*Zygote*

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# The protective effect of melatonin on the *in vitro* development of yak embryos against hydrogen peroxide-induced oxidative injury

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**Research Article**

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## Summary

Melatonin plays a critical role in several types of cells as an antioxidant to protect intracellular molecules from oxidative stress. The anti-oxidation effect of melatonin in yak embryos is largely unknown. We report that melatonin can protect the development of yak preimplantation embryos against oxidative stress induced by hydrogen peroxide ($H_2O_2$). Therefore, the quality of blastocysts developed from zygotes exposed to $H_2O_2$ was promoted. In addition, we observed that melatonin reduced $H_2O_2$-induced intracellular reactive oxygen species (ROS) levels and prevented mitochondrial dysfunction in zygotes. These phenomena revealed the effective antioxidant activity of melatonin to prevent oxidative stress in yak embryos. To determine the underlying mechanism, we further demonstrated that melatonin protected preimplantation embryos from oxidative damage by preserving antioxidative enzymes. Collectively, these results confirmed the anti-oxidation effect of melatonin in yak embryos that significantly improved the quantity and quality of blastocysts in the *in vitro* production of embryos in yaks.

## Introduction

The yak (*Bos grunniens*) is the principal meat and dairy animal in the Qinghai-Tibet plateau, as few other animals survive in this area. *In vitro* production (IVP) of embryos is a well established embryonic biotechnology with a variety of applications in basic and applied sciences (Sirard, 2017). IVP has made great progress in cattle and is becoming one of the most exciting and progressive procedures available for today's producers, however the efficiency of yak IVP is still low (Marinho *et al.*, 2015).

Although many advances have been made in cell culture techniques, the proportion of embryos that develop to the blastocyst stage is variable. Oxidative stress, a cellular condition caused by the accumulation of reactive oxygen species (ROS), is thought to contribute significantly to defective embryo development (Loren *et al.*, 2017; Tian *et al.*, 2017). Despite the fact that low concentrations of ROS have been shown to reduce DNA fragmentation and improve the developmental potential of porcine embryos, excessive ROS levels are one of the main determinants of embryo quality produced *in vivo* and *in vitro*, and are caused by exposure to elevated oxygen concentrations, light, and elevated concentrations of metabolites and substrates (Mata-Campuzano *et al.*, 2012).

One method that may help to overcome this problem is the supplementation of antioxidant compounds. The addition of antioxidants has been reported to decrease ROS levels and increase blastocyst production rates (Nikseresht *et al.*, 2017). Melatonin is a potent free radical scavenger and antioxidant. The beneficial effects of melatonin are evident, particularly with regard to its antioxidant properties, as ROS production is decreased compared with untreated controls (Mehrzadi *et al.*, 2016). Furthermore, as melatonin is amphiphilic, it can easily reach any cellular compartment, including the membrane, cytosol, and mitochondria (Asghari *et al.*, 2017). Importantly, it inhibits peroxidation, which is a common feature of other antioxidants. With regard to its free radical scavenging capacity, melatonin is five-fold more potent than glutathione (GSH) (Liu *et al.*, 2014). Although the antioxidant effect of melatonin has been reported in some mammalian cell models, there is currently no research regarding the effect of melatonin on yak preimplantation embryo development (Song *et al.*, 2016).

The aim of the present study was to determine whether melatonin has the ability to protect preimplantation yak embryos against oxidative injury and to determine the underlying mechanisms. We show, as far as we can ascertain for the first time, the embryo-protective effect of melatonin against oxidative injury. Furthermore, the improvement in embryo viability was associated with the decreased ROS levels, stable mitochondrial function, and modulation of the activity of enzymatic antioxidants.

## Materials and methods

### Animals and reagents

All animal experiments and treatments were approved and supervised by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. The ovaries of slaughtered cattle were obtained from Datong Abattoir in Qinghai, China.

Dulbecco's phosphate-buffered saline (DPBS) was purchased from HyClone Laboratories Inc. (Logan, UT, USA), follicle-stimulating hormone (FSH) from Bioniche Inc. (Belleville, Ontario, Canada) and fetal calf serum (FCS) from Gibco (Grand Island, NY, USA). All other chemicals and reagents were cell culture tested and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Synthetic oviductal fluid (SOF) medium was prepared according to a previous study.

### Oocyte collection and in vitro maturation (IVM)

Ovaries were collected from yaks at the abattoir from October to December, and transported to the laboratory in DPBS maintained at 28–34°C. Cumulus–oocyte complexes (COCs) were aspirated from follicles (2–8 mm diameter), and collected in DPBS supplemented with 6 mg/ml bull serum albumin (BSA) under a low-power (×20 magnification) stereomicroscope. Unselected COCs were rinsed three times in DPBS containing 5% (v/v) fetal calf serum (FCS) and twice in TCM-199 supplemented with 20% (v/v) fetal calf serum (FCS), 1 μg/ml estradiol-17β, 100 U/ml penicillin, and 100 μg/ml streptomycin. Only COCs with one or more layers of cumulus cells and an evenly granulated ooplasm were selected. Up to 30 COCs were placed in each culture well (Nunc Inc., Naperville IL, USA) containing 600 μl of maturation medium and covered with 300 μl mineral oil. The COCs were allowed to mature for approximately 24 h at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air.

### In vitro fertilization and embryo culture

Frozen semen was thawed and washed by centrifugation through a Percoll gradient (30%/45%) containing HEPES-buffered SOF medium supplemented with 5 mg/ml BSA, 50 μg/ml caffeine, 30 μg/ml glutathione and 20 μg/ml heparin (sperm medium) and centrifuged at 500 *g* for 10 min to separate motile sperm. After centrifugation, 120 μl of the concentrated sperm fraction was removed and placed into 200 μl of sperm medium and incubated at 38.5°C for 15 min.

Oocytes and sperm were then co-incubated for 24–26 h at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air. The putative zygotes were then washed three times in SOF medium. The number of cleaved zygotes was recorded 48 h post-insemination. The culture medium was changed at 96 h and blastocyst development was determined on days 7–9 post-insemination (Day 0 = insemination).

### Melatonin and hydrogen peroxide ($H_2O_2$) treatment

Zygotes were selected for the experiments. To determine oxidative damage, the zygotes were exposed to different concentrations of $H_2O_2$ (0–120 μM) for 15 min, thoroughly washed and then cultured in SOF until the day of blastocyst stage. For the melatonin dose–response study, zygotes were cultured with different concentrations of melatonin (0–100 μM) in the blastocyst stage throughout the development period, and the SOF medium containing melatonin at the specified concentrations was replenished after 72 h. To determine the protective effect of melatonin against oxidative stress, zygotes were preincubated with melatonin (0, 1, 10, and 50 μM) in dimethylsulfoxide (DMSO) or DMSO alone (0.1%) for 1 h. After preincubation, melatonin or DMSO was maintained and $H_2O_2$ was added for 15 min, and then washed out and the embryos were again cultured in medium containing melatonin or DMSO until day 7.

### Terminal-deoxynucleotidyl transferase mediated nick end labelling (TUNEL) assay

Embryo cell apoptosis was determined according to the manuscript's instructions. Briefly, blastocysts were washed three times (5 min each time) in phosphate-buffered saline (PBS) (Beyotime, China) containing 0.2% polyvinyl alcohol (PVA), fixed in Immunol Staining Fix Solution (Beyotime, China) for 30 min, and the embryos were then permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then equilibrated for 8 min after three washes. Samples were then incubated with rTdT incubation buffer (45 μl equilibration buffer, 5 μl nucleotide mix, and 1 μl rTdT enzyme) in the dark for 1 h at 37°C. The tailing reaction was terminated in 2 × standard saline citrate for 15 min.

### Immunofluorescence staining of embryos

Embryos were washed three times (5 min each) in PBS containing 0.2% PVA, and fixed in Immunol Staining Fix Solution (Beyotime, China) for 1 h. All steps were performed at room temperature unless otherwise stated. Embryos were permeabilized with 0.2% Triton X-100 in PBS for 30 min. After three washes, they were blocked in the Immunol Staining Blocking Solution (Beyotime, P0102) for 12 h at 4°C and then incubated with the first antibodies for 12 h at 4°C. Antibody against Caudal Type Homeobox 2 (CDX2; BioGenex, USA) was diluted 1:200 using Immunol Staining Primary Antibody Dilution Solution (Beyotime). After three washes, the embryos were treated with secondary antibodies of Alexa Fluor 555-labelled Goat Anti-Mouse IgG (Beyotime). Samples were analyzed using a Nikon eclipse Ti-S microscope equipped with a 198 Nikon DS-Ri1 digital camera (Nikon, Japan). The experiments were replicated three times. In each replicate, 10 to 15 embryos per group were processed.

### Detection of caspase-3 activity

Caspase-3 activity was determined using a caspase assay kit (Beyotime, China), which detected production of the chromophore *p*-nitroanilide after its cleavage from the peptide substrate DEVD-*p*-nitroanilide and LEHD-*p*-nitroanilide.

### Quantitative real-time PCR

A single day 7 blastocyst was used per sample, and 5–8 embryos were used from each group. Total RNA from the embryos was isolated using the Cells-to-Signal Kit (Ambion Co., USA) according to the manufacturer's protocol. The mRNA levels were quantified using SYBR Premix Ex*Taq*™ II (TaKaRa, Japan) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Carlsbad, CA, USA). Samples were denatured at 95°C for 1 min and then subjected to 40 cycles of amplification (95°C, 5 s; 60°C, 30 s). Fold changes in each gene were calculated using the $2^{-\Delta\Delta CT}$ method. All primers used in this study are shown in Table 1.

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**Table 1.** Primers for qRT-PCR

| Gene | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|---|---|---|
| *$H_2A$* | GTCTTGGAGTACCTGACCGC | AGTCTTCTTCGGGAGCAACA |
| *BAX* | TCTCCCCGAGAGGTCTTTTT | TGATGGTCCTGATCAACTCG |
| *BCL-2* | GGTATTGGTGAGTCGGATCG | CAAGACGACCCGAGGAGAA |
| *CASPASE-3* | GTTCATCCAGGCTCTTTG | TCTATTGCTACCTTTCG |

### Determination of ROS products

To measure ROS levels, zygotes were exposed to $H_2O_2$ for 15 min, and then each group was incubated in SOF supplemented with dichlorodihydrofluorescein diacetate (DCFH-DA; 2 mM, Molecular Probes) for 30 min at 37°C as previously described.

### Determination of adenosine triphosphate (ATP) content

The levels of ATP were measured with a luminometer (Bioluminat Junior, Berthold, Germany) and an ATP Bioluminescence Assay Kit (Beyotime).

### Detection of mitochondrial membrane potential (MMP)

MMP was determined by staining the zygotes with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; BioVision; MA, USA). The zygotes were incubated in SOF supplemented with JC-1 (1.25 μM) for 20 min at 37°C following exposure to $H_2O_2$.

### Oxidant and antioxidant capability assay

In total, 30–50 blastocysts from each group were lysed for 5 min, vortexed for 5 min, then frozen at −80°C and thawed at 37°C three times. The mixture was centrifuged at 10,000 *g* for 10 min at 4°C and placed on ice. The level of malondialdehyde (MDA), total antioxidant capacity (T-AOC), and glutathione (GSH) and the activities of glutathione peroxidase (GSH-Px) and catalase (CAT) were determined according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry Reagent Co., Nanjing, Jiangsu, China).

### Statistical analysis

Data [mean ± standard deviation (SD)] were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). Differences were analyzed using one-way analysis of variance (ANOVA) or the *t*-test. For statistical analysis of the percentage values, the $\chi^2$ test was performed. Differences with a *P*-value $< 0.05$ were considered to be statistically significant.

## Results

### Melatonin protected the development of yak preimplantation embryos from $H_2O_2$-induced oxidative stress

The dose effect of melatonin on embryonic development was first investigated. Hence, we used melatonin at different concentrations and compared the development of melatonin-treated embryos to controls cultured in DMSO. Table 2 shows that melatonin at 1, 10, and 50 μM, had no significant effect on the embryonic development, while melatonin at 100 μM reduced the rate of blastocysts formation. To induce oxidative stress, $H_2O_2$ was used to treat yak zygotes. When zygotes were treated with 40 μM $H_2O_2$, the rates of cleavage stage embryo and blastocyst formation decreased significantly (Table 3). In subsequent experiments, the effect of melatonin on the development of preimplantation embryos was evaluated following treatment of zygotes with 75 μM $H_2O_2$. Table 4 shows that the two-cell, blastocyst and hatched stage formation almost recovered to the levels in the control group at 10 μM melatonin, but when the concentration of melatonin reached 50 μM, the percentage of developmental embryos decreased, especially in the hatched stage. These findings indicated that the effect of melatonin on $H_2O_2$-injured embryos was not linear.

**Table 2.** Development of preimplantation yak embryos in the presence of melatonin

| Treatment (μM) | No. | No. (%) ≥2-cell | No. (%) blastocyst | No. (%) hatched stage |
|---|---|---|---|---|
| 0 (control) | 185 | 131 (70.8 ± 1.6) | 48 (25.7 ± 1.6) | 17 (8.7 ± 0.6) |
| 1 | 223 | 162 (72.7 ± 2.9) | 61 (27.1 ± 1.8) | 19 (8.4 ± 0.9) |
| 10 | 194 | 138 (71.1 ± 1.9) | 54 (27.6 ± 2.0) | 18 (9.2 ± 1.2) |
| 50 | 202 | 141 (70.4 ± 1.7) | 44 (22.4 ± 1.3) | 15 (7.4 ± 1.1) |
| 100 | 215 | 139 (65.2 ± 1.3) | 25 (12.2 ± 0.8)** | 3 (1.5 ± 0.3)** |

**$P < 0.01$ versus control; $\chi^2$ test was used.

### Melatonin protected the quality of blastocysts developed from zygotes exposed to $H_2O_2$

To determine whether the improvement in embryo development reflected enhanced blastocyst quality, we determined the cell numbers and ratio of inner-cell-mass cells (ICM)/total cell number (TCN) in blastocysts in each group; these parameters were the criteria used to assess blastocyst quality. As shown in Fig. 1 and Table 4, zygotes treated with $H_2O_2$ showed decreased numbers of total and ICM cells, as well as the ICM:TCN ratio of blastocysts, when compared with the control group. However, melatonin prevented these effects.

Apoptotic cell death is another criterion for evaluating blastocyst quality. As shown in Fig. 2(*A*, *B*), $H_2O_2$ treatment induced apoptosis in blastocysts compared with the control group. The number of TUNEL-positive cells decreased significantly in blastocysts derived from groups treated with all concentrations of melatonin. To determine the cause of the reduced apoptosis rate, we analyzed the relative expression levels of apoptosis-related genes. The results showed that $H_2O_2$ upregulated caspase-3

**Table 3.** Development of preimplantation yak embryos after treatment with $H_2O_2$

| Treatment (μM) | No. of zygotes | No. (%) ≥2-cell | No. (%) blastocyst | No. (%) hatched stage |
|---|---|---|---|---|
| 0 (control) | 228 | 160 (70.1 ± 1.6) | 58 (26.1 ± 1.4) | 19 (8.4 ± 0.9) |
| 20 | 196 | 136 (69.9 ± 2.5) | 47 (23.3 ± 1.4) | 15 (7.9 ± 0.6) |
| 40 | 214 | 143 (61.7 ± 1.8)* | 31 (14.5 ± 0.9)** | 5 (2.4 ± 0.4)** |
| 80 | 223 | 95 (42.7 ± 2.2)** | 11 (4.8 ± 1.1)** | 0 |
| 120 | 177 | 22 (12.3 ± 1.0)** | 0 | 0 |

*$P < 0.05$, **$P < 0.01$ versus control; $\chi^2$ test was used.

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**Table 4.** Protective effect of melatonin on the development of preimplantation yak embryos exposure to $H_2O_2$

| Groups | Conc. of $H_2O_2$ (μM) | Conc. of melatonin (μM) | No. of zygotes | No. (%) ≥2-cell | No. (%) blastocyst | No. (%) hatched stage |
|---|---|---|---|---|---|---|
| Control | 0 | 0 | 192 | 135 (70.5 ± 1.6) | 52 (26.9 ± 1.6) | 17 (8.9 ± 0.8) |
| $H_2O_2$ | 40 | 0 | 182 | 110 (61.1 ± 1.5)$^a$ | 25 (13.9 ± 1.0)$^b$ | 6 (2.3 ± 0.7)$^b$ |
| Melatonin + $H_2O_2$ | 40 | 1 | 214 | 138 (64.9 ± 0.9) | 37 (18.2 ± 1.4)$^c$ | 12 (5.7 ± 0.5)$^c$ |
| | 40 | 10 | 187 | 127 (68.7 ± 1.3)$^c$ | 44 (23.5 ± 1.1)$^d$ | 14 (7.5 ± 0.9)$^d$ |
| | 40 | 50 | 192 | 128 (67.0 ± 1.2)$^c$ | 43 (22.4 ± 0.8)$^d$ | 6 (3.5 ± 0.4) |

$^aP < 0.05$, $^bP < 0.01$ versus control; $^cP < 0.05$, $^dP < 0.01$ versus $H_2O_2$; $\chi^2$ test was used.

activity (Fig. 2*C*) and expression of the proapoptotic gene *Bax* (Fig. 2*D*), and downregulated antiapoptotic gene *Bcl-2* (Fig. 2*E*), which was preserved by melatonin. Collectively, these data suggested a protective effect of melatonin against apoptosis in blastocysts.

### *Melatonin reduced $H_2O_2$-induced intracellular ROS levels in zygotes*

To determine whether melatonin improved resistance to oxidative stress through regulation of ROS levels, the DCFH fluorescent reaction was performed to evaluate ROS production in zygotes. Compared with the control group, the DCFH fluorescence intensity was much higher in the $H_2O_2$-treated group, whereas it was markedly weakened in the presence of melatonin (Fig. 3*A*, *B*), indicating that melatonin can prevented the increase in ROS induced by $H_2O_2$.

### *Melatonin prevented mitochondrial dysfunction induced by $H_2O_2$ in zygotes*

Mitochondria are the major organelles that produce ROS, and mitochondrial dysfunction compromises embryo development. Therefore, we further investigated the ATP content and MMP in zygotes after $H_2O_2$ exposure. Melatonin treatment significantly recovered the decreased ATP content induced by $H_2O_2$ (Fig. 4*B*). Moreover, the MMP depolarized rapidly in zygotes following treatment with $H_2O_2$, as shown by enhanced green fluorescence and attenuated red fluorescence compared with the control group. However, exposure to melatonin significantly recovered MMP level (Fig. 4*A*, *C*), suggesting that melatonin may maintain mitochondrial function in zygotes following oxidative stress.

### *Melatonin protected preimplantation embryos from oxidative damage by preserving antioxidative enzymes*

$H_2O_2$ is a free radical generator and can lead to a decrease in antioxidant capacity, therefore we first determined the T-AOC in zygotes treated with $H_2O_2$ in the presence or absence of melatonin. $H_2O_2$ treatment decreased T-AOC and melatonin efficiently prevented this effect (Fig. 5*A*). In order to further investigate the effect of melatonin on antioxidant cellular defences, we evaluated the enzymatic and non-enzymatic antioxidants. Figure 5*B*, *C* shows that melatonin exposure significantly restored the decreased activity of GSH-Px and CAT induced by $H_2O_2$. In addition, $H_2O_2$ treatment resulted in a significant decrease in the level of GSH and the main non-enzymatic antioxidants in embryos, whereas melatonin treatment completely restored GSH level (Fig. 5*D*). These data suggested that melatonin may protect preimplantation embryos from oxidative injury by maintaining antioxidative enzymes.

## Discussion

During the development of preimplantation embryos *in vitro*, oxidative stress has a deleterious effect on fertilization and embryo quality (Kim *et al.*, 2017). The positive effects of antioxidants have been reported in relation to several reproductive aspects (Barbato *et al.*, 2017; Do *et al.*, 2017). The current study confirmed that the addition of melatonin significantly improved the development of yak zygotes to the blastocyst stage in the presence of $H_2O_2$ and decreased apoptosis in blastocysts, which suggested that melatonin had a protective effect against oxidative stress in yak preimplantation embryos.

In physiological conditions, cells can maintain their dynamic balance between ROS production and elimination. Low concentrations of ROS have been shown to reduce DNA fragmentation and improve the developmental potential of embryos (Takahashi *et al.*, 2013). However, an imbalance in these processes results in oxidative stress, and is associated with defective embryo development (Cheng *et al.*, 2011). The antioxidative capacity of melatonin has been shown in several other cell types, but this study is the first to report the effects of melatonin on the

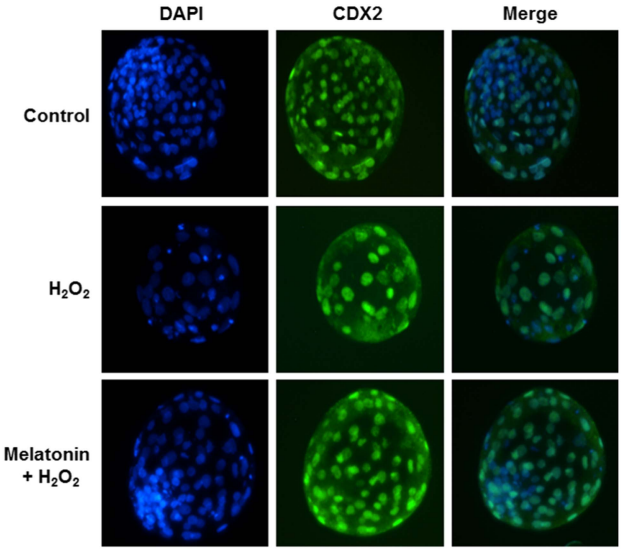


**Figure 1.** Immunostaining of Caudal Type Homeobox 2 (CDX2). Representative images of yak blastocysts was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and CDX2 (a marker for trophectoderm) after treatment with $H_2O_2$ (40 μM) or $H_2O_2$ (40 μM) plus melatonin (10 μM) during the zygote stage. Scale bar = 100 μm. $n$ = 29, 34, and 33 in the Control, $H_2O_2$ and Melatonin + $H_2O_2$ group, respectively.

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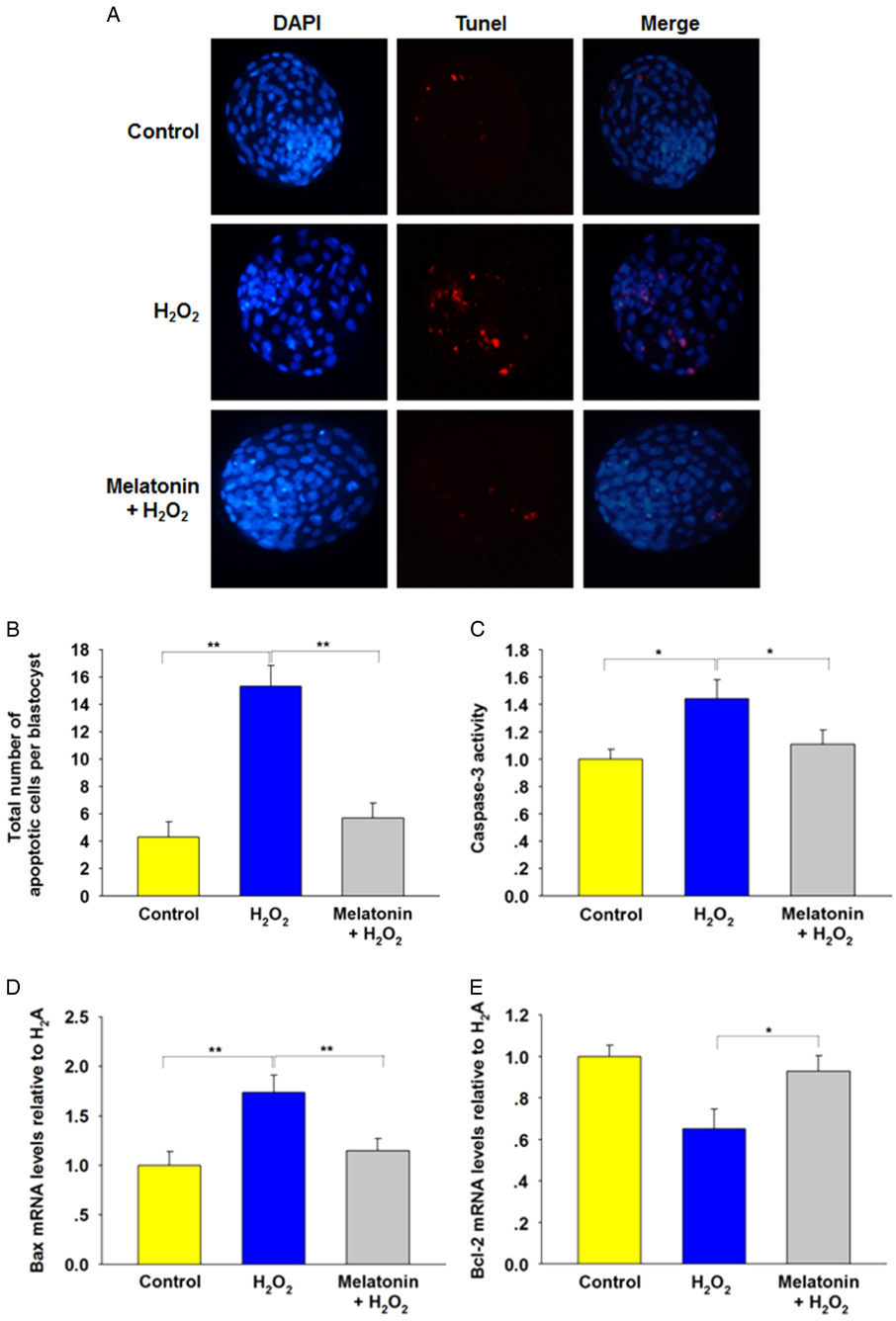


**Figure 2.** Incidence of apoptosis in blastocysts. The treatment concentrations of $H_2O_2$ and melatonin during zygote stage were 40 μM and 10 μM respectively. (*A*) TUNEL assay of blastocysts (red). Each sample was counterstained with DAPI to visualize DNA (blue). Scale bar = 100 μm. $n$ = 34, 31, and 36 in the Control, $H_2O_2$ and Melatonin + $H_2O_2$ group, respectively. (*B*) Total number of apoptotic cells in each blastocyst. Data are presented as mean ± standard deviation (SD). (*C*) Caspase-3 activity. (*D*) Relative expression levels of Bax. (*E*) Relative expression levels of Bcl-2. *$P < 0.05$, **$P < 0.01$.

development of yak preimplantation embryos under oxidative stress (Goswami and Haldar, 2014; Czechowska *et al*., 2015; Zhou *et al*., 2015). Melatonin exposure greatly improved the quality of $H_2O_2$-treated embryos, indicated by reduced oxidative damage, reduced apoptosis and increased blastocyst formation. The beneficial effects of melatonin may be attributed to its amphiphilic nature, thereby enabling it to penetrate all morphophysiological barriers and enter all subcellular compartments to scavenge intracellular ROS.

In addition, the current study demonstrated that not only the production rate but also embryo quality was increased by melatonin treatment. A high cell number/blastocyst ratio indicated that the embryo efficiently proceeded through mitosis and was likely to undergo to easy implantation resulting in pregnancy. The blastocysts produced following melatonin treatments had higher mean cell number/blastocysts than those in the $H_2O_2$-treated group. The ratio of ICM/TCN is another criterion for evaluating blastocyst quality (Moreno *et al*., 2015). The present results showed that $H_2O_2$ treatment decreased ICM cells and the ratio of ICM/TCN compared with the control group. Under oxidative stress, melatonin minimized the negative influence of $H_2O_2$ on embryo quality. Notably, we found that pretreatment with melatonin (100 μM) exhibits adverse effects on early yak embryo development *in vitro*, as shown by embryo development arrest.

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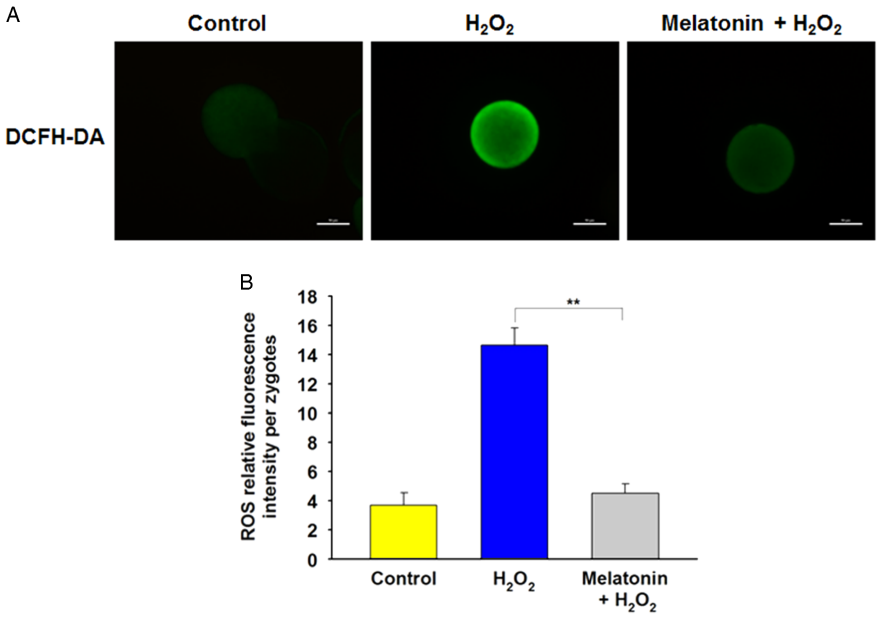


**Figure 3.** Melatonin decreases $H_2O_2$-induced intracellular ROS level in yak zygotes. (*A*) Representative photomicrographs of yak zygotes from $H_2O_2$ (40 μM), or $H_2O_2$ (40 μM) plus melatonin (10 μM) group stained with DCHFDA to detect ROS levels. (*B*) Quantification of relative fluorescence intensity of ROS per zygote. Scale bar = 100 μm. Data are presented as the mean ± standard deviation (SD) from three independent experiments. $n$ = 27, 31, and 28 in the Control, $H_2O_2$ and Melatonin + $H_2O_2$ group, respectively.

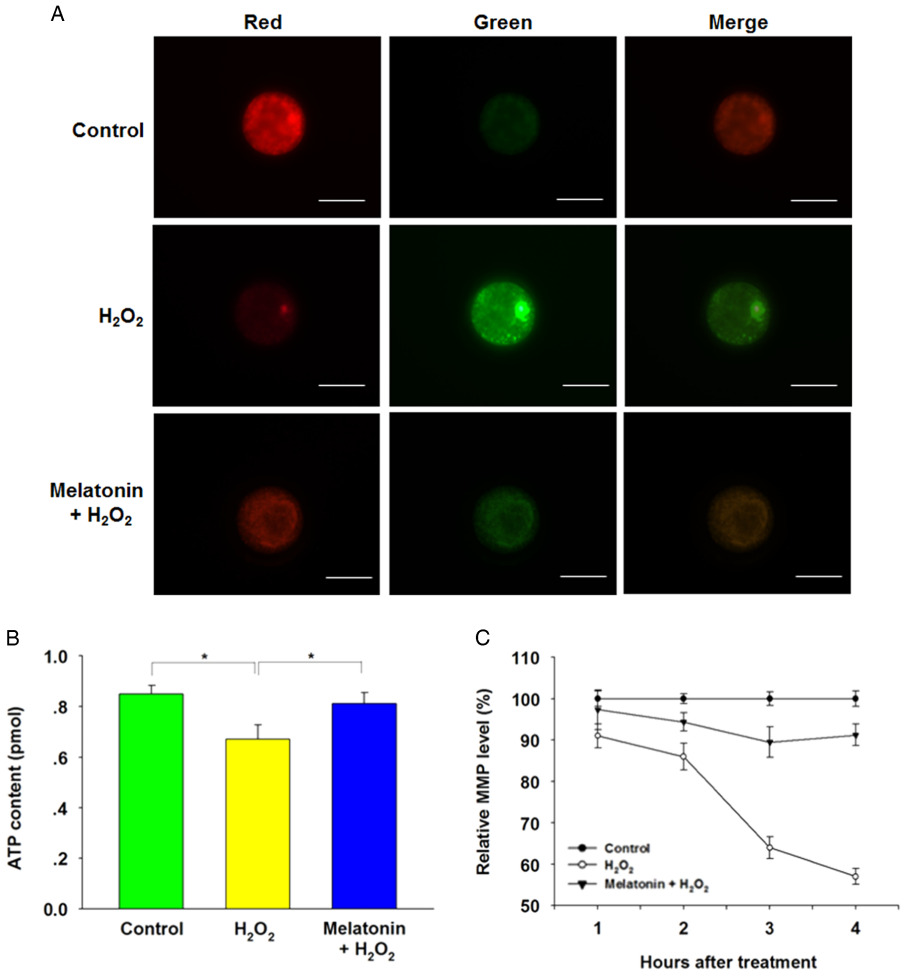


**Figure 4.** Melatonin prevented $H_2O_2$-induced mitochondrial dysfunction in yak zygotes. The treatment concentration of $H_2O_2$ and melatonin were 40 μM and 10 μM respectively. (*A*) Representative images of inner mitochondrial membrane potential (MMP) in yak zygotes. Zygotes were stained with JC-1. Red fluorescence represented J-aggregates (high polarized mitochondria) and green fluorescence represented monomer form of JC-1 (low polarized mitochondria). (*B*) ATP content in yak zygotes after treated with $H_2O_2$, or $H_2O_2$ plus melatonin. (*C*) The ratios of intensity of red /green fluorescence (MMP) were analyzed from the images. The average of MMP from zygotes in the control group at each time point was set at 100%. Scale bar = 100 μm. Data are presented as mean ± standard deviation (SD) from three independent experiments. $n$ = 19 for each group.

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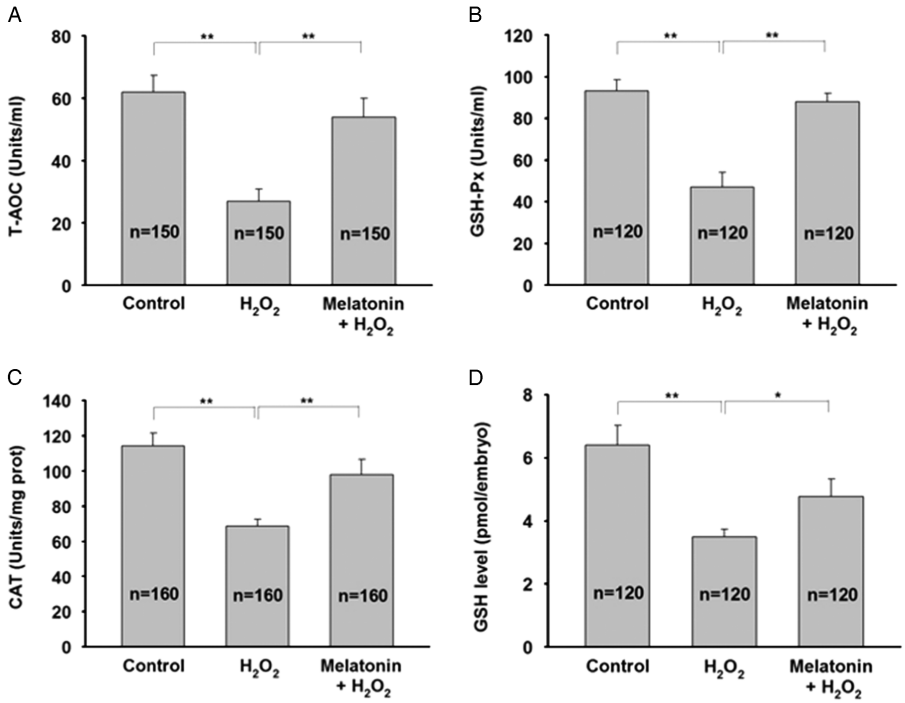


**Figure 5.** Melatonin attenuates $H_2O_2$-induced oxidative injury in yak zygotes by sparing antioxidative enzymes. Zygotes were pretreated with 10 μM melatonin for 1 h followed by addition of 40 μM $H_2O_2$ for 15 min, and then cultured in the presence of melatonin until day 7. (*A*) T-AOC activity, (*B*) GSH-Px activity, (*C*) CAT activity, and (*D*) GSH level were measured. Data are presented as mean ± standard deviation (SD) from three independent experiments. *n* indicates the total number of treated zygotes. $^*P < 0.05$, $^{**}P < 0.01$.

This is not surprising because relatively high concentrations of melatonin has the anti-proliferative effect in human leukemia, breast cancer and colon cancer cells. In fact, a current study also demonstrated that relatively low concentrations of melatonin exhibit beneficial effects on bovine oocyte maturation *in vitro*, while melatonin retards bovine oocyte maturation and embryo development at high concentrations (Tian *et al*., 2014).

In addition to scavenging ROS, another powerful antioxidant property of melatonin involves maintaining stable mitochondrial function (Areti *et al*., 2017; Maarman *et al*., 2017; Sharafati-Chaleshtori *et al*., 2017). During early embryo development, mitochondrial dysfunction may contribute to both cell cycle arrest and apoptosis (Chowdhury *et al*., 2017; Liu *et al*., 2017). As important parameters of mitochondrial function, MMP and ATP content have been used as indicators of cell apoptosis (Abe *et al*., 2013). In the present study, we found that administration of $H_2O_2$ led to dissipation of MMP and ATP content, and this effect was blocked by melatonin, which was consistent with the results of a previous study. Moreover, the results of the TUNEL assays supported our findings on the mRNA transcripts for genes related to apoptosis. Melatonin treatment was found to strongly downregulate caspase-3 activity and Bax expression. The expression of these genes was also lower in the melatonin treatment group compared with the $H_2O_2$ control. Bcl-2 is an important member of the anti-apoptotic family and plays a critical role in the regulation of apoptosis (Pan *et al*., 2015). In the current study, we observed that the Bcl-2 expression level in granulosa cells under thermal stress was significantly upregulated by melatonin treatment. These results indicated that melatonin was probably preventing apoptosis not only due to its antioxidant effects but also its ability to regulate gene expression.

In embryogenesis, GSH-Px, an enzymatic antioxidant, is highly expressed in most cells and tissues and protects the embryos from oxidative stress (Huo *et al*., 2017; Hamdy *et al*., 2017; Delwing-de Lima *et al*., 2017). In addition to GSH-Px, CAT is another enzymatic antioxidant expressed in embryos and its vital role as an antioxidant has been demonstrated using knockout and transgenic approaches in mice (Rahman *et al*., 2009; Avdesh *et al*., 2011). In addition to enzymatic defences, GSH has a non-enzymatic antioxidative defence and detoxifies $H_2O_2$ via the action of GSH-Px, and plays a prominent role in maintaining the redox status in embryos (Takahashi, 2012). Our results showed that melatonin not only strongly restored T-AOC and GSH-Px activity to levels similar to those in the controls under oxidative stress, but also partially recovered CAT activity. Moreover, we found that melatonin maintained intracellular GSH at high levels, which was consistent with the results of a previous study. Collectively, these data showed that melatonin may protect yak embryos against ROS by sparing antioxidative enzymes.

In summary, our results showed that under oxidative stress, yak zygotes exposed to melatonin maintained stable mitochondrial function, increased T-AOC, GSH-Px and CAT activities and decreased ROS levels, thereby improving embryo quality *in vitro*. These results provided a potential method for preventing oxidative damage in yak preimplantation embryos.

In conclusion, the results obtained from the studies provided new information regarding the mechanisms by which melatonin protects the development of yak preimplantation embryos from oxidative stress, which provides an important reference for *in vitro* embryo production of yak.

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**Ethical standards.** All animal experiments and treatments were approved and supervised by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. Ovaries of slaughtered cattle were obtained from Datong Abattoir, a local slaughterhouse located in Qinghai, China.

## References


**Abe Y, Sakairi T, Beeson C and Kopp JB** (2013) TGF-β1 stimulates mitochondrial oxidative phosphorylation and generation of reactive oxygen species in cultured mouse podocytes, mediated in part by the mTOR pathway. *Am J Physiol Renal Physiol* **305**, F1477–90.

**Areti A, Komirishetty P, Akuthota M, Malik RA and Kumar A** (2017) Melatonin prevents mitochondrial dysfunction and promotes neuroprotection by inducing autophagy during oxaliplatin-evoked peripheral neuropathy. *J Pineal Res* **62**, doi: 10.1111/jpi.12393.

**Asghari MH, Abdollahi M, de Oliveira MR and Nabavi SM** (2017) A review of the protective role of melatonin during phosphine-induced cardiotoxicity: focus on mitochondrial dysfunction, oxidative stress and apoptosis. *J Pharm Pharmacol* **69**, 236–43.

**Avdesh A, Wong P, Martins RN and Martin-Iverson MT** (2011) Memory function in a mouse genetic model of Alzheimer's disease. *J Alzheimers Dis* **25**, 433–44.

**Barbato V, Talevi R, Braun S, Merolla A, Sudhakaran S, Longobardi S and Gualtieri R** (2017) Supplementation of sperm media with zinc, d-aspartate and co-enzyme Q10 protects bull sperm against exogenous oxidative stress and improves their ability to support embryo development. *Zygote* **25**, 168–75.

**Cheng NH, Zhang W, Chen WQ, Jin J, Cui X, Butte NF, Chan L and Hirschi KD** (2011) A mammalian monothiol glutaredoxin, Grx3, is critical for cell cycle progression during embryogenesis. *FEBS J* **278**, 2525–39.

**Chowdhury SR, Ray U, Chatterjee BP and Roy SS** (2017) Targeted apoptosis in ovarian cancer cells through mitochondrial dysfunction in response to *Sambucus nigra* agglutinin. *Cell Death Dis* **8**, e2762.

**Czechowska G, Celinski K, Korolczuk A, Wojcicka G, Dudka J, Bojarska A and Reiter RJ** (2015) Protective effects of melatonin against thioacetamide-induced liver fibrosis in rats. *J Physiol Pharmacol* **66**, 567–79.

**Delwing-de Lima D, Sasso S, Dalmedico L, Delwing-Dal Magro D, Pereira EM and Wyse ATS** (2017) Argininic acid alters markers of cellular oxidative damage *in vitro*: Protective role of antioxidants. *Exp Toxicol Pathol* **69**, 605–11.

**Do GY, Kim JW, Park HJ, Yoon SB, Park JY, Yang SG, Jung BD, Kwon YS, Kang MJ, Song BS, Kim SU, Chang KT and Koo DB** (2017) Native plants (*Phellodendron amurense* and *Humulus japonicus*) extracts act as antioxidants to support developmental competence of bovine blastocysts. *Asian-Australas J Anim Sci* **30**, 1245–52.

**Goswami S and Haldar C** (2014) UVB irradiation severely induces systemic tissue injury by augmenting oxidative load in a tropical rodent: efficacy of melatonin as an antioxidant. *J Photochem Photobiol B* **141**, 84–92.

**Hamdy SM, Shabaan AM, Abdel Latif AKM, Abdel-Aziz AM and Amin AM** (2017) Protective effect of hesperidin and tiger nut against acrylamide toxicity in female rats. *Exp Toxicol Pathol* **69**, 580–8.

**Huo X, Liu C, Gao L, Xu X, Zhu N and Cao L** (2017) Hepatoprotective effect of aqueous extract from the seeds of *Orychophragmus violaceus* against liver injury in mice and HepG2 cells. *Int J Mol Sci* **18**, pii E1197.

**Kim MG, Kim DH, Lee HR, Lee JS, Jin SJ and Lee HT** (2017) Sirtuin inhibition leads to autophagy and apoptosis in porcine preimplantation blastocysts. *Biochem Biophys Res Commun* **488**, 603–8.

**Liu LF, Qin Q, Qian ZH, Shi M, Deng QC, Zhu WP, Zhang H, Tao XM and Liu Y** (2014) Protective effects of melatonin on ischemia-reperfusion induced myocardial damage and hemodynamic recovery in rats. *Eur Rev Med Pharmacol Sci* **18**, 3681–6.

**Liu J, Zhang Y, Chen L, Yu F, Li X, Dan T, Zhao J and Zhou S** (2017) Polyphyllin I induces G2/M phase arrest and apoptosis in U251 human glioma cells via mitochondrial dysfunction and the JNK signaling pathway. *Acta Biochim Biophys Sin (Shanghai)* **49**, 479–86.

**Loren P, Sanchez R, Arias ME, Felmer R, Risopatron J and Cheuqueman C** (2017) Melatonin scavenger properties against oxidative and nitrosative stress: impact on gamete handling and *in vitro* embryo production in humans and other mammals. *Int J Mol Sci* **18**, pii E1119.

**Maarman GJ, Andrew BM, Blackhurst DM and Ojuka EO** (2017) Melatonin protects against uric acid-induced mitochondrial dysfunction, oxidative stress, and triglyceride accumulation in $C_2C_{12}$ myotubes. *J Appl Physiol* **122**, 1003–10.

**Marinho LS, Sanches BV, Rosa CO, Tannura JH, Rigo AG, Basso AC, Pontes JH and Seneda MM** (2015) Pregnancy rates to fixed embryo transfer of vitrified IVP *Bos indicus*, *Bos taurus* or *Bos indicus* × *Bos taurus* embryos. *Reprod Domest Anim* **50**, 807–11.

**Mata-Campuzano M, Alvarez-Rodriguez M, del Olmo E, Fernandez-Santos MR, Garde JJ and Martinez-Pastor F** (2012) Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37°C in presence of several antioxidants. *Theriogenology* **78**, 1005–19.

**Mehrzadi S, Safa M, Kamrava SK, Darabi R, Hayat P and Motevalian M** (2016) Protective mechanisms of melatonin against hydrogen-peroxide-induced toxicity in human bone-marrow-derived mesenchymal stem cells. *Can J Physiol Pharmacol* **95**, 773–86.

**Moreno D, Neira A, Dubreil L, Liegeois L, Destrumelle S, Michaud S, Thorin C, Briand-Amirat L, Bencharif D and Tainturier D** (2015) *In vitro* bovine embryo production in a synthetic medium: embryo development, cryosurvival, and establishment of pregnancy. *Theriogenology* **84**, 1053–60.

**Nikseresht M, Toori MA, Rahimi HR, Fallahzadeh AR, Kahshani IR, Hashemi SF, Bahrami S and Mahmoudi R** (2017) Effect of antioxidants (β-mercaptoethanol and cysteamine) on assisted reproductive technology *in vitro*. *J Clin Diagn Res* **11**, BC10–14.

**Pan Y, Cui Y, Baloch AR, Fan J, He J, Li G, Zheng H, Zhang Y, Lv P and Yu S** (2015) Insulinlike growth factor I improves yak (*Bos grunniens*) spermatozoa motility and the oocyte cleavage rate by modulating the expression of Bax and Bcl-2. *Theriogenology* **84**, 756–62.

**Rahman M, Halade GV, Bhattacharya A and Fernandes G** (2009) The fat-1 transgene in mice increases antioxidant potential, reduces pro-inflammatory cytokine levels, and enhances PPAR-γ and SIRT-1 expression on a calorie restricted diet. *Oxid Med Cell Longev* **2**, 307–16.

**Sharafati-Chaleshtori R, Shirzad H, Rafieian-Kopaei M and Soltani A** (2017) Melatonin and human mitochondrial diseases. *J Res Med Sci* **22**, 2.

**Sirard MA** (2017) The influence of *in vitro* fertilization and embryo culture on the embryo epigenetic constituents and the possible consequences in the bovine model. *J Dev Orig Health Dis* **8**, 411–7.

**Song C, Peng W, Yin S, Zhao J, Fu B, Zhang J, Mao T, Wu H and Zhang Y** (2016) Melatonin improves age-induced fertility decline and attenuates ovarian mitochondrial oxidative stress in mice. *Sci Rep* **6**, 35165.

**Takahashi M** (2012) Oxidative stress and redox regulation on *in vitro* development of mammalian embryos. *J Reprod Dev* **58**, 1–9.

**Takahashi T, Inaba Y, Somfai T, Kaneda M, Geshi M, Nagai T and Manabe N** (2013) Supplementation of culture medium with l-carnitine improves development and cryotolerance of bovine embryos produced *in vitro*. *Reprod Fertil Dev* **25**, 589–99.

**Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, Xu J, Ji P and Liu G** (2014) Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. *J Pineal Res* **57**, 239–47.

**Tian X, Wang F, Zhang L, Ji P, Wang J, Lv D, Li G, Chai M, Lian Z and Liu G** (2017) Melatonin promotes the *in vitro* development of microinjected pronuclear mouse embryos via its anti-oxidative and anti-apoptotic effects. *Int J Mol Sci* **18**, pii E988.

**Zhou L, Zhao D, An H, Zhang H, Jiang C and Yang B** (2015) Melatonin prevents lung injury induced by hepatic ischemia-reperfusion through anti-inflammatory and anti-apoptosis effects. *Int Immunopharmacol* **29**, 462–7.