Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

H_2O_2-induced mild stress in relation with in vitro ovine oocyte developmental competence: implications for blastocyst apoptosis and related genes expression

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Received March 4, 2016; **Accepted** May 4, 2017; **Published** May 20, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.5.9

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Abstract: In this study, *in vitro* maturation was performed in presence of various concentrations (0, 10, 100, or 1000 µM) of H2O2. The intracellular glutathione (GSH) level, fertilization, cleavage, and blastocyst rates, total cell number, and apoptotic cell number and expression of *Bax*, *Bcl-2*, and *p53* genes in blastocyst-stage embryos were studied. At 10 µM H2O2 concentration, a higher GSH level was detected in comparison to the other groups while oocytes exposed to 1000 µM H2O2 had the lowest GSH level. Treatment of oocytes with 1000 µM H2O2 decreased the rate of two pronuclei formation as compared with other groups. A higher rate of blastocyst formation was seen in 100 µM H2O2 group as compared with the control group. However, exogenous H2O2 in maturation medium did not affect total cell numbers and apoptotic cell ratio at the blastocyst stage. Moreover, mRNA transcript abundance of *Bax*, *Bcl-2*, and *p53* genes was similar between blastocysts derived from H2O2-induced oocytes and control blastocysts. Treatment of oocytes with H2O2 at mild level during *in vitro* maturation had a positive effect on GSH level and this, in turn, may lead to improvement in preimplantation embryonic development.

Key words: Blastocyst; GSH level; Hydrogen peroxide; Oocyte; Apoptosis.

Introduction

Numerous observations suggest that *in vivo* culture conditions are more stable than their *in vitro* counterparts (1-8). The establishment of an optimal culture condition for pre-embryo development is intrinsically interesting and an area that has seen significant research attention over the past decade (9-13). In human assisted conception treatment, the *in vitro* culture condition is different from *in vivo* environments in that a higher concentration oxygen is available, and in this circumstance the oocytes/embryos show an excess generation of reactive oxygen species (ROS) in simple culture medium (14). An accepted concept among a multitude of others that may be responsible for altered cell functions *ex vivo* is the formation of oxidative stress within the oocyte/embryo culture medium (15, 16). Oxidative stress is the result of unregulated formation of ROS, such as nitric oxide, superoxide, hydrogen peroxide, and the highly reactive hydroxyl radicals (17).

Elevated ROS production has been shown to cause meiotic arrest and increase in degenerated oocytes, and oxidative stress induces apoptosis in zygotes (18). However, a certain concentration of ROS is required in many reproductive organs (19), for instance in the blastocyst, presence of adequate amount of ROS has been reported to be critical regulation of the balance between inner cell mass and trophectoderm (20).

Scavenger enzymes (superoxide dismutase, catalase, glutathione peroxidase) and lipid- and water-soluble antioxidant compound (ascorbic acid, glutathione, albumin, transferrin, etc.) in numerous studies have been regarded as cell effective defense against ROS (21-25). Among the known ROS, hydrogen peroxide (H2O2) has long half-life and membrane permeability properties and is most desirable to act as a signal either intra- or intercellularly. (26). H2O2 has been implicated in many stress conditions to investigate the effect of ROS on the molecular mechanisms underlying cellular plasticity (27-29). In this regard, recent prospective data demonstrate that H2O2 has profound and wide- ranging physiological effects. For instance, it has been noted that zygotes and blastocysts are less resistant to exogenous H2O2 than 9- to 16-cell embryos in bovine (30). Rat immature oocytes undergoing maturation in the presence of exogenous H2O2 have been shown to induce germinal vesicle breakdown (27, 31). However in that study, before morphological changes characteristic of oocyte apoptosis can be recognized, the first polar body extrusions were inhibited in mature oocytes (27).

These information suggest that stress at the mild level can be ultimately protective and may have functional implications in physiological responses (32). Activation of cell metabolism and enhancement of the physiological activity has been detected in organisms following exposure to a mild stress, and such stress treatment did

not cause any damage effects even at a longer duration of the stress factors (20).

It has been shown that exposure of oocytes to H2O2 in a short-term from 23 to 24 h of maturation period had no effect on fertilization, although blastocyst formation was improved (20). Moreover, the incidence of the apoptosis of 7 days old blastocyst was increased at the low level of H2O2 concentration, while incubation of oocytes with a high level of H2O2 fail to show any evidence of apoptosis in blastocysts at day 7 of development. To put this information into perspective, we evaluated the impact of different concentrations of H2O2 on oocyte intracellular glutathione (GSH) level, subsequent preimplantation embryo development, and expression of apoptosis-related markers in blastocysts.

Materials and Methods

Media and reagents

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA).

Collection of ovaries

Ovaries were obtained from ewes at an abattoir and transported to the laboratory within 2–3 h after harvest in thermos flask containing 0.9 % saline solution at a temperature of between 32 and 37 °C.

Oocyte collection

Cumulus-oocyte complexes (COCs) were isolated from antral ovarian follicles by the aspiration method. Oocytes with compact and thick cumulus and homogenous cytoplasm were randomly selected and employed randomly for the present study (33).

In vitro maturation

The COCs were rinsed three times in maturation medium consisting of bicarbonate-buffered TCM-199 with 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS), 5.5 mg/ml sodium pyruvate, 25 µg/ml gentamycin sulphate, 0.5 µg/ml FSH, 5.0 µg/ml LH and 1 µg/ml Estradiol. In vitro maturation (IVM) was performed by culturing 10 COCs for 24 h in 50 µl medium droplet covered with mineral oil at 39°C, 5% CO2 in air with maximum humidity (34).

Evaluation of intracellular GSH level

Intracellular GSH level was analyzed according to the methods of previous studies (35, 36). In brief, after maturation *in vitro*, the COCs were freed from their cumulus cells and incubated for 30 min in tyrodes medium plus 5 mg/ml poly vinyl alcohol containing 10 µM Cell Tracker blue. The oocytes were then rinsed in modified PBS (mPBS), transferred into 10 µL droplets, examined and imaged under an epifluorescence microscope (Nikon, Tokyo, Japan) with UV filters. All fluorescent images were captured and recorded as graphic files (Figure 1). The oocytes were evaluated for their fluorescence intensities using ImageJ software (http://rsb.info.nih.gov/ij).

In vitro fertilization

Following maturation *in vitro*, a number of COCs were rinsed three times in HEPES-buffered synthetic oviductal fluid (HSOF) and 10 COCs were cultured in 44 µl drops covered with equilibrated mineral oil. The medium used during fertilization was SOF supplemented with 2% (v/v) estrous sheep serum (OSS), 4 IU/ml heparin, and PHE (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine). Straws with frozen sperm (Nahadehaye Dami Jahed, Tehran, Iran) were thawed and used for fertilization *in vitro*. To separate the viable and motile sperm for *in vitro* fertilization, a swim-up method was applied and sperm with high motility were added to COCs at a final concentration of 2×10^6 spermatozoa/ml in fertilization medium. Sperm and oocytes were coincubated under mineral oil at 39°C, 5% CO2, 5% O2, and 90% N2 in air with maximum humidity for approximately 18-20 h (37).

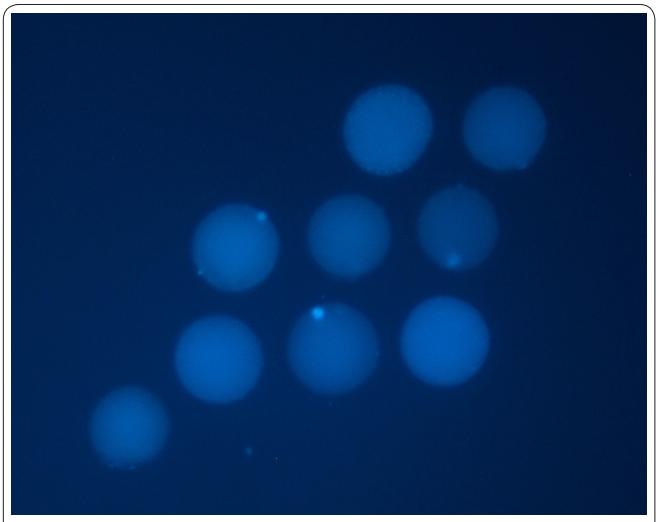

Figure 1. Images of oocytes shown for the intracellular glutathione (GSH) content after in vitro maturation (IVM) based on fluorescence intensity (pixel/oocyte).

Evaluation of pronuclei formation

To evaluate the rate of fertilization and observation of the presence of two pronuclei, denuded zygotes were fixed at 20–22 hpi in 2% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde and subjected to Hoechst 33258 staining (20).

In vitro culture

After 18-20 h, presumed zygotes were pipetted through flame-polished Pasteur pipettes to remove excess sperm and cumulus cells. Ten cumulus-free zygotes were rinsed three times in Charles Rosencrans (CR1aa) medium supplemented with 12% heat inactivated OSS and then transferred into the 50 µL of the same medium under mineral oil in 5% CO2 in humidified air atmosphere at 38.7°C (33).

Apoptosis assay with TUNEL

Apoptosis was detected in blastocysts-stage embryos using terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) method. The In Situ Cell Death Detection Kit (TMR red; Roche, Mannheim, Germany) was used for this purpose. Blastocysts were rinsed three times in PBS containing 10% FBS and then fixed in 4% paraformaldehyde for 2 h at room temperature. Cell membranes permeabilization was carried out by treating the blastocysts with 0.1% Triton X-100 in

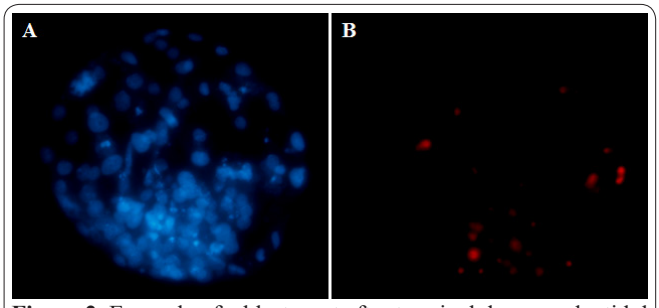

Figure 2. Example of a blastocyst after terminal deoxynucleotidyl transferasemediated dUTP nick end labelling (TUNEL) assay labelling followed by 4_,6-diamidino-2-phenylindole (DAPI) staining. Blastocyst was stained with DAPI (A) combined with TUNEL labelling (B) for counting total cell number and apoptotic cells, respectively.

0.1% citrate solution for 1 h at room temperature. Fixed blastocysts were then incubated in TUNEL reaction medium for 1 h in the dark at 38.5°C. After stopping the reaction, the blastocysts were rinsed and the cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 1 mg/ml) for 10 min at room temperature. The blastocysts were then mounted onto a slide under a coverslip and the total cell numbers (Figure 2A) and apoptotic cells number (Figure 2B) were analyzed using a fluorescence microscope (37).

RNA extraction, reverse transcription, and real-time PCR

Total blastocyst RNA was extracted using TRI-reagent. Then, synthesis of cDNA was performed using M-MuLV reverse transcriptase and random hexamer primers, as described in manufacturer's instructions (Fermentas; St. Leon-Rot, Germany). PCR amplification was applied by standard method with Taq DNA polymerase with denaturation at 94°C for 15 s, annealing at 60–61°C for 30 s depending on the melting temperature of each primer, and extending for 45 s at 72°C. The cycle's number of PCR amplification differed between 30 and 40, depending on the amount of a particular mRNA. A list of PCR primer sequences and product lengths are listed in Table 1.

Real-time PCR reactions were performed in a 25 µl reaction volume with a Rotor Gene 6000 (Corbett Life Science, Sydney, Australia), by adding 0.4 µM of final concentration for each primer, 2 µl template, 12.5 µl 2× SYBR Premix Ex Taq, and distilled water make up a final volume of 25 µl. Real-time PCR was carried out in two steps with the following thermal cycling parameters: at 95°C for 3 min for initial enzyme activation, followed by 40 amplification cycles (each 5 s at 95°C, and 20 s at 60°C with fluorescence detection) and a final step of melting curve analysis. Reactions were performedin duplicate, and the average value of the duplicate was used for quantification. Differences in relative gene expression between groups we quantified using the $2^{-\Delta\Delta Ct}$ method. Expression of *YWHAZ* was used as an internal reference gene (37).

Experimental design

GSH level of oocytes after exposure to H2O2-induced mild stress

H2O2 was added to the pyruvate-free maturation medium after 23 h of maturation, for 1 h. It is well known that pyruvate is able to to neutralize the effect of peroxide. The concentrations of 0 (Control Negative; C-), 10 (H2O2-10), 100 (H2O2-100), and 1000 (H2O2-1000) µM H2O2 have been chosen for induction of mild stress in this study. A group of oocytes also cultured in maturation medium without exposure to the H2O2 in the presence of pyruvate for 24 h (Control Positive; C+).

To determine the effect of exogenous H2O2 in maturation medium on oocyte intracellular level of GSH after IVM, cumulus cells were removed soon after maturation *in vitro* and intracellular GSH levels were analyzed for the C+, C-, H2O2-10, H2O2-100, and H2O2-1000 groups.

Fertilization rate and developmental competence of oocytes after exposure to H2O2-induced mild stress

After maturation, COCs from C+, C-, H2O2-10, H2O2-100, and H2O2-1000 groups were inseminated and the rate of fertilization was evaluated in a cohort of presumable zygotes of each group after denuding, fixing and staining by observation of the presence of two pronuclei.

Rates of cleavage and blastocyst development of fertilized oocytes were recorded for the groups at days 3 and 8 post-fertilization, respectively. The blastocysts derived from H2O2-100 treated (n = 20) and Control (n = 19) oocytes were stained with TUNEL and DAPI. The total cell number and apoptotic cells number were then observed under a fluorescence microscope for each group and compared.

Exposure of oocyte to H2O2-induced mild stress in relation to the expression of apoptosis-related genes in blastocysts

Blastocysts derived either from control, or H2O2-100 oocytes were evaluated for the expression of pro-apoptotic (*Bax*, and *p53*) and antiapoptotic (*Bcl-2*)

Table 1. Details of primers used for real time PCR quantitative analysis.

Gene name	GenBank accession number	Primer Sequences	Annealing temperature (°C)	Product size (bp)
Bax	NM_173894.1	F:5′-GCATCCACCAAGAAGCTGAG-3′ R:5′-CCGCCACTCGGAAAAAGAC-3′	61	130
Bcl-2	NM_001166486.1	F:5′-ATGTGTGTGGAGAGCGTCA-3′ R:5′-AGAGACAGCCAGGAGAAATC-3′	60	182
p53	NM_174201.2	F:5′-AGGGGAAAGCAGGGCTCACTCT-3′ R:5′-GGGATATGGGTGGGGATGTCAA-3′	60	151
YWHAZ	NM_174814.2	F:5′-GAAGAGTCCTACAAAGACAGCACGC-3′ R:5′-AATTTTCCCCTCCTTCTCCTGC-3′	60	115

F, forward; PCR, polymerase chain reaction; R, reverse.

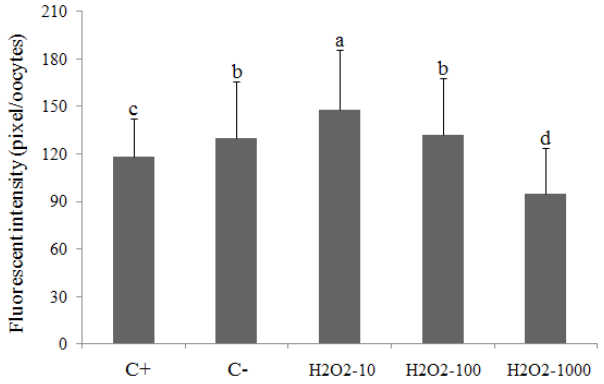

Figure 3. Effect of exogenous H2O2 on intracellular glutathione (GSH) content [Mean (± SD)] of sheep oocyte after *in vitro* maturation. [a,b,c,] [d]Different letters within the bars indicate a significant difference (P<0.05).

genes. Three biological replicates, each containing three blastocysts were used for extraction of RNA, reverse transcription-PCR, and real-time PCR.

Statistical analyses

Comparisons between means values were carried out using ANOVA followed by Duncan's multiple range test. Gene expression data were assessed using General Linear Model of the Statistical Analysis System software package version 8.0 (SAS Institute Inc., NC, USA). A value of P<0.05 were considered to be significant.

Results

GSH level of oocytes after exposure to H2O2-induced mild stress

The highest (P<0.05) GSH level was found after treatment of oocytes with 10 µM H2O2 while treatment of oocytes with 1000 µM H2O2 resulted in lowest (P<0.05) level of GSH when compared with other groups (Figure 3). We detected a lower level of GSH in C+ group than in C- and H2O2-100 groups.

Fertilization rate and developmental competence of oocytes after exposure to H2O2-induced mild stress

Fertilization rate were not changes in groups containing different concentrations of H2O2, except for the highest H2O2 concentration (Figure 4). Fertilization rate of H2O2-1000 group was significantly (P<0.05) lower when compared with other stressed groups as well as control groups.

The cleavage rate was not altered (P>0.05) when H2O2 was added into the maturation medium, except at the concentration of 1000 µM that resulted in a lowest cleavage rate (P<0.05) in comparison to the other groups. In addition, our data showed that oocytes from H2O2-100 group produced more (P<0.05) blastocyst in comparison with C+ and C- groups (Table 2).

Neither total cell number of yielded blastocysts nor the apoptotic cells ratio was influenced (P>0.05) by exogenous H2O2 in maturation medium (Table 3).

Exposure of oocyte to H2O2-induced mild stress in relation to the expression of apoptosis-related genes in blastocysts

Results for relative expression of *Bax*, *Bcl-2*, and, *p53* genes in blastocysts are presented in Figure 5. The expression of apoptosis-related (*Bax*, *Bcl-2*, and, *p53)* genes was not affected by treatment of the oocytes with

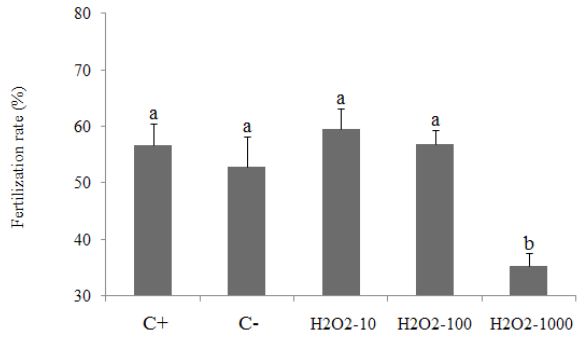

Figure 4. Effect of H2O2 treatment during in vitro maturation on oocyte fertilization rate in ovine. [a,b]Different letters within the bars indicate a significant difference (P<0.05).

Table 2. Effect of different H2O2 concentrations on embryonic developmental potential of sheep oocytes after IVF (cumulative results of four replicates).

Groups	No. Oocytes	Embryo development	
		Cleaved n (Mean ± SEM)	Blastocyst n (Mean ± SEM)
C+	118	63 (53.4 ± 1.8)[a]	29 (24.5 ± 2.5)[b]
C-	100	50 (50.1 ± 2.5)[a]	22 (22.2 ± 3.1)[b]
H2O2-10	118	62 (52.5 ± 5.3)[a]	31 (26.4 ± 1.5)[ab]
H2O2-100	137	77 (56.2 ± 3.3)[a]	44 (32.2 ± 1.8)[a]
H2O2-1000	109	33 (29.8 ± 2.4)[b]	6 (4.8 ± 1.9)[c]

C+: Control medium, C-: Control medium without sodium pyruvate, H2O2-10, H2O2-100 and H2O2-1000: Control medium without sodium pyruvate supplemented with 10, 100 and 1000 µM H2O2, respectively.
[a,b,c]Different superscripts within the columns indicate a significant difference (P < 0.05).

Table 3. Mean (± SD) total cell number and apoptotic cell number in blastocysts derived from H2O2-treated and control oocytes.

Groups	No. Blastocysts	Embryo Quality	
		Blastocyst Nuclei	Apoptotic cells
Control	19	107.3 ± 9.2	7.3 ± 2.8
H2O2-100	20	103.1 ± 10.4	9.1 ± 4.3

Control: Control medium, H2O2-100: Control medium without sodium pyruvate and supplemented with 100 µM H2O2.
No differences (P>0.05) were detected among groups.

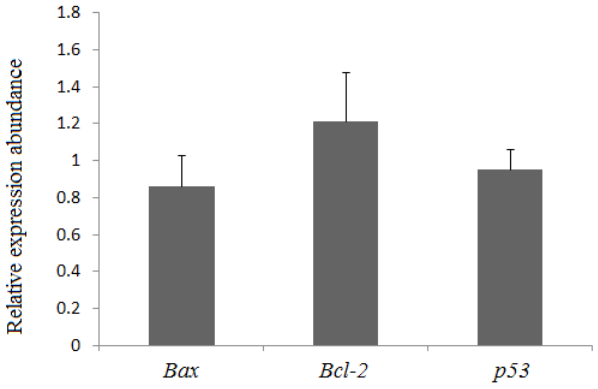

Figure 5. Relative mRNA expression of *Bax*, *Bcl-2*, and *p53* transcripts of H2O2 treated oocytes (H2O2-100) compared with control oocytes (C+) after IVM. Data are given as the mean±SD. No difference (P >0.05) was observed among treated and untreated oocytes.

the H2O2 during maturation period.

Discussion

A range of 0, 10, 100, or 1000 μM H2O2 were added to the maturation medium *in vitro* to study the effect of mild stress on cytoplasmic maturation by analyzing intracellular GSH levels, after period of maturation. Our data revealed that addition of 10 μM H2O2 to the IVM medium improved the cytoplasmic maturation of ovine oocytes, as evidenced by the higher GSH level. In contrast, GSH did not affect in bovine oocytes after treatment with 0.01–100 μM H2O2 (20). In defense against H2O2, GSH formation is a part of an enzymatically driven redox process (38). The GSH is a thiol with the predominant low-molecular-weight that is present in many organisms and has been proposed to protect cells from oxidative damage such as that formed by H2O2 (39). Thiol compounds like glutathione function as innate antioxidants that provide adequate protons to convert hydrogen H2O2 to water (40). The GSH in the reduced form is critical for detoxification of hydrogen peroxide in the cell (41). Previous evidence for oxidative stress in cryopreserved mammalian oocytes is represented by the observations of decreased glutathione (GSH) and increased levels of hydrogen peroxide (42). In this study also, we observed a similar hydrogen peroxide-induced decrease in GSH level when higher (1000 μM) level of H2O2 under mild stress, was added into the maturation medium. From such observations, it can be speculated that formation of GSH may be stimulated in mild levels of stress as higher values were observed for lower concentration of H2O2 in our findings. Such oxidative stress and its moderate up-regulation in GSH level might lead to more efficient ROS defense of oocytes under mild stress conditions. High levels of antioxidants like catalase, superoxide dismutase, and GSH may control the generation of ROS as well as lipid peroxidation and play the role in the formation of good quality oocytes, thereby, improve the fertilization rate and developmental ability (20).

The higher GSH level observed in H2O2-exposed oocytes at the mild levels raises a question of whether differences in the enzymatic antioxidant might be reflected in higher fertilization and development rates to blastocyst stage. To study the effect of H2O2 concentration at mild levels on sheep oocyte fertilization rate, developmental potential and blastocyst quality, cleavage and blastocyst rates as well as blastocyst cell number and the ratio of the apoptotic cells were evaluated post-fertilization. Fertilization rate of H2O2-1000 was lower than than the other groups and indicated the detrimental effect of the H2O2 treatment at this level. Our data also indicated that adding H2O2 at the level of 100 μM to the maturation medium improved the developmental competence of sheep oocytes, as determined by blastocyst production following insemination *in vitro*. Similar study in bovine also showed that H2O2-induced mild stress during maturation *in vitro* led to an increased developmental potential up to the blastocyst stage. Exposure of mature cumulus–oocyte complexes (COCs) to a high hydrostatic pressure has initiated an argument about how acquisition of stress tolerance by a mild stressor occurs despite its unknown mechanism of effect (43). The ability of high hydrostatic pressure to enhance oocyte stress tolerance has been found in cattle (44) and pig embryos (43, 45, 46). Similarly, an increase in embryonic development has been found following 1 h osmotic stress in porcine oocytes following activation of the stressed-oocytes exposed to sodium chloride, sucrose or trehalose (47). Heat shock protein (HSP) mRNA has been proposed to be involved in induction of stress tolerance (20, 45, 48), but up to now its precise mechanism has not been well understood and studied in detail.

In the current study, addition such amounts of H2O2 to the medium of maturation had no impact on apoptotic cell ratio of day 7 blastocysts, as determined by the TUNEL assay used to detect DNA degradation in apoptotic cells. This is line with the previous finding suggest that a high H2O2 pulse has been found not to alter the apoptotic cell ratio (20), whereas low concentrations of H2O2 was able to increase apoptosis in blastocyst-stage embryos.

A cluster of proteins like Bcl-2 family are involved in regulation of apoptotic mechanism, which can either oppose or promote apoptosis (49). The *Bcl-2* (suppressor) and the *Bax* (inducer) proteins can act autonomously in regulating death of cell (49, 50). The *p53* (tumour suppressor) protein is accumulated in the oocyte, and acts as a transcription factor that responds to a variety of cellular stresses, including in cell-cycle control, DNA damage repair, and induction of cell cycle arrest or apoptosis (37). In this regard, the buffering of *Bcl-2* family of proteins in oxidative stress has been shown in several previous studies. To examine further whether H2O2-induced mild stress is related to the apoptosis of blastocyst and to elucidate the molecular background involved, we analyzed the mRNA expression of *Bax*, *Bcl-2*, and *p53* in blastocysts. No differences in expression levels were detectable for the genes *Bax*, *Bcl-2*, and *p53* in RNA derived from H2O2-treated and control blastocysts. Apoptosis should not have been occurring following exposure of COCs to 100 μM H2O2, since neither the TUNEL assay, or the apoptosis-related transcripts (*Bax*, *Bcl-2*, and *p53*) did indicate an increase of apoptosis status.

In conclusion, H2O2 treatment at the sub-lethal level has enhanced the developmental potential of treated

oocytes without reducing the quality of the derived blastocysts. Further investigation is required to understand the cytoplasmic and molecular events that leading up to a higher competence that can be observed in oocytes following exposure to the mild stress.

Acknowledgments
The authors thank the members in laboratories and department of animal science, Sari Agricultural Sciences and Natural Resources University, and department of biotechnology, University of Tehran for the excellent technical support. This work was supported by Iran National Science Foundation, INSF [grant number: 93024412].

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