Sodium selenite improves the in vitro follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity

A. Abedelahi¹, M. Salehnia¹,*, A.A. Allameh², and D. Davoodi³

¹Department of Anatomy, Tarbiat Modares University, PO Box 14115-111, Tehran, Iran ²Department of Biochemistry, Tarbiat Modares University, Tehran, Iran ³Department of Molecular and Cellular Biology, Agricultural Biotechnology Research Institute of Iran, Karaj, Iran

*Correspondence address. Tel: +98-2182883871; Fax: +98-2188006544; E-mail: mogdeh@dr.com; salehnim@modares.ac.ir

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BACKGROUND: The aim of this study was to investigate the effect of sodium selenite (SS) on reactive oxygen species (ROS) production, total antioxidant capacity (TAC) and glutathione peroxide (GPx) activity of cultured pre-antral follicles derived from vitrified and non-vitrified ovarian tissue.

METHODS: Immature mouse ovaries were vitrified, and mechanically isolated pre-antral follicles from vitrified and non-vitrified samples were cultured in TCM 199 medium supplemented with different concentrations (0, 5 and 10 ng/ml) of SS. Follicular, oocyte and embryo development was assessed. In parallel, ROS, TAC and GPx levels were analyzed after 0, 12, 24, 48, 72 and 96 h of culture.

RESULTS: Development rates of follicles, oocytes and embryos were significantly higher in SS-supplemented groups (P < 0.005). ROS production was increased, and TAC levels and GPx activities were decreased after 24 h of culture of pre-antral follicles in vitrified and non-vitrified groups, whereas in the presence of SS, ROS production was decreased and TAC levels and selenium-dependent GPx-specific activities were increased after 96 h of culture. Vitrified and non-vitrified samples responded in a similar manner.

CONCLUSION: SS caused an increase in follicular TAC level and GPx activity and a decrease in ROS level, thus improving the in vitro development of follicles.

Key words: glutathione peroxidase / ovarian tissue / pre-antral follicles / reactive oxygen species / total antioxidant capacity

Introduction

There are many factors in culture conditions which control oocyte and follicular maturation. Oocyte growth and maturation appear to be affected by nutritional imbalances, hormonal disturbances and physical conditions of the microenvironment, such as oxidative stress (Nasr-Esfahani et al., 1990; Demeestere et al., 2005; Murray et al., 2008).

Oxygen concentration is higher in in vitro cultures than in vivo conditions, and free radicals are produced during aerobic metabolism of the cells (Goto et al., 1993; Agarwal et al., 2006; Chwa et al., 2006). Also, during cryopreservation, some physical and chemical conditions lead to changes in membrane properties and cause lipid peroxidation (Ahn et al., 2002). It has been shown that cryopreservation increases the reactive oxygen species (ROS) production in semen (Wang et al., 1997). Frozen cells are in a hypoxic state that leads to excess electron production, thus leading to the formation of ROS (Wang et al., 1997). Rahimi et al. (2003) demonstrated that the cryopreservation of human ovarian tissue using less rapid methods results in an increase in ROS levels and apoptosis just after warming.

There are controversial reports about the function of ROS in reproduction (Guérit et al., 2001; Dalvit et al., 2005; Agarwal et al., 2006; Jancar et al., 2007; Hammadeh et al., 2008; Morado et al., 2009). Low concentrations of ROS molecules are essential for gamete maturation, fertilization (Dalvit et al., 2005; Hammadeh et al., 2008; Morado et al., 2009).
2009) and cell proliferation (Dalvit et al., 1998; Rahimi et al., 2003). In contrast, high concentrations of ROS have been reported to induce oxidative stress which causes cell membrane damage and DNA fragmentation in somatic cells and leads to cell apoptosis (Dumont et al., 1999; Tatimoto et al., 2000; Bedaiwy et al., 2004).

Oxidative stress and a high concentration of ROS are related and induce apoptotic cell death in various cell types by both the death receptor and mitochondrial apoptotic pathways (Chandra et al., 2000; mitogen-activated protein kinase (MAPK) and caspase pathways are then induced by a variety of apoptotic stimuli (Schulze-Osthoff et al., 1998).

During in vitro maturation (IVM) of oocytes, ROS are produced (Cetica et al., 2001; Kodaman and Behrman 2001; Combelles et al., 2009) and it has been shown that cumulus cells play a major role in the antioxidant defense of oocyte using glutathione peroxidase activity (GPx) and catalase activities. Since these enzymes are active in mature oocyte, it seems that oocytes have also their own capacity to control ROS production during maturation by their antioxidant system (Cetica et al., 2001; Kodaman and Behrman 2001; Combelles et al., 2009).

An increase in ROS production in granulosa cells seems to have a deleterious effect on oocyte fertilization, embryo quality and the implantation rate; it also seems that germ cells, in comparison with somatic cells, are more susceptible to ROS and oxidative stress (Nasr-Esfahani et al., 1990; Bedaiwy et al., 2004; Jancar et al., 2007). Bedaiwy et al. (2004) showed that high levels of ROS in culture media are associated with low rates of embryo development and blastocysts formation.

There is a balance between ROS production and its scavenger system within the cells to prevent or minimize free radicals damage, including enzymatic and non-enzymatic mechanisms. Selenium is an antioxidant and a trace element, which is incorporated into the catalytic site of antioxidant enzymes, such as GPx, and is involved in cell growth and development by protecting cells against the toxic and damaging effects of ROS (Yoon et al., 2002; Patrick, 2004; Papp et al., 2007; Abedelahi et al., 2008).

The effect of sodium selenite (SS) on the IVM of mouse pre-antral follicles has been previously reported by our group (Abedelahi et al., 2008). We demonstrated that SS can improve the in vitro growth and maturation of mouse pre-antral follicles in the medium supplemented with fetal bovine serum (FBS), but no studies have described its effect on ROS production after vitrification and or the IVM of pre-antral follicles. ROS measurements, in combination with total antioxidant capacity (TAC) analysis, are important for the assessment of quality and growth of cultured follicles and would increase our knowledge of follicle development, particularly female gamete maturation. Thus, the aim of the present study was to assess the effects of SS, as an antioxidant agent, on ROS levels, the activity of GPx and the total enzymatic antioxidant capacity of follicles during growth in in vitro culture of mouse pre-antral follicles derived from vitrified and non-vitrified samples.

Materials and Methods

Animals and experimental design
Female NMRI mice, 12–14 days old (n = 100), were cared for and used according to the Guide for the Care and Use of Laboratory Animals of Tarbiat Modares University and housed under controlled conditions (12-h light/12-h dark) with free access to water and food. Mice were sacrificed by cervical dislocation, and the ovaries were dissected and randomly divided into two non-vitrified and vitrified groups. After mechanical isolation of pre-antral follicles, some of them were reserved for maturation and developmental assessment, and the others were reserved for analysis of ROS levels, GPx activity and TAC.

All chemicals were purchased from Sigma-Aldrich (Hamburg, Germany), unless otherwise stated.

Vitrification and warming
The vitrification procedure was based on a method used previously in our laboratory (Salehnia et al., 2002). Briefly, ovarian tissues were vitrified with the medium containing 40% ethylene glycol (V/V), 30% Ficoll 70 (W/V) and 1 M sucrose supplemented with bovine serum albumin (BSA). Warmed ovaries were equilibrated for 30 min in tissue culture medium (TCM 199; Gibco, Grand Island, NY, USA) supplemented with 5% FBS before follicle isolation.

Follicle isolation and culture
Isolation and culture of pre-ovulatory follicles were adapted from previously described methods (Haidari et al., 2006). The isolated pre-antral follicles from vitrified and non-vitrified ovaries were cultured for up to 14 days for maturation and developmental assessment or for 96 h for biochemical analyses and confocal microscope studies.

The culture medium consisted of TCM-199 supplemented with 0.33 mM sodium pyruvate, 100 mM/l/ml of recombinant follicle-stimulating hormone (or Gonal-f; Serono, Geneva, Switzerland), 1% insulin and transferrin (Gibco), 100 μg/ml of penicillin, 50 μg/ml of streptomycin, 5% FBS and 0, 5 or 10 ng/ml of SS (Abedelahi et al., 2008). Media without SS were considered the control.

In vitro ovulation induction
On Day 12 of culture, final oocyte maturation and ovulation were induced by the addition of 1.5 IU/ml human chorionic gonadotrophin (hCG; Organon, Grienkenweg, The Netherlands) to the media. Released oocytes were scored as germinal vesicle (GV), germinal vesicle breakdown (GVBD) when the GV was absent, and as metaphase II (MII) when the first polar body was extruded. The proportions of GV, GVBD and MII were assessed 48 h after hCG addition in all groups (Haidari et al., 2008).

In vitro fertilization and embryo culture
Spermatozoa were extracted from the cauda epididymis of 7–8-week-old male NMRI mice and capacitated for 1.5 h in T6 medium supplemented with 5 mg/ml of BSA.

The collected MII oocytes from all experimental and control groups were transferred (n = 264) to T6 medium containing capacitated spermatozoa, and after insemination, they were cultured for 120 h (Haidari et al., 2008).

Blastocyst staining
The embryos at an expanded stage were stained by incubation in a 250 μl droplet of T6 containing 0.1% toluidine blue for 60 s. The stained blastocysts were mounted on glass microscope slides, and cell counting was performed under a light microscope in two or three planes.
Detection of ROS production in cultured follicles using a spectrofluorometer

To measure ROS levels in vitrified and non-vitrified cultured follicles, 10 follicles were pooled from different times of study (0, 12, 24, 48, 72, 96 h; n = 60 ÷ 5) at least for five repeats, then washed three times with phosphate buffer saline (PBS) and incubated in 40 mmol/l of Tris–HCl buffer (pH = 7.0) containing 5 μmol/l of 2²,7²-dichlorodihydrofluorescein diacetate (DCHFDA; Merck) at 37°C for 30 min. Then, the medium was removed and follicles were washed three times with PBS and immediately homogenized in 100 μl of Tris–HCl buffer (40 mmol/l, pH 7.0) and sonicated at 50 W for 1 min, centrifuged at 10 000 g for 20 min at 4°C, and supernatants were collected. Fluorescence was monitored in the supernatant using a spectrofluorometer at 488-nm excitation and at 525-nm emission (LeBel et al., 1992).

Data were expressed as μM H2O2 and the mean dichlorofluorescein (DCF) fluorescence intensity (means ± SEM). The analysis for each sample was duplicated.

Determination of ROS intensity in cultured follicles using a laser scanning confocal microscope

The intensity of ROS in intact cultured follicles was assessed on the basis of the method of Tsai-Turton and Luderer (2006).

In each study group at different times of culture, at least five follicles were incubated within 100 μmol/l of DCHFDA (Merck) in TCM 199 for 30 min, then washed again with PBS. Also, follicles treated with 5% H2O2 (for 30 min) were considered a positive control group for oxidative stress.

The DCF fluorescence intensity was viewed using a Zeiss LSM 510 META laser scanning confocal microscope and at 488 nm for excitation and at 525 nm emission (LeBel et al., 1992).

TAC assay

In each study group, 10 follicles corresponding to different times of culture (0, 12, 24, 48, 72, 96 h) were pooled and homogenized in 100 μl Tris–HCl buffer (40 mmol/l, pH 7.0) and sonicated at 50 W for 1 min and then centrifuged at 10 000 g for 20 min at 4°C, and supernatants were prepared. These supernatants were used for TAC, GPx and total protein analysis. All experiments were repeated at least five times.

The ferric reducing/antioxidant power (FRAP) assay was used for the assessment of TAC in cultured pre-antral follicles. This assay was performed using tripyridyltriazine (Merck) reagent as described before by Benzie and Strain (1996). For the assay, 50 μl of supernatant was added to 1 ml of freshly prepared FRAP reagent in a semi-micro plastic cuvette and incubated at 37°C for 10 min. The absorbance of the blue-colored complex was read against a reagent blank at 593 nm with readings every 20 s for 10 min. Standard solutions of Fe²⁺ in the range of 100–1000 μM were prepared from ferrous sulfate in distilled water.

Total protein measurement

The protein content of the supernatant from cultured pre-antral follicles was estimated by the Bradford method (Bradford, 1976). BSA was used as a standard.

GPx activity

Supernatants were prepared as described earlier at different intervals of the culture period, and the GPx activity of cultured follicles was determined spectrophotometrically on the basis of the method of Paglia and Valentine (1967) by coupling the oxidation of glutathione and nicotine adenine dinucleotide phosphate (NADPH) using glutathione reductase.

The reaction mixture contained 50 mmol/l Tris–HCl (pH 7.0), 1 mmol/l EDTA, 1 mmol/l sodium azide, 1 IU/ml of purified glutathione reductase, 1 mmol/l GSH, 0.2 mmol/l NADPH and 1.2 mmol/l cumene hydroperoxide (for Se-independent GPx activity) or 0.25 mmol/l H2O2 (for Se-dependent GPx (Se-GPx) activity) as substrate.

One unit of enzyme activity was defined as 1 μmol of NADPH oxidized/min/mg protein or units/mg protein. The analysis for each sample was duplicated.

Statistical analysis

The results are expressed as the mean ± SD. Statistical analysis was performed using the statistical package for social sciences (SPSS-15). One-way analysis of variance was used to compare the mean values of different treatment groups after various times in culture and was followed by a multiple comparison post hoc Tukey LSD test. Independent and paired t-tests were used to compare the values of different groups.

Results

In vitro follicular maturation

Percentages of MI oocytes derived from cultured pre-antral follicles in non-vitrified samples in the absence and presence of SS is summarized in Table I. These rates were significantly different, both in vitrified and non-vitrified samples, in SS-free conditions compared with SS-supplemented groups (P < 0.001). Also the rates of antrum formation (Table I) were significantly lower in SS-free conditions than in SS-supplemented groups in both vitrified and non-vitrified samples (P < 0.05).

Fertilization rate and embryo development

Fertilization and developmental rates of inseminated oocytes derived from cultured isolated follicles in non-vitrified and vitrified groups in the presence of 0, 5 and 10 ng/ml of SS are shown in Table II.

There were significant differences in the fertilization rate and blastocyst formation rate in between the selenium-free group and selenium-supplemented groups (P < 0.005) both in vitrified and non-vitrified samples; however, there were no significant differences between vitrified and non-vitrified samples.

The mean cell number per blastocyst derived from vitrified samples were not significantly different compared with that in the respective non-vitrified groups, but the mean cell number per blastocyst was significantly different in the group without SS compared with groups treated with 5 or 10 ng/ml of SS (P < 0.005).

ROS production in cultured pre-antral follicles observed by spectrofluorometry

ROS levels in follicles derived from non-vitrified and vitrified samples after 0, 12, 24, 48, 72 and 96 h culture in the presence and absence of SS are shown in Fig. 1. Maximum ROS levels in the absence of SS in the control group (non-vitrified) were observed at 72 and 96 h of culture, whereas maximum ROS levels in the presence of 5 and 10 ng/ml of SS occurred at 24 h, and 12 and 24 h of culture, respectively (P < 0.05). ROS levels following treatment with 10 ng/ml of SS after 96 h of culture completely returned to the baseline and
there were no significant differences between the ROS levels at the end and the beginning of the culture ($P < 0.05$).

ROS levels were also increased in the absence of SS during the culture period up to 96 h in follicles derived from vitrified samples ($P < 0.001$), and maximum levels of ROS in the presence of 5 and 10 ng/ml SS occurred at 24 and 48 h of culture in vitrified samples ($P < 0.05$).

There were significant differences between ROS levels in vitrified and non-vitrified samples at all concentrations of 5 or 10 ng/ml SS and at all durations of culture ($P < 0.005$), except at 96 h when ROS levels were similar in vitrified and non-vitrified groups.

Confocal microscopic observation of ROS

Follicles in both groups of the study at the beginning of the culture were considered negative for a DCF fluorescent reaction, as seen in Fig. 2A and E.

All follicles which were cultured in selenium-free medium showed a high intensity of ROS up to 96 h of culture in non-vitrified (Fig. 2A–D) and vitrified samples (Fig. 2E–H). Images of pre-antral follicles from non-vitrified (Fig. 2I–P) and vitrified (Fig. 2Q–X) ovaries cultured in the presence of 5 and 10 ng/ml of SS were almost similar and showed that the DCF fluorescent intensity was still high in both groups at 12–48 h, whereas it was low after 96 h of culture. The fluorescence was localized in the outer granulosa and theca cells in all treatment groups.

TAC in cultured pre-antral follicles

Figure 3 shows TAC levels in cultured follicles derived from both vitrified and non-vitrified ovaries after 0, 12, 24, 48, 72 and 96 h of culture in the presence or absence of SS. TAC levels were decreased in the absence of SS in pre-antral follicles derived from vitrified samples during the culture period up to 96 h ($P < 0.001$). Maximum TAC levels in the presence of 5 or 10 ng/ml of SS in the non-vitrified group occurred at the beginning of culture and 96 h later ($P < 0.05$). TAC levels in pre-antral follicles treated with 10 ng/ml of SS after 96 h of culture completely increased to the level at the beginning of culture and there were no significant differences between the end and beginning of the culture ($P < 0.05$). Maximum TAC levels in vitrified samples in the absence or the presence of 5 ng/ml of SS occurred at the beginning of the culture, and the TAC level was significantly decreased up to 96 h of culture ($P < 0.05$) in both groups. However, in the vitrified group treated with 10 ng/ml of SS, TAC levels were significantly decreased from 12–72 h of culture, then at 96 h of culture, the TAC level returned to the level at the beginning of culture ($P < 0.05$).

There were no significant differences between TAC levels in vitrified and non-vitrified samples in the absence of SS; however, it was

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**Table I** Developmental rates of cultured follicles isolated from vitrified and non-vitrified ovaries in the presence of different concentrations of sodium selenite (SS).

<table>
<thead>
<tr>
<th>Group</th>
<th>SS (ng/ml)</th>
<th>Number of survived (%)</th>
<th>Number of degenerated (%)</th>
<th>Number of MII (%)</th>
<th>Antrum formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>104/140 (74.28)*</td>
<td>36/140 (25.71)*</td>
<td>12/104 (11.55)*</td>
<td>20/104 (19.23)*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>85/108 (78.70)</td>
<td>23/108 (21.30)</td>
<td>24/85 (28.23)</td>
<td>23/85 (27.05)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>137/152 (90.13)</td>
<td>15/152 (9.87)</td>
<td>43/137 (31.38)</td>
<td>40/137 (29.19)</td>
</tr>
<tr>
<td>Vitrified</td>
<td>0</td>
<td>73/105 (69.52)*</td>
<td>32/105 (30.47)*</td>
<td>8/73 (10.95)*</td>
<td>14/73 (19.17)*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>92/122 (75.40)</td>
<td>30/122 (24.59)</td>
<td>16/92 (17.39)*</td>
<td>24/92 (26.08)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>122/138 (88.40)</td>
<td>16/138 (11.59)</td>
<td>37/122 (30.32)</td>
<td>32/122 (26.62)</td>
</tr>
</tbody>
</table>

Five experimental replicates were performed for each group.

*Significant differences compared with concentrations of SS within the same group ($P < 0.001$; $^*P < 0.05$).

*Significant differences compared with treatments with 10 ng/ml SS in the vitrified group ($P < 0.001$).

**Table II** Fertilization and developmental rates of oocytes derived from cultured follicles in vitrified and non-vitrified ovaries in the presence of different concentrations of sodium selenite (SS).

<table>
<thead>
<tr>
<th>Group</th>
<th>SS (ng/ml)</th>
<th>Number of fertilized oocytes (%)</th>
<th>Number of two cell (%)</th>
<th>Number of morulas (%)</th>
<th>Number of blastocysts (%)</th>
<th>Number of hatched (%)</th>
<th>Number of blastocysts (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>21/42 (50.4)*</td>
<td>8/21 (38.09)*</td>
<td>2/21 (9.52)*</td>
<td>1/21 (4.76)*</td>
<td>0</td>
<td>59 ± 3.56*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>31/41 (75.60)</td>
<td>22/31 (70.96)</td>
<td>13/31 (41.93)</td>
<td>9/31 (29.03)</td>
<td>7/31 (22.58)</td>
<td>86 ± 3.74</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>39/49 (79.59)</td>
<td>31/39 (78.48)</td>
<td>16/39 (41.02)</td>
<td>14/39 (35.89)</td>
<td>10/39 (25.64)</td>
<td>90 ± 4.32</td>
</tr>
<tr>
<td>Vitrified</td>
<td>0</td>
<td>17/47 (36.17)*</td>
<td>5/17 (29.41)*</td>
<td>1/17 (5.88)*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29/44 (65.90)</td>
<td>18/29 (62.06)</td>
<td>10/29 (34.48)</td>
<td>8/29 (27.85)</td>
<td>6/29 (20.68)</td>
<td>85.25 ± 4.99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37/41 (90.24)</td>
<td>28/37 (75.67)</td>
<td>14/37 (37.83)</td>
<td>12/37 (32.43)</td>
<td>9/37 (24.32)</td>
<td>88.50 ± 5.06</td>
</tr>
</tbody>
</table>

There is no significant difference between vitrified and non-vitrified groups.

Five experimental replicates were performed for each group.

*Significant differences compared with concentrations of SS within the same group ($P < 0.005$).
**Se-GPx activities**

In order to determine the contribution of SS to GPx activity of cultured follicles, Se-GPx and Se-independent GPx-specific activities were measured in follicles in all groups of the study.

Specific activities of Se-GPx in cultured pre-antral follicles are demonstrated in Fig. 4. The activity of Se-GPx in the non-vitrified group in the absence of SS was significantly decreased during the culture period right up to 96 h ($P < 0.001$); however, the activity of Se-GPx in SS-treated groups at 96 h of culture reached the same levels as at the beginning of the culture. In vitrified samples, the activity of Se-GPx was significantly decreased up to 96 h of culture ($P < 0.05$), but in the group supplemented with 10 ng/ml of SS at 96 h, this activity was significantly increased to the same levels as at the beginning of the culture ($P < 0.05$).

The activity of Se-GPx in vitrified groups in comparison with non-vitrified samples was significantly lower in vitrified samples at 72 and 96 h of culture in the presence of 5 ng/ml of SS and at 48 h of culture in the presence of 10 ng/ml of SS ($P < 0.05$). TAC levels were similar in vitrified and non-vitrified groups after treatment with 10 ng/ml of SS after 96 h of culture.

**Selenium-independent GPx activities**

Selenium-independent GPx-specific activities in cultured follicles derived from vitrified and non-vitrified ovaries are shown in Fig. 5A and B. There were no significant differences in the Se-independent GPx specific activities of cultured follicles in vitrified and non-vitrified samples during the culture period. The mean Se-GPx activity in the cultured pre-antral follicles was 2-fold higher than that of the Se-independent GPx activity.

**Discussion**

Our observations showed that there were not any differences in the rate of follicular maturation and embryo development and blastocyst cell number between vitrified and non-vitrified samples. These data confirm that ovarian vitrification exerts no harmful effects on the subsequent development of follicles, as shown previously (Haidari *et al*., 2008).

Effects of SS on the maturation and development of isolated follicles in fresh samples have been shown before (Abedelahi *et al*., 2008), but this study is the first report to evaluate effects of SS on IVM of follicles derived from vitrified samples.

Effects of SS on improving the IVM of follicles may be due to several pathways. One suggestion is that SS is a proliferating factor and it not only enhances the growth of oocytes, but also increases cell proliferation in theca and granulosa cells.

In agreement with this suggestion, the *in vitro* studies of Basini and Tamanini (2000) showed that SS stimulated proliferation of bovine granulosa cells and had some stimulatory effects on estradiol secretion. Uhm *et al.* (2007) showed that the addition of SS to the culture media increased the blastocyst rate and cell number, decreased the apoptotic index and increased the expression of GPx in porcine embryos. Several investigations have also shown that selenium can either stimulate or inhibit cell growth, and these effects depend on its concentration, forms of the selenium and the cell types (Menter *et al.*, 2000; Zeng, 2002; Asfour *et al.*, 2009).

The other explanation for effects of SS in improving IVM conditions is related to antioxidant actions of SS in reducing the ROS concentration.

In agreement with this and our previous results, Ebert *et al.* (2006) demonstrated that selenite supplementation of bone marrow stromal cells culture media was effective in restoring the antioxidative capacity and in reducing cell damage.

Selenium plays on intracellular signaling including protein kinase C, nuclear factor-kappa B and inhibitor of apoptosis protein (Gopalkrishna *et al.*, 1997). The selenium function is dose-dependent and in other reports has shown that selenium supplementation actually prevents the generation of cancer (Cho *et al.*, 1999; Kim *et al.*, 2001).

There is some evidence that SS contributes to apoptosis and G2/M cell cycle arrest in cancer cells by MAPK and caspase pathways (Rayman, 2005; Ranawat and Bansal, 2009; Zhao *et al.*, 2009). It has also been suggested that selenite inhibits the AP-1 factor thus preventing cell growth (Syprou *et al.*, 1995).

Our data showed for the first time that ROS levels are increased in cultured follicles derived from vitrified and non-vitrified ovaries up to 96 h, but in the presence of SS, this level was decreased and returned...
to the baseline level. The antioxidant capacity was decreased in cultured follicles after 24 h but increased in the presence of SS and returned to the baseline level after 96 h of culture.

Many of the biological actions of selenium have been attributed to powerful antioxidant properties, including direct quenching of ROS, chelation of metal ions and regeneration of Se-GPx antioxidants.

This observation is in agreement with Saito et al. (2003), who showed that the removal of selenium from the culture medium induces ROS production and cell death. Yeo and Kang (2007) investigated the prevention of cell death by selenium using cultured brain-derived neural progenitor cells (NPCs) and they showed that selenite inhibits H$_2$O$_2$-induced apoptosis of NPCs and its in vivo function was...
ROS production in vitrified cultured follicles

Figure 3 Total antioxidant capacity (TAC) levels in pre-antral follicles derived from non-vitrified control ovaries (A) and vitrified samples (B) during 96 h of culture. TAC levels in follicles at different times of culture within the same group in the absence of SS (a) (P < 0.001) or in the presence of 5 ng/ml SS (b) (P < 0.001) or 10 ng/ml of SS (c) (P < 0.05).

Figure 4 Selenium-dependant glutathione peroxidase (Se-GPx) activity in pre-antral follicles derived from non-vitrified control ovaries (A) and vitrified samples (B) during 96 h of culture. The maximum GPx activity of cultured follicles in the absence of SS (a) (P < 0.001) or in the presence of 5 ng/ml SS (b) or 10 ng/ml of SS (c) (P < 0.05) at different culture times within the same group.

Figure 5 Selenium-independent GPx activity in pre-antral follicles derived from non-vitrified control ovaries (A) and vitrified samples (B) during 96 h of culture. There were no significant differences between the groups.

associated with the inhibition of H2O2-induced ROS production. Confocal microscopic studies were used in our experiments only to support our biochemical findings and determine the localization of ROS in cultured follicles. Our observations showed that ROS mainly localized in granulosa and theca cells and there were no signs of ROS production in oocytes.

GPx has been used as an indicator of Se function in cultured pre-antral follicles. Selenium forms the catalytic center of antioxidant enzymes, such as cytosolic GPx or membrane-bound GPx (Patrick, 2004). Low levels of Se-GPx activity in pre-antral follicles when cultured in selenium-free media and the high concentration of ROS may be the major factors responsible for follicular regression in the current study. The decrease in ROS production during IVM of follicles in the presence of SS compared with the control demonstrated that the Se-GPx acts as an enzymatic ROS scavenger in follicular cells.

In agreement with our data, Saito et al. (2003) showed that selenium deficiency decreases activities of cytosolic GPx and phospholipid-hydroperoxide-GPx, increases lipid peroxidation in the membranes and eventually induces cell death, whereas the addition of selenite to cultured cells causes an increase in Se-GPx activities (Sandström and Marklund, 1990; Kayanoki et al., 1996). Our explanation for differences in follicular ROS and TAC levels in vitrified samples compared with the control demonstrated that the Se-GPx acts as an enzymatic ROS scavenger in follicular cells.

In agreement with our data, Saito et al. (2003) showed that selenium deficiency decreases activities of cytosolic GPx and phospholipid-hydroperoxide-GPx, increases lipid peroxidation in the membranes and eventually induces cell death, whereas the addition of selenite to cultured cells causes an increase in Se-GPx activities (Sandström and Marklund, 1990; Kayanoki et al., 1996). Our explanation for differences in follicular ROS and TAC levels in vitrified samples compared with the control demonstrated that the Se-GPx acts as an enzymatic ROS scavenger in follicular cells.

We conclude that oxidative stress plays an important role in the cryoinjury damage and SS may be effective in recovery from the cryoinjures; however, more study with complementary techniques is required.

Cryopreservation causes a change in the cell membrane fluidity and induces mitochondrial malfunction (Ahn et al., 2002; Salehnia et al.,...
in cryopreservation induces an excess of electron production and ATP deprivation in mitochondria, leading to ROS formation (Ahn et al., 2002).

In agreement with our suggestion, Rahimi et al. (2003) showed that an increase in ROS levels just after vitrification and warming could reduce tissue quality. However, our observations in this study and our previous study have shown that this change in vitrified samples is reversible (Mazoochi et al., 2008; Mazoochi et al., 2009).

In conclusion, our results showed that culture of pre-antral follicles in the presence of SS increases follicular TAC levels and Se-GPx activities and decreases ROS levels, thereby improving the developmental rate of mouse follicles in vitro. These results introduce a potential improvement in the method to preserve the fertility potential of infertile women by IVM of isolated follicles with subsequent vitrification of ovarian tissue.

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Authors’ Roles
All authors contributed to the study design and to drafting the paper. M.S. led the study together with A.M.A. and A.A.A. D.D. provided excellent technical assistance in confocal scanning microscopy.

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