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Addition of MitoTEMPO to the maturation medium improves *in vitro* maturation competence of bovine oocytes

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Abstract

The effects of MitoTEMPO, a mitochondria-targeted antioxidant, and its non-targeted parent, TEMPO, on bovine oocyte maturation competence have not been determined so far. Hence, our study was aimed to investigate the effects of supplementing maturation medium with different concentrations of MitoTEMPO (0.00, 0.10, 1.00 and 10.00 μM) or TEMPO (0.00, 5.00, 10.00 and 15.00 mM) on *in vitro* maturation (IVM) and fertilization (IVF) of bovine oocytes. The oocytes after IVM and IVF were evaluated for the signs of nuclear maturation and normal fertilization. The average number of spermatozoa penetrated per oocyte and the level of intracellular reactive oxygen species (ROS) were also evaluated. The results showed that percentages of bovine oocytes reached the metaphase II stage of meiosis were significantly higher in the 1.00 μM MitoTEMPO group compared to the control group (without antioxidant supplementation). The normal fertilization rate also tended to be greater in this group than the control group. In comparison with the control group, the medium supplementation with 1.00 μM MitoTEMPO led to a significant decrease in the intracellular ROS level. The average number of spermatozoa penetrated per oocyte was not significantly different among the antioxidant-treated and the non-treated groups. The TEMPO addition to the maturation medium affected neither the rate of maturation/fertilization nor the level of intracellular ROS in bovine oocytes. Based on these results, we concluded that MitoTEMPO at a concentration of 1.00 μM had beneficial effects on the quality and fertilization potential of bovine oocytes.

Introduction

Despite scientific efforts to enhance the efficiency of producing *in vitro* bovine embryos, only a low percentage of oocytes develop to blastocysts, probably because of suboptimal *in vitro* culture conditions.[1] *In vitro* oocyte maturation and fertilization are currently performed in cell culture incubators with approximately 20.00% atmospheric oxygen concentration, which is about three times higher than its concentration within the lumen of the reproductive tract in females (3.00-9.00%).[2] It seems likely that exposure of cells to such high tension of oxygen increases the production of reactive oxygen species (ROS).[2,3] Besides, manipulation, light and its temperature, medium constituents and sperm may boost ROS production, and thereby decrease the defense mechanisms of cells cultured *in vitro*.[4,5] As a result, an imbalance in the amount of ROS production and elimination leads to oxidative stress and cell injuries, such as cell membrane damage,[6] DNA fragmentation,[7] mitochondrial dysfunction,[8] inhibition of sperm–oocyte fusion[9] and meiotic arrest.[10] Therefore, to diminish the harmful effects of oxidative stress and ROS on oocytes and embryos, it would be required to include antioxidant(s) into the culture media.

To eliminate excessive ROS, a wide variety of antioxidants have been included in bovine *in vitro* maturation (IVM) and *in vitro* culture (IVC) media,[2,11-17] however, some of them, such as catalase, superoxide dismutase, vitamins E and C and N-acetyl cysteine failed to improve the maturation of oocytes and the developmental competence of preimplantation embryos in cattle.[2,11,18-21] This could be because of inability of these antioxidants to cross the membranes.

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It has been reported that conventional antioxidants may not arrive at the intracellular site(s) of ROS production.[22] Large proteins like catalase and superoxide dismutase are not able to cross the membranes, and also highly lipophilic antioxidants such as vitamin E and coenzyme Q tend to remain in the cell membranes.[22] Consequently, these antioxidants are not effective in eliminating intracellular ROS. Hence, it is necessary to guide antioxidants to intracellular sites, specifically to mitochondria, to protect cells from intracellular free radicals.

Currently, an effective method for targeting antioxidants to mitochondria (the major site of ROS production) is their conjugation to the triphenylphosphonium (TPP^+), a lipophilic cation. This cation has the capacity to easily permeate lipid bilayers and consequently accumulates inside mitochondria.[23] Mito-TEMPO, a targeted mitochondrial antioxidant, is formed by conjugating TEMPO to the TPP+.[24] It has an activity like that of superoxide dismutase.[24] It has been recently reported that MitoTEMPO has a positive effect on the quality and oocytes developmental competence in pigs.[25] However, there were no reports on where MitoTEMPO and its non-targeted parent antioxidant, TEMPO, were applied as antioxidant agents in culture media for producing *in vitro* bovine embryos. Hence, our study was aimed to determine the effects of the enrichment of the maturation medium with different concentrations of MitoTEMPO or TEMPO on IVM, subsequent fertilization and the intracellular ROS level of bovine oocytes.

Materials and Methods

Materials. All chemicals, reagents, and media were purchased from Sigma-Aldrich (St. Louis, USA) and Gibco (Grand Island, USA) unless otherwise stated.

The retrieval and selection of bovine oocyte. Bovine ovaries were transferred from abattoir to the laboratory in a normal saline solution containing 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at approximately 31.00 - 35.00 ˚C within 2 hr of the collection. Upon arrival at the laboratory, collected ovaries were rinsed thrice with sterile saline solution maintained at 37.50 ˚C. An 18-gauge needle attached to a low-pressure vacuum pump (MEDAP; Sekretsauger, Tilburg, the Netherlands) was used to aspirate the immature cumulus-oocyte complexes (COCs) from visible follicles of bovine ovaries.[26] Retrieved COCs were then put in a plate containing oocyte collection medium [OCM; consisting of tissue culture medium 199 (TCM199) with Hanks' salts supplemented with 2.00% (v/v) fetal bovine serum (FBS; containing 2.00 U mL^{-1} heparin), 50.00 μg mL^{-1} gentamycin, and 1.00 mM glutamine]; Those COCs with more than three-layer of intact compact cumulus and a homogeneous ooplasm were selected for IVM, and washed thrice in OCM.[27]

Experimental design. Two separate experiments were designed to investigate the effects of the inclusion of three different concentrations of MitoTEMPO or TEMPO in the oocyte maturation medium (OMM) on the quality and maturation competence of oocytes retrieved from the bovine ovaries. In each experiment, selected COCs were randomly distributed among the four groups in culture plates containing 50.00 μL-drops of the OMM (10 oocytes per each drop) supplemented without any antioxidant (the untreated control group) or with different concentrations of MitoTEMPO (0.10, 1.00 and 10.00 μM) or TEMPO (5.00, 10.00 and 15.00 mM) antioxidant. The MitoTEMPO and TEMPO are water-soluble antioxidants, therefore, we used the OMM as the solvent. The levels of antioxidants used in the present study were adopted from previous studies of porcine and mouse oocytes.[25,28] It is noteworthy that before distributing the COCs, all droplets were covered with mineral oil and preincubated under conditions of IVM for at least 3 hr. Nuclear staining with Hoechst 33342 dye (Sigma-Aldrich) was accomplished to assess the maturation and fertilization status of bovine oocyte after IVM and *in vitro* fertilization (IVF). An assessment of the levels of intracellular ROS in oocytes was carried out by measuring fluorescence intensity emitted by the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye (Sigma-Aldrich). Both experiments were independently replicated four times.

***In vitro* maturation of the oocytes.** Oocyte maturation was conducted in a humidified atmosphere containing 5.00% CO_2 at 38.50 ˚C for 24 hr. The OMM used in this study was prepared according to the previous literature methods[27] with some modifications and was consisted of TCM199 with Earle's salts supplemented with 10.00% (v/v) FBS, 22.00 μg mL^{-1} sodium pyruvate, 2.00 μg mL^{-1} estradiol, 20.00 μg mL^{-1} follicle-stimulating hormone (FSH; Sigma-Aldrich), 50.00 μg mL^{-1} gentamicin sulfate and 1.00 mM glutamine.

***In vitro* fertilization of the oocytes.** After maturation of bovine oocytes with or without presence antioxidants, the media used for IVF were the same in all groups and prepared using previously reported methods with some modifications.[29] Following IVM, maturated COCs were rinsed with HEPES-buffered Tyrode's albumin- lactate-pyruvate (TALP) and, groups of 10-18 treated or non-treated COCs were then transferred to 4-well plates containing pre-equilibrated IVF-TALP medium (250 μL per well) supplemented with 48 μL PHE (12.00 μL per well; a normal saline solution of dissolving penicillamine, hypotaurine, and epinephrine at concentrations of 0.50 mM, 0.25 mM and 25.00 mM, respectively) and covered with mineral oil. As previously described,[27] the Percoll gradient (45.00% over 90.00%) was used to isolate motile spermatozoa from a pool of cryopreserved semen from two bulls. Percoll-purified spermatozoa were washed with the Sperm-TALP medium via centrifugation at 1,100 rpm

for 5 min. Then, spermatozoa were resuspended in 500 µL IVF-TALP medium and counted in a hemocytometer. Finally, spermatozoa were added to each fertilization well in an appropriate volume to obtain a final concentration of 1.00×10^6 per mL. Co-incubation of gametes for 18 hr was conducted in similar conditions as applied for IVM.

Nuclear staining for the assessment of maturation and fertilization status. To assess the rates of maturation and fertilization and polyspermy index, a group of denuded unfertilized/fertilized oocytes from each group were stained with Hoechst 33342 after IVM and IVF, according to the procedures mentioned previously with some modifications.[30,31] Briefly, the oocytes, which had been stripped of their surrounding cumulus cells using 0.10% hyaluronidase (100 IU mL^{-1}) and/or vortex, were washed thrice in phosphate buffered saline – polyvinyl-pyrrolidone solution (PBS/PVP; 1.00 mg mL^{-1}) and fixed in 100 µL-drops of PBS containing 4.00% (w/v) para-formaldehyde under dark condition and at room temperature for an hour. Thereafter, the denuded oocytes were re-washed thrice in PBS-PVP drops and stained in darkness with Hoechst 33342 at room temperature for 10 min. After transferring to glass slides, stained oocytes were evaluated under a fluorescent microscope (BX51; Olympus, Tokyo, Japan), and images were acquired using an Olympus DP72 digital camera attached to the fluorescent microscope. The presence of one polar body and chromosomes in the metaphase-II stage of meiosis (MII) were considered as the signs of the nuclear maturation (Fig. 1). The meiotic maturation rate was then calculated with respect to the total number of oocytes in each group cultured for IVM. The normal fertilization rate calculated by counting the number of inseminated oocytes showed both two (a male and a female) pronuclei or their fusion (Fig. 1). The average number of spermatozoa penetrated per oocyte was considered as the polyspermy index and calculated via dividing the total number of penetrated spermatozoa by the total number of fertilized oocytes (Fig. 1).

Assessment of the levels of intracellular ROS. The denuded mature oocytes from each group were stained with a solution of 10.00 µM H_2DCFDA (diluted in dimethyl sulfoxide) in 1.00% PBS/PVA (polyvinyl alcohol) following incubation in the dark, for 30 min, at 38.50 ˚C and under 5.00% CO_2 atmosphere and maximum humidity conditions, as described by Sovernigo *et al.*[3] After the incubation period, stained oocytes were rinsed thrice in droplets of PBS/PVA, placed on a glass slide along with a drop of PBS/PVA (10.00 µL) and photographed immediately using an DP72 Olympus digital camera attached to the fluorescent micro-scope. ImageJ software (National Institutes of Health, Bethesda, USA) was used to analyze the fluorescence intensity as an indicator of intracellular ROS level in oocyte. The final average intensities for each group were determined by the average values of all measurements subtracted from the average values of the background signal intensities.

Statistical analysis. All parameters, except intracellular ROS and the average number of spermatozoa penetrated per oocyte, were analyzed using the GENMOD procedure of SAS (version 9.4; SAS Institute, Cary, USA). The generalized linear model (GLM) was used to analyze the variances of intracellular ROS, and the average number of spermatozoa penetrated per oocyte. The mean values were compared by the test of Tukey. In all cases, p values equal to or less than 0.05 were considered statistically significant. The data are expressed as the mean for the rates of maturation and fertilization or the mean ± standard error of the mean (SEM) for the average number of spermatozoa penetrated per oocyte and the level of intracellular ROS.

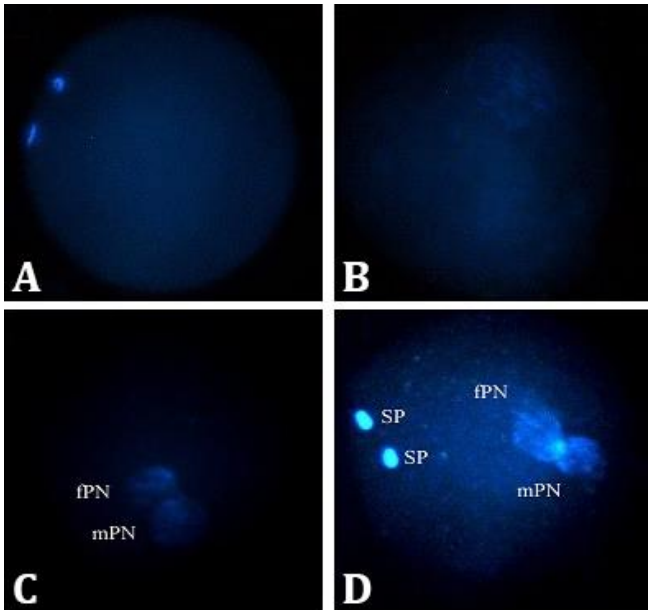

Fig. 1. Assessments of the maturation and fertilization status of bovine oocytes after *in vitro* maturation and *in vitro* fertilization by Hoechst staining under a fluorescence microscope at 20×. A) The mature oocyte; B) The immature oocyte; C) The normal fertilization; D) The fertilized oocyte with three spermatozoa. fPN: Female pronucleus, mPN: Male pronucleus, SP: Spermatozoa.

Results

Effect of MitoTEMPO or TEMPO supplementation during IVM on maturation status of bovine oocyte. The percentage of oocytes reached the MII stage after 24 hr of IVM was significantly higher (p = 0.05) in the 1.00 µM MitoTEMPO-treated group in comparison with that of the control group (Table 1). The 10.00 µM MitoTEMPO-treated group tended (p = 0.07) to have a higher percentage of MII oocytes than the control group (Table 1). Supplementation of IVM medium with TEMPO at different concentrations did not show such an increase in the percentage of oocytes matured to the MII stage ($p >$ 0.05; Table 1).

Table 1. Effects of supplementing MitoTEMPO and TEMPO antioxidants in the *in vitro* maturation medium on the nuclear maturation rate of bovine oocytes.

Parameters	MitoTEMPO concentration (µM)			
	0.00	**0.10**	**1.00**	**10.00**
Number of total oocytes	66	64	66	63
Metaphase II oocytes (%)	72.58[b]	78.11[ab]	86.43[a]	85.79[ab]
Immature oocytes (%)	27.41[a]	21.88[ab]	13.56[b]	14.20[ab]
	TEMPO concentration (mM)			
	0.00	**5.00**	**10.00**	**15.00**
Number of total oocytes	58	59	56	56
Metaphase II oocytes (%)	74.44	77.93	76.84	76.47
Immature oocytes (%)	25.55	22.06	23.15	23.52

The mean data of four independent repeats are shown here. [a,b] Values without a common superscript within each row differ significantly ($p \leq 0.05$).

Effect of MitoTEMPO or TEMPO supplementation during IVM on the subsequent fertilization of bovine oocytes. No significant differences ($p > 0.05$) were found in the percentage of the normal fertilization, and the average number of spermatozoa penetrated per oocyte among the antioxidant-treated and control groups (Table 2). However, 1.00 µM MitoTEMPO tended ($p = 0.06$) to increase the percentage of normal fertilization more than the control group (Table 2).

Effect of MitoTEMPO or TEMPO supplementation during IVM on the level of intracellular ROS. Oocytes derived from supplementing 1.00 µM MitoTEMPO in the IVM medium showed significantly ($p < 0.05$) a reduction of intracellular ROS levels compared to those of the control group (Fig. 2). The MitoTEMPO at the concentration of 10.00 µM tended to reduce the intracellular ROS level more than the control group ($p = 0.07$). Also, there was no significant difference in this parameter among oocytes derived from 0.10, 1.00 and 10.00 µM MitoTEMPO-treated groups ($p > 0.05$; Fig. 2). The TEMPO antioxidant also had no effect on the level of intracellular ROS in bovine oocyte matured *in vitro* for 24 hr ($p > 0.05$; Fig. 2).

Discussion

Aiming to improve bovine oocyte quality through counteracting the harmful effects of oxidative stress, TEMPO and its mitochondria-targeted form, MitoTEMPO, were supplemented in the oocyte IVM medium as antioxidants. The impacts of the different concentrations of those antioxidants were evaluated on bovine oocyte nuclear maturation, ROS level, subsequent fertilization and the average number of spermatozoa penetrated per oocyte *in vitro*. Our results found that supplementation of 1.00 µM MitoTEMPO in the IVM medium improved maturation and fertilization rates of the oocytes and promoted a reduction in the level of ROS relative to the non-treated control group. Although not significant, but in the oocytes of 10.00 µM MitoTEMPO group, a slight increase in the level of intracellular ROS was observed compared to the oocytes of the 1.00 µM MitoTEMPO group. The prooxidant effect of TEMPO has been reported elsewhere[32] and could be a reason for observing this result. Conversely, extracellular antioxidant of TEMPO at the different concentrations had no effect on either *in vitro* oocyte maturation/fertilization or ROS levels of bovine oocytes. Overall, our results indicated the effectiveness of the mitochondria-targeted form of TEMPO (MitoTEMPO) at the level of 1.00 µM in neutralizing intracellular ROS and improving bovine oocyte quality. However, further study is required to clarify the effect of this type of antioxidant on embryo development *in vitro*.

It has been reported that *in vitro* developmental competence of oocytes obtained from abattoir bovine ovaries is low,[33] attributing to not only their intrinsic factors but also *in vitro* culture conditions.[34] The exposure of oocytes to certain *in vitro* situations (e.g., visible light and atmospheric oxygen during the essential manipulations and incubation) leads to depleting intracellular anti-oxidant levels through increasing the ROS production, which in turn disturbs the intracellular redox equilibrium and decreases their developmental competence.[3]

Table 2. Effects of supplementing MitoTEMPO and TEMPO antioxidants in the *in vitro* maturation medium on the fertilization status of bovine oocytes.

Parameters	MitoTEMPO concentration (µM)			
	0.00	**0.10**	**1.00**	**10.00**
Number of oocytes inseminated	56	54	57	55
Normal fertilization (%) ǂ	62.95	66.80	78.63	75.03
The average number of spermatozoa penetrated per oocyte	1.41	1.39	1.25	1.27
	TEMPO concentration (mM)			
	0.00	**5.00**	**10.00**	**15.00**
Number of oocytes inseminated	54	54	55	53
Normal fertilization (%) ǂ	66.42	71.09	68.99	66.87
The average number of spermatozoa penetrated per oocyte	1.36	1.27	1.37	1.40

The mean data of four independent repeats are shown here. There were no significant differences in fertilization status among the groups ($p > 0.05$) but the percentage of normal fertilization tended to be higher in the 1.00 µM MitoTEMPO group compared to the control group ($p = 0.06$).

ǂ Percentage of inseminated oocytes showing a male and a female pronuclei.

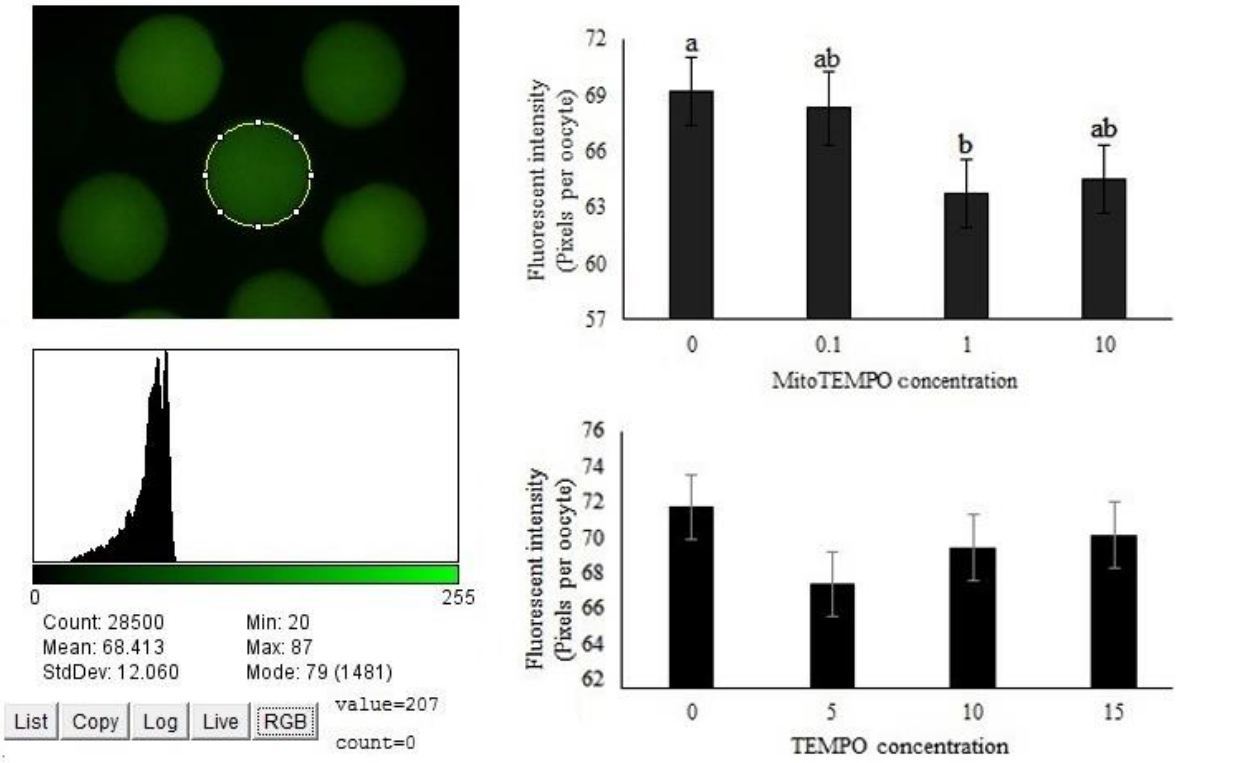

Fig. 2. The mean fluorescence intensity of the intracellular reactive oxygen species (ROS) in bovine oocytes maturated with or without presence of different concentrations of MitoTEMPO and TEMPO. The intensity of the green fluorescence in each oocyte following staining with the 2',7'-dichlorodihydrofluorescein diacetate was quantified by ImageJ software. Data were expressed as the mean of three independent replicates ± SEM.

[ab] Different letters indicate significant statistical differences among treatments ($p < 0.05$).

Hence, ROS-derived oxidative stress inevitably threatens oocytes cultured *in vitro*, suggesting the addition of antioxidants in culture media. In our study, MitoTEMPO supplementation in the IVM medium reduced the level of intracellular ROS during maturation and subsequently increased the percentage of oocytes reached the MII stage while TEMPO did not have such effects. This was probably because of direct targeting of MitoTEMPO to mitochondria (where it was considered as the main source of ROS in the cell)[35] and scavenging mitochondrial superoxide anions. Another probable reason was that the cumulus cells with isolating the oocyte from the culture medium could provide a defending role against oxidizing agents,[2] suggesting no need for using extracellular antioxidants such as TEMPO. In addition, albumin present in the IVM medium is able to trap extracellular ROS.[36]

The MitoTEMPO has been reported to increase the meiotic maturation rate and reduce the intracellular ROS level in porcine oocytes,[25] which was in agreement with our observations. An inhibition in the production of ROS with MitoTEMPO in two mouse models of diabetes has also been reported.[24] In another study, the addition of MitoTEMPO to human semen extender led to improve post-thaw sperm parameters, antioxidant system and mitochondrial membrane potential, and to decrease malondialdehyde content.[37] In contrary, the results of our study were different from those of other studies that reported no influences of antioxidant additions in IVM medium on nuclear maturation of bovine oocyte (resveratrol, cysteamine, carnitine, vitamin C and quercetin,[3] vitamins E and C,[11] anthocyanin[38]), probably because those antioxidants were not able to cross the membranes and did not accumulate in the intracellular compartments of cell to neutralize intracellular ROS.

Oxidative stress-triggered by excessive generation of ROS has been shown to involve in mitochondrial dysfunction leading to insufficient ATP availability and in damage to intracellular molecules such as nucleic acids, proteins, and lipids in the oocytes.[2,39] These changes, in turn, impair several maturation events, including the completion of nuclear meiosis,[40,41] the cytoplasm maturation[41] and the formation and function of meiotic spindles.[42] The MitoTEMPO has been shown to improve the mitochondrial function in the porcine oocyte[25] and human spermatozoa[37] through oxidative stress reduction. Based on these reports, the possibility of such an effect can be expected in bovine oocytes of MitoTEMPO group, which experienced lower oxidative stress suggesting an improvement in oocyte maturation.

It has been found that pronuclear formation has a negative correlation with the level of intracellular ROS.[43] Also, IVC conditions have been shown to exacerbate the formation of ROS, which in turn depletes intracellular GSH content in the oocyte.[44] In our experiment, the normal fertilization evaluated by the presence of two (a male and a female) pronuclei or their fusion within the oocyte tended to be enhanced in the presence of 1.00 μM MitoTEMPO. In one study, the addition of green tea polyphenols to the maturation medium of bovine oocyte has been shown to have a positive effect on the proportion of pronuclear formation through the decrease of oxidative stress and the increase of intracellular glutathione (GSH) level.[45] In another study, it has been shown that melatonin considered as a mitochondria-targeted antioxidant improved the *in vitro* fertilization capacity of bovine oocyte by decreasing the ROS level and increasing intracellular GSH and ATP levels.[46] Glutathione participates in the decondensation of the sperm chromatin and the transformation of the sperm head into a male pronucleus, suggesting that oxidative stress and GSH depletion could influence the pronuclear formation.[47] In addition, a previous study has reported that the buthionine sulfoximine treatment during bovine oocyte IVM through GSH depletion results in inhibiting the formation of a male pronucleus during fertilization.[47] Until now, and to our knowledge, no one has studied the effect of MitoTEMPO on oocyte GSH and ATP contents. Hence, according to findings above, it seems that an improvement in the normal fertilization in our study may be related to the reduction in the level of intracellular ROS and/or the increase of GSH and ATP contents.

In conclusion, the results of our study indicated that the supplementation of 1.00 μM MitoTEMPO in the IVM medium improved maturation and fertilization outcome of bovine oocytes, which was related to the decrease of oxidative stress injury. Additionally, TEMPO, as an extracellular antioxidant, had no effect on oocyte maturation or fertilization *in vitro*. Further studies are required to determine the molecular mechanisms of MitoTEMPO action in bovine oocyte and its effect on bovine embryo development.

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Conflict of interest

The authors declare no conflict of interest.

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