ORIGINAL ARTICLE

Veterinary Research Forum. 2020; 11 (4) 377 – 383
doi: 10.30466/vrf.2018.93471.2255
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

The effect of sodium selenite on apoptotic gene expression and development of *in vitro* cultured mouse oocytes in comparison with *in vivo* obtained oocytes

Maziar Malekzadeh Kebria[1], Mojdeh Salehnia[1]*, Saeed Zavareh[2,3], Seyyed Saeed Moazzeni[4]

[1] Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran; [2] Department of Cellular and Molecular Biology, School of Biology, Damghan University, Damghan, Iran; [3] Institute of Biological Sciences, Damghan University, Damghan, Iran; [4] Prevention of Metabolic Research Disorder Center, Research Institute for Endocrine Disorder, Shahid Beheshti University of Medical Science, Tehran, Iran.

Article Info

Article history:

Received: 10 September 2018
Accepted: 04 December 2018
Available online: 15 December 2020

Keywords:

Apoptosis genes
Germinal vesicle oocytes
In vitro maturation
Sodium selenite

Abstract

In vitro maturation (IVM) of oocytes is widely used in assisted reproduction technologies. The present study aimed to improve the *in vitro* oocyte maturation and its development through enriching the culture media with sodium selenite (SS). Moreover, the effects of SS on the expression of the oocytes apoptosis-related genes were assessed. In this study, male and female NMRI mice were used and after collecting their germinal vesicle (GV) oocytes, they were cultured with SS (experimental group) and without SS (control group). Collected metaphase II oocytes (MII) from the fallopian tube were considered as *in vivo* group. After *in vitro* culture, the oocytes were assessed in terms of nuclear maturation. The MII oocytes were inseminated and the development was examined until the blastocyst stage. Also, oocytes were subjected to the molecular analysis for evaluating the expression of BAX, BCL2, P53, and BAD genes using the real-time RT-PCR. The maturation rate was significantly increased in the SS supplemented group compared to the control one. The developmental rate of the embryos was significantly higher for both of the *in vivo* and SS supplemented groups rather than the control one, however, no significant difference was seen between these rates of the experimental and *in vivo* groups. Real-time RT-PCR did not show any significant differences in the expression of the apoptosis-related genes for all of the studied groups. The p53 gene was not expressed in any of groups. Sodium selenite improved the oocyte developmental competence but did not change the expression of the apoptosis-related genes in MII oocytes.

Introduction

Approximately, 8.00-10.00% of couples who suffer from infertility, are candidates for the assisted reproduction techniques (ART).[1] *In vitro* maturation (IVM) of oocytes is one of the important methods in ART with limited success. IVM has several advantages such as the elimination of the expensive gonadotropin treatment, therefore, it is cost-effective. Also, it can be considered as the only choice for certain conditions such as the patients who suffer from polycystic ovarian syndrome, estrogen-sensitive cancer recurrence, and those who require rapid fertility reservations.[2,3] Nevertheless, the embryonic development and pregnancy rates in the patients who undergo IVM, are significantly lower than those of patients undergoing *in vitro* fertilization (IVF) with oocyte obtained from *in vivo* condition. Therefore, efforts have been focused on the improvement of IVM protocol to increase its efficiency.[2]

Recent findings have shown that oxidative stress (OS) is one of the challenges during IVM.[4] Oxidative stress is defined as the imbalance between the oxidative agents production and antioxidant capacity by overcoming these oxidative agents.[5] The OS is an inevitable phenomenon occurs during the IVM due to the high oxygen concentration compared to *in vivo* condition, which increases the production of the reactive oxygen species (ROS).[6] The ROS, as the potent oxidizing agent, can react with all compounds of cells such as DNA, proteins, lipids, carbohydrates, and damage them.[7] Therefore, if the ROS production is not controlled, biomolecules will undergo severe damage and OS may develop. Also, OS can induce

***Correspondence:**

Mojdeh Salehnia. PhD
Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
E-mail: salehnim@modares.ac.ir

cellular apoptosis in both mitochondria-dependent and mitochondria-independent pathways[8,9] Apoptosis as the programmed cell death is required for the tissue homeostasis. However, its dysregulation yields compromising tissue integrity and cell fate.[10] Several proteins are considered as the critical regulators of apoptosis having both the anti-apoptotic (BCL-2, BCL-XL, and BCL-w) and pro-apoptotic (BAX, BAK, BAD, BIM, and BID) functions.[11,12]

Supplementation of the culture medium with antioxidants could control the excessive ROS level during the in vitro maturation.[4-6,13-20] Therefore, it might be effective for the appropriate maturity of the cytoplasm and nucleus of the oocytes.[6,13,21,22]

Selenium is a rare element and is located in the catalytic site of the enzymatic antioxidants such as glutathione peroxidase which is essential for maintaining the physiological activity of the body.[9,23] Selenium protects the cells existent in the culture medium in the form of sodium selenite (SS) by reducing the free radicals and increasing the enzymatic antioxidant against oxidative damage.[24] It has been proved that the selenium absence increases cell death and induces apoptosis.[23,25-27] It seems that the short-term effect of SS is mainly an anti-apoptotic one.[28] Furthermore, this anti-apoptotic effect of SS is induced through inhibition of H_2O_2-induced ROS generation, which has been shown using cultured brain-derived neural progenitor cells.[28] Previous studies indicated that SS increased the total antioxidant capacity level and decreased the ROS level in the cultured ovarian follicles which in turn improved the in vitro development of the follicles.[24,29] In this regard, Ghorbanmehr *et al*. illustrated that SS increases the copy number of the oocyte mtDNA via decreasing the OS.[30] It seems that the destructive effects of ROS are diminished by the SS treatment.[24,28,30,31] However, the precise mechanism of SS on the improvement of mouse oocytes has not been specified yet. Thus, this study was conducted to know whether supplementation of the culture medium with SS as an antioxidant could modify the expression of the apoptotic and anti-apoptotic genes of the mouse oocyte.

Materials and Methods

Chemicals. Unless otherwise stated, all the chemicals reagents were obtained from Sigma-Aldrich (Cambridge, UK) and all the media were prepared using Milli-Q deionized water.

Animals. Adult female (n = 60) and male (n = 20) National Medical Research Institute (NMRI) mice (6-8 weeks old) were used under the guideline for care and use of the animal laboratory of Tarbiat Modares University. Research and all of the animal experiments were approved by the Animal Ethics Committee, Tarbiat Modares University (IACUC No: 1395/496). They were housed under a 12 hr light:12 hr dark regimen (light on at 7:00), at a temperature of 22.00 ± 2.00 ˚C, and relative humidity of 45.00 ± 2.00% for at least one week before use. Animals were provided with food and water, both available *ad libitum*.

Experimental Design. After collecting the oocytes at the germinal vesicle (GV) stage, they were randomly categorized into two groups. First, the control group consisting of the GV oocytes undergoing IVM without any intervention of SS (SS-) and second, the experimental group consisting of the GV oocytes undergoing IVM in the presence of 10.00 ng mL^{-1} SS (SS+). Also, the metaphase II oocytes (MII) were obtained from the fallopian tube after inducing the ovulation and considered as *in vivo* group. Some of the MII oocytes of each group were inseminated and used for evaluating the developmental capability and the others were considered for the molecular analysis.

Germinal vesicle oocytes collection. The female mice (n = 40) were primed with an intraperitoneal injection of 10.00 IU pregnant mare's serum gonadotropin (PMSG; Intervet, Sydney, Australia). After 48 hr, the mice were sacrificed by cervical dislocation. As previously reported,[32,33] the anesthesia has adverse effects on oocyte quality, thus, in the present study, the anesthetic was not applied before ovarian tissue extraction. Their ovaries were removed and placed in α-MEM medium (Gibco-BRL) supplemented with 10.00% (v/v) fetal bovine serum (FBS; Gibco-BRL Carlsbad, USA), 0.23 mM sodium pyruvate, 50.00 μg mL^{-1} penicillin, and 75.00 μg mL^{-1} streptomycin. Under the view of a stereo microscope, the immature GV oocytes were released from large antral follicles by puncturing with a sterile needle. The selection of the GV oocytes (n = 1067) was performed based on the morphological parameters including characteristics of a cumulus-oocyte complex structure, oocyte cytoplasm, perivitelline space, and zona pellucida, as described before.[34,35]

***In vivo* MII oocyte collection.** To achieve the MII oocytes, the ovulation induction was carried out by the intraperitoneal injection of 10.00 IU PMSG followed by 10.00 IU human chorionic gonadotropin (hCG; Sereno, Geneva, Switzerland) 48 hr later. After that, the mice (n = 20) were sacrificed by cervical dislocation 12-16 hr later and MII oocytes were obtained from the fallopian tube through the flushing method. Cumulus cells were enzymatically removed using 0.01% hyaluronidase. The selection of the MII oocytes was performed based on the morphological parameters including characteristics of a cumulus-oocyte complex structure, oocyte cytoplasm, polar body, perivitelline space, zona pellucida, and simultaneous meiotic spindle, as described before.[34,35]

***In vitro* maturation of the GV oocytes.** As previously mentioned, *in vitro* maturation of the GV oocytes was accomplished with some modifications.[36] Briefly, GV oocytes were cultured in 10.00 μL drops of α-MEM supplemented with 50 μg mL^{-1} penicillin, 75.00 μg mL^{-1}

streptomycin, 0.23 mM sodium pyruvate, 10.00% FBS, 75.00 mIU mL^{-1} recombinant follicle-stimulating hormone, 10.00 IU mL^{-1} hCG, and 10.00 ng mL^{-1} SS according to the experimental design under mineral oil at 37.00 ˚C, 100% humidity in 5.00% CO_2 for 16 hr. The rates of degeneration, GV, germinal vesicle breakdown (GVBD), and MII oocytes were evaluated using an inverted microscope. MII oocytes were collected (n = 604) for IVF and the others used for the molecular analysis (n = 90).

***In vitro* fertilization.** The IVF was performed with some modification as previously described.[36] Briefly, cauda epididymis of adult male NMRI mice (n = 20) were squeezed out into a 500-µl drop of human tubal fluid (HTF) medium supplemented with 5.00 mg mL^{-1} bovine serum albumin (BSA) under mineral oil. Freshly released spermatozoa were incubated in a 37.00 ˚C with 5.00% CO_2 for 90 - 120 min. Capacitated sperms (1.00 × 10^6 per mL) were added to IVF media consisting of HTF medium supplemented with 15.00 mg mL^{-1} BSA. Both collected MII oocytes from *in vivo* and *in vitro* condition were transferred to the IVF media. After 4-6 hr, the oocytes were transferred to 10.00 µL drops of HTF medium with 5.00 mg mL^{-1} BSA. Embryos were daily observed under an inverted microscope and the developmental rates of embryos that reached 2-cell, 4-cell, morula, and blastocyst stage were calculated until 120 hr (Fig. 1).

RNA extraction and cDNA synthesis. Total RNA was extracted from the MII oocytes (n = 45 in each group) using the trizol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's guidance. Extracted RNA was dissolved in diethylpyrocarbonate-treated water and any DNA contamination was eliminated, as well. RNA quality was evaluated using spectrophotometry (Eppendorf, Hamburg, Germany). The ratio of A260 to A280 nm was calculated and samples with this ratio value of 1.90 to 2.00 were considered as acceptable and used for the reverse transcription. The cDNA was synthesized using a cDNA synthesis kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. In brief, Oligo dT was used for cDNA synthesis from 1.00 µg of total RNA, and reverse transcriptase reaction was incubated at 42.00 ˚C for 60 min. After the inactivation of reverse trans criptase enzyme, the product was stored at – 20.00 ˚C.

Real-time RT-PCR. The gene-specific primers for BAX, BCL2, P53, and BAD genes were designed using the Primer-BLAST tool in NCBI (Table 1). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was considered as the housekeeping gene (Table 1). The primers were analyzed using Oligo Analyzer. Real-time RT-PCR was performed on an ABI Step One machine (Applied Biosystem, Foster, USA) using Quanti-Tect SYBR Green RT-PCR kit (Qiagen, Courtaboeuf, France). The thermal program of the real-time RT-PCR was set to an initial denaturation for 5 min at 95.00 ˚C followed by 40 cycles of denaturation at 95.00 ˚C for 15 sec and the annealing and extension at 58.00 ˚C for 30 sec, and 72.00 ˚C for 15 sec. The $2^{-\Delta\Delta Ct}$ formula was applied for assessing the results. The specificity of the real-time RT-PCR was evaluated by implementing the melting curve analysis.

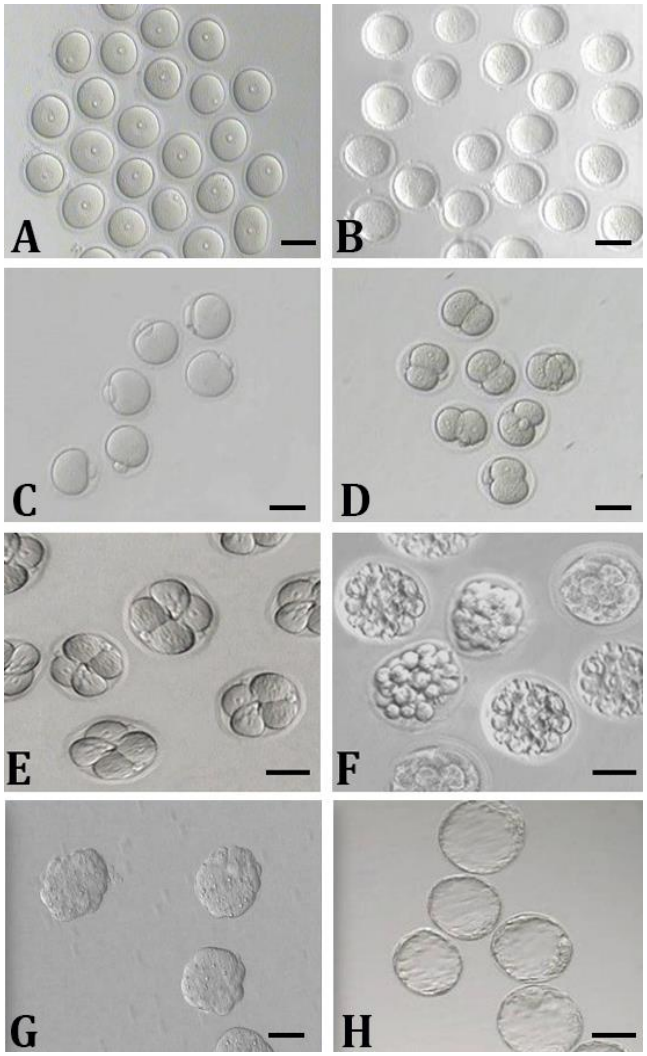

Fig. 1. The Developmental stages of *in vitro* maturation, fertilization, and cleavage of mouse germinal vesicle (GV) oocytes. **A)** GV oocytes, **B)** Germinal vesicle breakdown oocytes, **C)** metaphase II oocytes, **D)** 2-cell embryo, **E)** 4-cell embryo, **F)** morula, **G)** compact morula, and **H)** blastocyst, (Scale bars = 100 µm).

Statistical analysis. All data were presented as mean ± SD and analyzed by SPSS software (version 24.0; IBM Corp., Armonk, USA). A Mann–Whitney U test was conducted to compare the rates of maturation (GV, GVBD, and MII) and survival of the studied groups. Differences among the groups in terms of the rates of fertilization, embryo development stages, and gene expression levels were statistically analyzed by one-way ANOVA and post hoc Tukey's HSD test. A *p*-value of less than 0.05 was considered statistically significant.

Table 1. Primer sequence.

Genes	Length	Primer sequence	Product size (bp)	Temperature (˚C)	Gene code
BCL2	21 19	F:5′-GGTGTTCAGATGTGCGTTCA-3' R: 5′-CGTCGTGACTTCGCAGAG-3'	135	66	NM-177410
P53	20 21	F:5′-AGAGACCGCAGTACAGAAGA-3' R: 5′-GCATGGGCATCCTTTAACTC-3'	227	65	NM-011640
BAD	21 20	F:5′-CGCTTAGAACTGGAGGGAGGA-3' R: 5′-CACTCGGCTCAAACTCTGGG-3'	99	69	NM-0011285423.1
BAX	21 21	F:5′-CGCCGAAATGGAGATGAACTG-3′ R: 5′-GCAAAGTAGAAGAGGGCAACC-3'	160	67	NM-0075227.3
GAPDH	23 22	F: 5'-TGACATCAAGAAGGTGGTGAAGC-3' R: 5'-CCCTGTTGCTGTAGCCGTATTC-3'	203	62	XM_001476707.5

Results

The survival and developmental rates. After 16 hr of culture, the survival rate of the oocytes for the experimental group (SS+) was measured as 91.00%, while, it was equal to 93.00% for the control one (SS-). Therefore, there was no significant difference between these two groups. The rates of GV oocytes which reached GVBD and MII stages for the SS+ group were measured as 15.00% and 65.00%, respectively. However, these values for SS- group were 9.00% and 76.00%, respectively (Table 2). There was no significant difference between these two groups regarding the rate of GVBD, however, the rate of MII in the SS+ group was significantly higher than that of SS- one ($p < 0.05$).

Embryo development. The developmental rates of embryos to 2-cells, 4-cells, morula, and blastocyst stage were estimated as 51.00%, 57.00%, 30.00%, and 21.00% for SS- group and 64.00%, 67.00%, 48.00%, and 40.00% for SS+ group, respectively. These rates were significantly higher for both *in vivo* (62.00%, 64.00%, 44.00%, and 30.00%, respectively) and experimental groups in comparison with the control one ($p < 0.05$), however, there were no considerable difference between these rates of the experimental and *in vivo* groups (Table 3).

Real time RT-PCR. The relative mRNA expression levels of apoptosis-related genes were evaluated by real-time RT-PCR. As shown in Figure 2, the mRNA expression of BAX, BAD, and BCL2 is detectable for all of the studied groups. Although the mRNA quantities are changing, a comparison of the relative gene expression levels indicated no significant differences among the studied groups ($p > 0.05$). Also, the results obtained via real-time RT-PCR, showed no significant difference among the ratio of the mRNA expression levels of BAX to BCL2 corresponding to the studied groups ($p > 0.05$). Besides, the mRNA expression of the P53 gene was not identified for any of the experimental groups.

Discussion

In this study, the effect of SS was investigated on the developmental capability of *in vitro* matured oocytes. The obtained results illustrated that 10.00 ng mL^{-1} SS, improved the developmental capability of mice oocyte during *in vitro* culture. It has been stated in the literature that the effect of SS is dose-dependent. Xiao *et al.* showed a high concentration of SS increases apoptosis.[37] The previously performed studies showed that supplementing the maturation medium with 10.00 ng mL^{-1} SS improves

Table 2. The survival and developmental rates of mouse oocytes after 16 hr *in vitro* maturation in the presence and absence of sodium selenite. Data are presented as No. and (mean ± SD).

Groups	Total Number of oocytes	Survived	GV	GVBD	MII
SS-	516	470 (91.10 ± 2.17)	86 (18.62 ± 4.98)	73 (15.46 ± 3.63)	311 (68.89 ± 4.15)
SS+	551	514 (93.15 ± 0.84)	72 (13.76 ± 3.87)	51 (9.64 ± 4.72)	391 (76.58 ± 4.67)*

The percentage of survived oocytes was calculated to the total number of oocytes, and the percentage of GV, GVBD, and MII oocytes was calculated to the survived oocytes.

SS+: *in vitro* maturation with sodium selenite; SS-: *in vitro* maturation without sodium selenite; GV: germinal vesicle; GVBD: germinal vesicle breakdown; MII: Metaphase II. * Indicates significant differences with other groups in the same column ($p < 0.05$).

Table 3. The developmental rate of embryos (n) derived from MII oocytes in studied groups. Data are presented as No. and (mean ± SD).

Groups	Total MII oocyte	2-cells	4-cells	Morula	Blastocyst
In vivo	338	212 (62.2 ±14.81)	138 (64.14 ± 5.51)	97 (44.99 ± 8.38)	67 (30.43 ±10.95)
SS-	263	137 (51.21 ± 5.67)*	80 (57.16 ± 7.43)*	44 (30.05 ± 9.42)*	33 (21.90 ±12.48)*
SS+	341	219 (64.34 ± 4.32)**	148 (67.23 ± 1.78)**	106 (48.40 ± 3.47)**	88 (40.15 ± 2.99)**

The percentages of 2-cell embryos were calculated to the number of inseminated oocytes and the percentages of 4-cell, morula, and blastocyst embryos were calculated to the 2-cell embryos.

SS+: *in vitro* maturation with sodium selenite; SS-: *in vitro* maturation without sodium selenite; MII: metaphase II. * indicates significant differences with *in vivo* group in the same column ($p < 0.05$) and ** indicates significant differences with SS- group in the same column ($p < 0.05$).

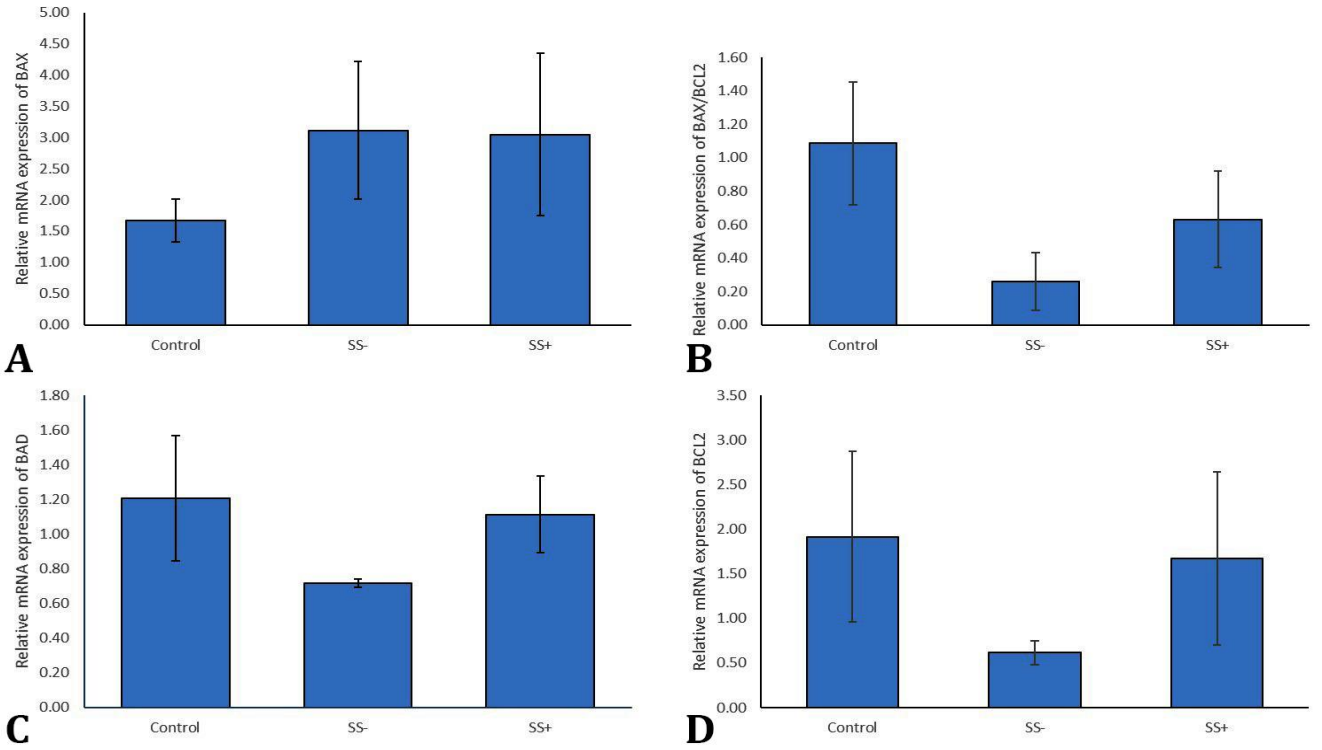

Fig. 2. Real-time RT-PCR results of relative mRNA expression of **A)** BAX, **B)** BAX/BCL2, **C)** BAD, and **D)** BCL2 in different experimental groups SS+: *in vitro* maturation with sodium selenite SS-: *in vitro* maturation without sodium selenite.

the maturation of the GV oocytes and pre-antral follicle during *in vitro* culture.[24,30] There are several explanations for these results. First, SS acts via reducing the ROS levels and increasing the total antioxidant capacity. [24] Therefore, the antioxidant properties of the determined dosage of SS improves the oocytes maturation and embryo development as well.[30] In this regard, Uhm *et al.* observed that by supplementing the pig embryo culture medium with 25.00 ng mL^{-1} SS, the glutathione peroxidase levels increase and ROS levels decrease which in turn led to an increment in the rate of the blastocyst formation.[9] Second, it was demonstrated by Ghorbanmehr *et al*. that SS increased the oocyte mtDNA copy number by OS inhibition and it was associated with better oocyte developmental competence.[30] Third, SS may improve the oocyte development in other routes, by inducing cell proliferation.[38,39] Basini and Tamanini reported an increase in the estradiol level while supplementing the culture media with SS which resulted in the granulosa cell proliferation and an improvement in the oocyte development.[40] Fourth, it was demonstrated that SS acted as a survival factor through increasing the level of extracellular signal-regulated protein kinases 1 and 2 (*ERK1/2*) protein. The *ERK1/2* had a critical role in cell proliferation and survival one through increasing the *intracellular* calcium.[9] In the second part of the present study, attempts were made to show if SS acted as a survival factor by changing in the pattern of apoptosis-related gene expression (BAX, BAD, P53, and BCL2). The present results indicated that the supplementation of the culture media with 10ng/ml SS could not alter the oocyte pro and anti-apoptotic genes expression in comparison with SS non-treated control and *in vivo* obtained MII oocytes. The findings of the present study might be due to several reasons including: the GV oocytes were cultured for 16 hr and the expression of apoptosis-related genes were evaluated in the MII oocytes, therefore, the alteration in the gene expression might have occurred earlier at the GV or GVBD stages. Another reason might be attributed to the quality of the studied MII oocytes for the molecular analysis. The MII oocytes were in good quality and reached to the higher stage of development.[41] Therefore, the changes in the apoptosis-related genes expression were not detectable in these MII oocytes. Finally, SS with 10.00 ng mL^{-1} might have led to an improvement in the oocyte development in another pathway rather than the apoptosis inhibition as mentioned earlier.[9,24,29,30,38,39,42,43] In this regard, Park *et al.* showed that the improvement of the embryonic development was not related to the decline in the expression of the pre-apoptotic genes such as BAX.[42]

In conclusion, our data clarify that SS could improve the oocyte maturation from GV to MII stage, as well as the development of their embryos to the blastocyst stage. However, it did not change the expression of the apoptosis-related genes in the MII oocyte.

Acknowledgments

The author would like to express their special thanks to Mr. Shahram Pour Bayranvand for technical assistance.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Szamatowicz M. Assisted reproductive technology in reproductive medicine - possibilities and limitations. Ginekol Pol 2016;87(12):820-823.
2. Smitz J, Cortvrindt R. Oocyte *in-vitro* maturation and follicle culture: current clinical achievements and future directions. Hum Reprod 1999;14(suppl 1):145-161.
3. Chian RC, Lim JH, Tan SL. State of the art in in-vitro oocyte maturation. Curr Opin Obstet Gynecol 2004;16(3):211-219.
4. Combelles CMH, Gupta S, Agarwal A. Could oxidative stress influence the in-vitro maturation of oocytes? Reprod Biomed Online 2009;18(6):864-880.
5. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 2005; 3(1):28. doi: 10.1186/1477-7827-3-28.
6. Agarwal A, Said TM, Bedaiwy MA, et al. Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 2006;86(3):503-512.
7. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis 2005;15(4):316-328.
8. Rahimi G, Isachenko E, Sauer H, et al. Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. Reprod Fertil Dev 2003;15(6):343-349.
9. Uhm SJ, Gupta MK, Yang JH, et al. Selenium improves the developmental ability and reduces the apoptosis in porcine parthenotes. Mol Reprod Dev 2007;74(11): 1386-1394.
10. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35(4):495-516.
11. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9(1):47-59.
12. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. J Cell Mol Med 2003;7(3):249-257.
13. Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol 2010;42(10):1634-1650.
14. Hatami S, Zavareh S, Salehnia M, et al. Total oxidative status of mouse vitrified pre-antral follicles with pre-antral follicles with pre-treatment of alpha lipoic acid. Iran Biomed J 2014;18(3):181-188.
15. Hatami S, Zavareh S, Salehnia M, et al. Comparison of oxidative status of mouse pre-antral follicles derived from vitrified whole ovarian tissue and vitrified pre-antral follicles in the presence of alpha lipoic acid. J Obstet Gynaecol Res 2014;40(6):1680-1688.
16. Hatami S, Zavareh S, Salehnia M, et al. The impact of alpha lipoic acid on developmental competence of mouse vitrified pre-antral follicles in comparison to those isolated from vitrified ovaries. Iran J Reprod Med 2014;12(1):57-64.
17. Hosseinzadeh E, Zavareh S, Lashkarbolouki T. Anti-oxidant properties of coenzyme Q10-pretreated mouse pre-antral follicles derived from vitrified ovaries. J Obstet Gynaecol Res 2016;43(1):140-148.
18. Kashka RH, Zavareh S, Lashkarbolouki T. Augmenting effect of vitrification on lipid peroxidation in mouse preantral follicle during cultivation: Modulation by co-enzyme Q_{10}. Syst Biol Reprod Med 2016; 62(6):404-414.
19. Rahnama A, Zavareh S, Ghorbanian MT, et al. The Effects of cAMP-elevating agents and alpha lipoic acid on *in vitro* maturation of mouse germinal vesicle oocytes. J Reprod Infertil 2013;14(4):173-183.
20. Talebi A, Zavareh S, Kashani MH, et al. The effect of alpha lipoic acid on the developmental competence of mouse isolated preantral follicles. J Assist Reprod Genet 2012;29(2):175-183.
21. Gupta S, Sekhon L, Kim Y, et al. The role of oxidative stress and antioxidants in assisted reproduction. Curr Womens Health Rev 2010; 6(3):227-238.
22. Halliwell B. Cell culture, oxidative stress, and anti-oxidants: avoiding pitfalls.Biomed J 2014;37(3):99-105.
23. Saito Y, Yoshida Y, Akazawa T, et al. Cell death caused by selenium deficiency and protective effect of antioxidants. J Biol Chem 2003;278(41):39428-39434.
24. Abedelahi A, Salehnia M, Allameh A, et al. 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. Hum Reprod 2010; 25(4):977-985.
25. Ma YM, Ibeanu G, Wang LY, et al. Selenium suppresses glutamate-induced cell death and prevents mitochondrial morphological dynamic alterations in hippocampal HT22 neuronal cells. BMC Neurosci 2017;18(1):15. doi: 10.1186/s12868-017-0337-4.
26. Milner JA: Rationale and possible mechanisms by which selenium inhibits mammary cancer, in: Vitamins and minerals in the prevention and treatment of cancer. Fl, USA CRC Press, 2018,110-127.
27. Vinceti M, Filippini T, Del Giovane C, et al. Selenium for preventing cancer. The Cochrane Library 2018. doi:10.1002/14651858.CD005195.pub4.

28. Yeo JE, Kang SK. Selenium effectively inhibits ROS-mediated apoptotic neural precursor cell death *in vitro* and *in vivo* in traumatic brain injury. Biochim Biophys Acta 2007; 1772(11-12):1199-1210.
29. Abedelahi A, Salehnia M, Allameh A. The effects of different concentrations of sodium selenite on the *in vitro* maturation of preantral follicles in serum-free and serum supplemented media. J Assist Reprod Genet 2008;25(9-10):483-488.
30. Ghorbanmehr N, Salehnia M, Amooshahi M. The effects of sodium selenite on mitochondrial DNA copy number and reactive oxygen species levels of *in vitro* matured mouse oocytes. Cell J. 2018;20(3):396-402.
31. Xiong X, Lan D, Li J, et al. Selenium supplementation during *in vitro* maturation enhances meiosis and developmental capacity of yak oocytes. Anim Sci J 2018;89(2):298-306.
32. Alsalili M, Thornton S, Fleming S. The effect of the anaesthetic, propofol, on *in-vitro* oocyte maturation, fertilization and cleavage in mice. Hum Reprod 1997;12(6):1271-1274.
33. Hayes MF, Sacco AG, Savoy-Moore RT, et al. Effect of general anesthesia on fertilization and cleavage of human oocytes *in vitro*. Fertil Steril 1987;48(6): 975-981.
34. Lazzaroni-Tealdi E, Barad DH, Albertini DF, et al. Oocyte scoring enhances embryo-scoring in predicting pregnancy chances with IVF where it counts most. PloS ONE 2015; 10(12): e0143632.doi:10.1371/journal. pone.0143632.
35. Lasiene K, Vitkus A, Valaneiūte A, et al. Morphological criteria of oocyte quality. Medicina (Kaunas) 2009; 45(7):509-515.
36. Zavareh S, Salehnia M, Saberivand A. Comparison of different vitrification procedures on developmental competence of mouse germinal vesicle oocytes in the presence or absence of cumulus cells. Int J Fertil Steril 2009;3(3):111-118.
37. Xiao R, Qiao JT, Zhao HF, et al. Sodium selenite induces apoptosis in cultured cortical neurons with special concomitant changes in expression of the apoptosis-related genes. Neuro Toxicology 2006;27(4):478-484.
38. Hwang JT, Kim YM, Surh YJ, et al. Selenium regulates cyclooxygenase-2 and extracellular signal-regulated kinase signaling pathways by activating AMP-activated protein kinase in colon cancer cells. Cancer Res 2006;66(20):10057-10063.
39. Modarres SZ, Heidar Z, Foroozanfard F, et al. The effects of selenium supplementation on gene expression related to insulin and lipid in infertile polycystic ovary syndrome women candidate for *in vitro* fertilization: a randomized, double-blind, placebo-controlled trial. Biol Trace Elem Res 2018;183(2): 218-225.
40. Basini G, Tamanini C. Selenium stimulates estradiol production in bovine granulosa cells: possible involvement of nitric oxide. Domest Anim Endocrinol 2000;18(1):1-17.
41. Melka MG, Rings F, Hölker M, et al. Expression of apoptosis regulatory genes and incidence of apoptosis in different morphological quality groups of *in vitro*-produced bovine pre-implantation embryos. Reprod Domest Anim 2010;45(5):915-921.
42. Park SH, Cho HS, Yu IJ. Effect of bovine follicular fluid on reactive oxygen species and glutathione in oocytes, apoptosis and apoptosis-related gene expression of *in vitro*-produced blastocysts. Reprod Domest Anim 2014;49(3):370-377.
43. Said RS, Nada AS, El-Demerdash E. Sodium selenite improves folliculogenesis in radiation-induced ovarian failure: a mechanistic approach. PloS ONE 2012; 7(12):e50928. doi:10.1371/journal.pone.0050928.