 5), the number of blastocysts obtained per donor mouse and the average number of young per recipient after embryo transfer were significantly lower in restraint-stressed mice that were not injected with hCG compared with those in unstressed control mice, and EGCG injection increased these parameters in oocytes from restraint-stressed mice to levels observed in oocytes from control mice.

**Table 3** Pregnancy and birth of live young after embryo transfer of blastocysts derived from IVF of oocytes from unstressed control or restraint-stressed mice that had been matured in TCM-199 medium with or without C+C supplementation.

<table>
<thead>
<tr>
<th>Mouse treatments</th>
<th>C+C</th>
<th>Percentage of term pregnancy</th>
<th>Live young per pregnant recipient</th>
<th>Percentage of live young/embryos transferred</th>
<th>Birth weight of young (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>75.0 (9/12)a</td>
<td>4.4 ±0.4 (40/9)a</td>
<td>22.2 ±0.4 (40/180)a</td>
<td>2.3 ±0.1a</td>
</tr>
<tr>
<td>Stressed</td>
<td>−</td>
<td>42.9 (6/14)a</td>
<td>2.8 ±0.5 (17/6)a</td>
<td>8.1 ±0.5 (17/210)a</td>
<td>2.2 ±0.1a</td>
</tr>
<tr>
<td>Stressed</td>
<td>+</td>
<td>69.2 (9/13)a</td>
<td>4.1 ±0.6 (37/9)a</td>
<td>19.0 ±0.6 (37/195)a</td>
<td>2.3 ±0.1a</td>
</tr>
</tbody>
</table>

aValues without a common letter in their superscripts differ (P<0.05, ANOVA) within columns. Oocytes that were recovered from unstressed mice and cultured for IVM without C+C supplementation were used as controls. C+C, combined supplementation of cysteamine (100 μM) and cystine (200 μM).

**Figure 2** Levels of ROS and glutathione after the maturation of oocytes from restraint-stressed mice in α-MEM without C+C (CC−) or with C+C (CC+) supplementation. GSX, GSH, GSSG, and G:G represent total, reduced, oxidized glutathione, and ratio of GSH:GSSG respectively. a,b,c,d,eValues without a common letter above their bars differ (P<0.05, independent t-test).
In each treatment, 84–94 oocytes were observed. Values with a different letter above their bars differ significantly (P<0.05, ANOVA).

restraint-stressed mice that showed increased oxidative stress, addition of antioxidants to IVM medium significantly improved blastocyst formation and postimplantation development of oocytes from restraint-stressed mice. Furthermore, C+C supplementation to IVM medium reduced MII aneuploidy and ROS levels while increasing glutathione levels in oocytes from restraint-stressed mice. However, although all the antioxidants added to IVM medium improved the developmental competence of oocytes from restraint-stressed mice to some extent, the best results were obtained only with C+C supplementation. The synthesis of glutathione is highly dependent on the availability of cysteine in the medium (Meister & Tate 1976, Chance et al. 1979). In vivo, cysteine is produced from cystine in the reaction catalyzed by the enzyme cystine reductase. In vitro, however, the synthesis of glutathione may be impaired because of a deficiency of cysteine in the culture medium and its high instability and easy auto-oxidation to cystine (Bannai 1984, Sagara et al. 1993). Furthermore, our previous study has shown that neither mouse cumulus cells nor the oocyte itself could use cystine or cysteamine when added alone, but cysteamine could reduce cystine to cysteine in a cell-free medium (Zhou et al. 2010). Therefore, cystine and cysteamine were used in combination in this study to promote the synthesis of glutathione in oocytes from restraint-stressed mice.

It has been reported that dietary NAC is efficacious in supplying cysteine in support of chick growth (Dilger & Baker 2007) and that NAC is available as a precursor for cysteine to support growth and protein (nitrogen) accretion in piglets administered a parenteral solution (Shoveller et al. 2006). However, several studies including the present study have observed little or no effect of NAC on oocyte quality when supplemented to IVM medium (Ali et al. 2003, Choe et al. 2010). By contrast, it was found that whereas cysteine was metabolized by oocytes despite the presence of cumulus cells that separated oocytes from the extracellular medium, NAC was not metabolized at all (de Matos et al. 1997). Because vitamins E and C and dithiothreitol (data not shown) tested in this study are ‘pure’ antioxidants, which do not promote the synthesis of glutathione, the results of the present study that C+C supplementation was the best to increase the competence of oocytes from restraint-stressed mice suggest that glutathione might promote the maturation of oocytes not only by acting as an antioxidant but also by other long-term actions. 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 GSSG (Gardiner & Reed 1995a, 1995b).

The results of the present study indicated that whereas C+C supplementation to α-MEM medium during IVM significantly improved the developmental potential of oocytes from both restraint-stressed and unstressed control mice, C+C supplementation to TCM-199 medium had only a mild promoting effect on oocytes from control mice. This suggests that oocytes suffered more severe oxidative stress or glutathione insufficiency during maturation in α-MEM than in TCM-199 medium. In other words, it suggests that C+C supplementation overcome not only the oxidative stress induced by restraint stress but also the oxidative stress induced by \textit{in vitro} culture. It is known that \textit{in vitro} cultures are maintained under concentrations of \textit{O}_2 that are higher than those that occur \textit{in vivo}, resulting in increased production of ROS. Thus, the addition of cysteamine and/or cystine to the routine maturation medium was found to improve the maturation and development of oocytes by increasing their GSH content (de Matos et al. 1995, Abeydeera et al. 1998, Zhou et al. 2008, 2010). Compared with our modified α-MEM, which had only a simple composition, TCM-199 medium is much more complicated and, particularly, contains components such as cystine and glutathione that can be used by oocytes to alleviate glutathione insufficiency or oxidative stress.

This study showed that injecting restraint-stressed mice with the antioxidant EGCG significantly improved the blastocyst formation and postimplantation development of their oocytes. EGCG is a polyphenol derived from green tea. As a ROS scavenger, EGCG is considered to have potentially positive effects on numerous physiological systems. For example, it prevents cell death (Reznichenko et al. 2005) and chromosomal damage induced by ROS (Sugisawa & Umegaki 2002).

However, studies on its effect on oocytes are limited. Wang et al. (2007) showed that green tea polyphenols at certain concentrations (15 mM) in IVM medium had beneficial effects on subsequent embryo development of bovine oocytes and that the effect was correlated with the intracellular levels of GSH. Roth et al. (2008) observed that while 2-h hyperthermia application after hCG injection in mice disrupted the competence of oocytes, EGCG injection improved the developmental competence of these oocytes. The present study is thus more meaningful for human IVF, because it shows for the first time that psychological stress applied during the FSH-priming period impairs the developmental potential of oocytes and that \textit{in vivo} administration of EGCG improves the quality of the embryos that develop from these oocytes. Furthermore, the results of the present study indicated that the beneficial effect of EGCG injection on oocytes was dose dependent, with a too high concentration being harmful. Spinaci et al. (2008) found that high EGCG concentrations could affect IVM and IVF in pigs. Thus, we have provided evidence that excessive EGCG concentrations could induce negative reproduction-related consequences also \textit{in vivo}.

In conclusion, although it is known that patients undergoing IVF often suffer from anxiety and concern, it is not known whether the IVF-associated psychological stress would impair the competence of oocytes by causing oxidative stress. Although it has been suggested that antioxidant supplementation may be effective in controlling the production of ROS and may be explored as a potential strategy to cure reproductive disorders, studies on its use to overcome the detrimental effect of oxidative stress on oocytes are limited. In this study, female mice were subjected to restraint stress for 24 h starting 24 h after eCG injection, to mimic the human IVF-associated distress taking place sometime during the

### Table 4 Effects of (−)-EGCG injection in restraint-stressed mice on \textit{in vitro} blastocyst development following Sr\textsuperscript{2+} activation of oocytes matured in α-MEM.

<table>
<thead>
<tr>
<th>Injection of restraint-stressed mice with</th>
<th>Percentage of four-cell embryos/activated oocytes</th>
<th>Percentage of blastocysts/ four-cell embryos</th>
<th>Number of cells per blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed controls</td>
<td>88.5±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.1±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.4±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nothing</td>
<td>73.3±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2±1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.1±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saline (vehicle)</td>
<td>73.5±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.9±6.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.1±2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG (35 mg/kg)</td>
<td>76.6±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.1±2.9&lt;sup&gt;ab,c&lt;/sup&gt;</td>
<td>26.9±1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG (65 mg/kg)</td>
<td>90.7±3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.5±4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.2±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG (100 mg/kg)</td>
<td>70.9±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.2±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.3±1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Values without a common letter in their superscripts differ (<i>P</i>&lt;0.05, ANOVA) within columns. Each treatment was repeated four to five times, and in each replicate, 30–40 oocytes were observed. Oocyte activation rates ranging from 89 to 93% did not differ among the treatments.

### Table 5 Effects of EGCG (65 mg/kg) injection in restraint-stressed mice on \textit{in vivo} embryo development in oocytes.

<table>
<thead>
<tr>
<th>Injection of restraint-stressed mice with</th>
<th>Donor mouse</th>
<th>Blastocysts obtained per donor mouse</th>
<th>Number of cells per blastocyst</th>
<th>Recipient mouse</th>
<th>Average number of young per recipient</th>
<th>Birth weight of young (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed controls</td>
<td>12</td>
<td>19.3±2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.3±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>9.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nothing</td>
<td>15</td>
<td>9.6±1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.1±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td>5.9±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG (65 mg/kg)</td>
<td>13</td>
<td>17.9±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.1±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>8.7±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values without a common letter in their superscripts differ significantly (<i>P</i>&lt;0.05, ANOVA) within columns.
FSH stimulation period in the follicular phase. The effects of restraint stress and antioxidant supplementation on oocyte oxidative stress and in vitro and in vivo development were then studied. The results showed that restraint stress applied during the prematuration period impaired the developmental potential of oocytes by increasing oxidative stress and that the addition of antioxidants to IVM medium or maternal antioxidant injection overcame the detrimental effect of restraint stress-induced oxidative stress on oocytes. The data reported herein are helpful when making attempts to increase the chances of a successful outcome following human IVF.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by grants from the National Basic Research Program of China (Nos. 2012CB944403) and the China National Natural Science Foundation (Nos. 31272444 and 30972096).

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