

Using cysteine/cystine to overcome oxidative stress in goat oocytes and embryos cultured *in vitro*

ZHENGRONG ZHOU^{1,2*}, RUO-XIN JIA^{1*}, GUOMIN ZHANG¹, YONGJIE WAN¹, YANLI ZHANG¹, YIXUAN FAN¹, ZIYU WANG¹, PAN HUANG² and FENG WANG¹

¹Jiangsu Livestock Embryo Engineering Laboratory, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095; ²Department of Histology and Embryology, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013, P.R. China

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Abstract. Assisted reproductive techniques expose gametes to excessive concentrations of reactive oxygen species. The present study aimed to evaluate the effects of oxidative stress on apoptosis in goat oocytes and embryonic development. The results demonstrated that the addition of 100 μM hydrogen peroxide (H_2O_2) into media produces an oxidative environment during oocyte maturation. The number of cumulus cells positive for terminal deoxynucleotidyl transferase UTP nick end labeling, and the activity of caspase 3 in mature oocytes were increased, compared with the control group ($P < 0.05$). In addition, the expression levels of mitochondrial regulators, including peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) and nuclear respiratory factor-1 (NRF-1) were increased in the oxidative oocytes, compared with those in the control group ($P < 0.05$). The ratio of the proapoptotic gene, B cell lymphoma (Bcl-2)-associated X protein (BAX), to the anti-apoptotic gene, BCL-2, was higher in the H_2O_2 group, compared with the control group ($P < 0.05$). To overcome oxidative stress in oocytes and embryos cultured *in vitro*, 200 μM cysteine and 200 μM cystine were added to the media, thereby increasing the concentration of intracellular glutathione (GSH) and assisting in maintaining the redox state of the cells. In conclusion, cysteine and cystine reduced the oxygen tension caused by H_2O_2 , thereby providing a novel strategy for optimizing *in vitro* embryonic development systems.

Introduction

Mammalian oocytes are exposed to oxidative conditions (1). The oxidative modification of cell components caused by the increased production of reactive oxygen species (ROS) (2) compromises membrane integrity, and causes structural and functional changes in proteins, as well as damage to nucleic acids (3). ROS accelerate oocyte aging, lower oocyte quality, and induce apoptosis in oocytes and early embryonic cells (3). Therefore, increased ROS production negatively affects cell function and contributes significantly to several diseases, including those which damage reproduction and fertility (4). Accumulating evidence has demonstrated that oocytes are major sources of ROS, as they use oxygen to produce energy through mitochondrial oxidative phosphorylation (5). ROS production is higher during oocyte *in vitro* maturation (IVM) than during *in vivo* maturation (5). The major ROS, H_2O_2 , is produced intracellularly during several pathophysiologic processes, and causes oxidative damage. Exposure to exogenous H_2O_2 (25-300 μM) reportedly induces apoptosis in rat oocytes (1) and mouse zygotes (6). However, whether direct H_2O_2 exposure induces apoptosis in goat oocytes cultured *in vitro* remains to be elucidated.

Evidence has shown that glutathione (GSH) is involved in maintaining cellular redox states. Thus, the GSH content of oocytes protects the zygote and early embryos from oxidative damage prior to genomic activation (7). The addition of cysteine to oocyte culture media has been shown to increase intracellular levels of GSH in buffaloes (8,9), pigs (10) and goats (11). However, the effects of supplementation with a specific concentration of cysteine depend on the dose, the species and the maturation medium (2). The cystine content of TCM-199 (83.2 mM), a widely used medium, is sufficient for oocyte IVM, although the mechanism underlying its antioxidative capacity remains to be elucidated (12). The balance between ROS production and antioxidant defense is critical to the function of granulosa cells and oocytes (13,14). The oocyte environment has a marked effect on the outcomes of assisted reproductive technologies (15). Cysteine is unstable and readily oxidized into cystine in the medium; therefore, the addition of cysteine and cystine to the medium decreases levels of oxidative stress on oocytes cultured *in vitro*.

Correspondence to: Mr. Yongjie Wan, Jiangsu Livestock Embryo Engineering Laboratory, College of Animal Science and Technology, Nanjing Agricultural University, Weigang 1, Nanjing, Jiangsu 210095, P.R. China
E-mail: lhwswan@126.com

*Contributed equally

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The present study aimed to determine the effects of H₂O₂, with and without cysteine and cystine, on the apoptosis of mature goat oocytes cultured *in vitro*. The effects examined included DNA damage, caspase-3 activity, mitochondria regulators and the gene expression of B cell lymphoma-2-associated X protein (BAX). In addition, the intracellular concentration of GSH in oocytes and their subsequent parthenogenetic embryonic development capacities were determined.

Materials and methods

Reagents. The chemicals used to culture the oocytes and embryos in the present study were obtained from Sigma-Aldrich (St. Louis, MO, USA), whereas media (including, TCM199, FBS, formaldehyde and Triton X-100) were from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA). The RNeasy[®] Micro kit (cat. no. 74004) was purchased from QIAGEN (Valencia, CA, USA). SYBR Premix Ex Taq (cat. no. DRR420A) and the PrimeScript RT reagent kit with gDNA Eraser (cat. no. DRR047S) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The One-Step Terminal Deoxynucleotidyl Transferase UTP Nick End Labeling (TUNEL) apoptosis assay kit (cat. no. C1088), the GSH and GSSG assay kits (cat. no. S0053), and the Caspase 3 activity assay kit (cat. no. C1116) were purchased from Beyotime Institute of Biotechnology (Haimen, China). All other chemicals were obtained commercially and were of reagent grade. All experiments were performed in accordance with the Guidelines for the Care and Use of Animals of the College of Animal Science and Technology, Nanjing Agricultural University (Nanjing, China) (16).

Preparation of H₂O₂, cystine and cysteine working solutions. The working solutions of H₂O₂ (Sigma-Aldrich; cat. no. H3410), cystine (Sigma-Aldrich; cat. no. C7602) and cysteine (Sigma-Aldrich; cat. no. C5360) were prepared freshly prior to use. Briefly, 3.5 μ l of 30% H₂O₂ was diluted in 1 ml of IVM culture media (30 mM). Subsequently, 10 and 12 μ l of this solution were further diluted in 3 ml of culture media (100 and 120 μ M). The 100 μ M H₂O₂ solution was diluted into further concentrations of 60 and 80 μ M. The cysteine stock solution (20 mM) and the cystine stock solution (100 mM) were diluted in culture media to obtain final concentrations of 100, 200 and 500 μ M. Thus, four IVM culture conditions were used for the oocytes in the present study, as follows: i) control (IVM media without chemical agent), ii) H₂O₂ (IVM media with H₂O₂), iii) CC (IVM media with cystine and cysteine), and iv) H₂O₂ and CC (IVM media with H₂O₂ and CC).

Oocyte collection. Goat ovaries were collected from a slaughterhouse (Haimen goat Research Center; Haimen, Nantong, China), delivered to the laboratory within 3-4 h and washed with normal saline. The connective tissues and the attached oviducts were removed. The ovaries were transferred into Petri dishes and the 2-6 mm follicles were sectioned. Cumulus-oocyte complexes (COCs) appropriate for IVM were selected for IVM culture. The base medium for IVM comprised modified TCM199 supplemented with 10 μ g/ml follicle-stimulating hormone luteinizing hormone, estrogen

(all Sigma-Aldrich) and 10% FBS. This media was further supplemented with 100 μ M cysteine and 100 μ M cystine or 100 μ M H₂O₂, according to the experimental design. The selected COCs were cultured for 20-22 h at 38.5°C under a saturated 5% CO₂ atmosphere.

Oocyte recovery. Following IVM, the COCs were treated with 0.1% hyaluronidase to strip their cumulus cells by repeated pipetting through a narrow-bore pipette in culture media. Good-quality MII oocytes with a uniform cytoplasm and a well-extruded polar body were selected to assess for parthenogenetic activation.

In vitro culture. The parthenogenetic embryos were treated with TCM 199 containing 5 μ M ionomycin for 5 min, incubated in TCM 199 containing 2 mM 6-DMAP for 4 h, and washed three times in 100 μ l of M16 medium. The oocytes were then transferred into 100 μ l M16 droplets under mineral oil and cultured for 6 days at 38.5°C under 5% CO₂. Cleavage and development to the blastocyst stage were observed on day 2.5 (day of parthenogenetic activation=day 0.5) and day 6.5, respectively. Blastocysts, which developed on day 6.5, were fixed to examine their cell numbers. The total number of blastocysts were determined under a Nikon Eclipse TE2000 fluorescence microscope (Nikon Corporation, Tokyo, Japan) following Hoechst 33342 staining (Sigma-Aldrich; cat. no. B-2261).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Mature oocytes (30 oocytes per group) were collected to analyze the expression levels of genes associated with the mitochondria and apoptosis in goat oocytes. Total mRNA was extracted using the RNeasy[®] Micro kit, according to the manufacturer's protocol. For the RT, total mRNA with a final volume of 20 μ l (containing 0.5 mg oligo-dT, 1X RT buffer, 10 mM dithiothreitol and 10 mM dNTP) was reverse transcribed at 37°C for 50 min and at 70°C for 15 min, and the products were stored at 4°C until use. The qPCR was performed using a qPCR reagent kit with gDNA Eraser. The total reaction volume of 20 μ l contained 2 μ l of 5X gDNA Eraser Buffer, 1 μ l of gDNA Eraser, 1 μ g of total RNA, 4 μ l of 5X RT Buffer, 1 μ l of qPCR enzyme mix, 1 μ l of RT Primer Mix, and sufficient nuclease-free H₂O. The qPCR was performed at 42°C for 2 min, 37°C for 15 min, followed by a denaturation step at 85°C for 15 sec and cooling on ice. The primer sequences for each gene are shown in Table I. The tests were performed in triplicate, and the mRNA levels in each sample were normalized to the mRNA level of GAPDH.

Analysis of gene expression levels using qPCR. All transcripts were quantified by qPCR using an ABI 7300 PRISM system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification products of PGC-1 α , NRF-1, BAX, Bcl-2 and GAPDH were detected using SYBR green staining. The GenBank accession numbers and primer sequences used to amplify the target genes are presented in Table I.

The qPCRs were run in triplicate in a total volume of 20 μ l, consisting of SYBR Premix Ex Taq, ROX Reference Dye, 200 nM of each gene-specific primer and 100 ng equivalent cDNA. The amplification conditions were as follows: DNA polymerase activation at 95°C for 30 sec, followed by

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene and reference sequence (GenBank accession no.)	Amplicon primer sequence	Annealing size (bp)	Temperature (°C)
NRF-1 (AY368269)	F: 5'-AGGCTGGGGCAAAGAAAG-3' R: 5'-CCAACCTGGATAAGCGAGAC-3'	303	58.0
PGC-1 α (AY321517)	F: 5'-CACCCACAACCTCCTCCTCAT-3' R: 5'-GCCTTCCTTTCCTCGTGTC-3'	232	58.0
BAX (XM_002701934.1)	F: 5'-GCATCCACCAAGAAGCTGAG-3' R: 5'-CCGCCACTCGGAAAAAGAC-3'	130	58.0
BCL2 (NM_001166486.1)	F: 5'-ATGTGTGTGGAGAGCGTCA-3' R: 5'-AGAGACAGCCAGGAGAAATC-3'	182	58.0
GAPDH (NM001034034)	F: 5'-CGACTTCAACAGCGACTCAC-3' R: 5'-CCCTGTTGCTGTAGCCAAATTC-3'	118	58.0

NRF-1, nuclear respiratory factor-1; PGC-1 α , proliferator-activated receptor γ coactivator-1 α ; BCL2, B cell lymphoma 2; BAX, Bcl-2-associated X protein; F, forward; R, reverse.

40 amplification cycles of 95°C for 5 sec and 58°C for 31 sec. At the end of the amplification cycles, melting curve analysis was performed to verify gene-specific amplification. The comparative CQ method (17) was used to quantify the relative expression levels of the target genes. For ease of comparison, the average expression level of each gene from the control group was set as one (16).

Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay. The TUNEL method was performed using a One-Step TUNEL Apoptosis Assay kit, as previously described. Briefly, the control and experimental oocytes (20 oocytes per group) undergoing shrinkage were fixed in 3.7% formaldehyde in PBS for 1 h at 18-20°C. Following fixation, the oocytes were washed in PBS and permeabilized by incubation in 0.1% (v/v) Triton X-100 for 2 min at 4°C. The oocytes were then washed twice in PBS and incubated with the TUNEL labeling media in the dark for 1 h at 37°C. Following counterstaining with 10 μ g/ml Hoechst 33342 for 10 min at 37°C to label all the nuclei, the oocytes were mounted with light coverslip compression and observed under a microscope.

GSH and oxidized glutathione (GSSG) assay. The GSH concentrations were measured in oocytes matured *in vitro* under four IVM culture conditions, according to the GSH and GSSG Assay kit. The oocytes (30 oocytes per group) were carefully denuded by repeated pipetting in 0.1% (w/v) hyaluronidase (Sigma-Aldrich) and washed several times in polyvinyl alcohol-PBS to remove any trace of materials with thiol groups. The oocytes were stored in a microtube at -20°C until they were assayed (30 oocytes per tube). All procedures complied with the manufacturer's protocol. Briefly, GSH was assayed using 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. The GSSG was measured by measuring the 5-thio-2-nitrobenzoic acid (TNB) produced from the reaction of reduced GSH with DTNB. The rate of TNB formation was measured at 412 nm using a Multiskan™

FC Microplate Photometer (Thermo Fisher Scientific, Inc.), over 3 min. The reduced GSH concentration in the sample was calculated by subtracting the GSSG from GSH.

Caspase-3 activity assay. Caspase-3 activity was analyzed in the control and experimental oocytes using a Caspase-3 Activity kit, with Ac-DEVD-pNA as the colorimetrically-specific substrate. Briefly, 30 oocytes from each group were washed twice in PBS and then lysed in 100 μ l of chilled lysis buffer (Gibco) at 4°C for 10 min. The lysate was centrifuged at 20,000 x g at 4°C for 10 min, and the supernatant was incubated for 7 h at 37°C with 10 μ l of caspase-3 substrate. Substrate cleavage was measured using an Hitachi Fluorescence Spectrophotometer F-7000 (Hitachi, Ltd., Tokyo, Japan) at 405 nm and was corrected to the protein content in the lysate. The caspase-3 activity is presented as the fold increase in optical density/30 oocytes.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean of triplicate samples. All percentages were subjected to arcsine square root transformation prior to statistical analysis. The data were analyzed using either Student's *t*-test or one-way analysis of variance followed by a multiple *t*-test. All statistical analysis was performed using SPSS (version 17.0; SPSS, Inc. Chicago, IL, USA). *P*<0.05 was considered to indicate a statistically significant difference.

Results

Optimal concentrations of H₂O₂ and CC for oxidative stress and goat parthenogenetic embryonic development. H₂O₂ induces ROS generation, causing oxidative stress. CC is usually used to improve GSH synthesis, as GSH protects cells against the destructive effects of ROS. To determine the optimal concentrations of H₂O₂ and CC, the present study evaluated goat oocyte maturation rate and parthenogenesis blastocyst formation rate. The oocytes treated with 100 μ M H₂O₂ exhibited significantly decreased maturation rates and blastocyst rates,

Table II. Effect of oxidative stress on goat oocyte development following parthenogenetic activation.

Treatment	Oocytes examined (n)	Maturation rate	Cleavage rate	Blastocyst formation rate	Cells/blastocysts (n)
Control	768	509 (65.71±2.74) ^a	321 (62.37±1.16) ^a	67 (20.57±0.61) ^a	92±2.1 ^a
H ₂ O ₂	854	464 (49.47±2.65) ^b	255 (65.86±4.69) ^a	35 (13.47±1.11) ^b	75±3.6 ^b
CC	1024	786 (78.36±2.83) ^c	500 (66.82±4.52) ^a	113 (23.06±0.59) ^a	99±3.4 ^a
H ₂ O ₂ +CC	760	524 (69.96±3.51) ^a	317 (62.88±1.90) ^a	63 (19.19±0.53) ^a	90±2.7 ^a

Data in brackets are the mean percentage ± standard error of the mean. Data not in brackets indicate the number of oocytes or embryos. Superscript letters are statistically different (P<0.05); ^aP<0.05 vs. control group; ^bP<0.05 vs. control group, CC group and H₂O₂+CC group; ^cP<0.05 vs. control group, H₂O₂ group and H₂O₂+CC group. H₂O₂, hydrogen peroxide; CC cystine and cysteine.

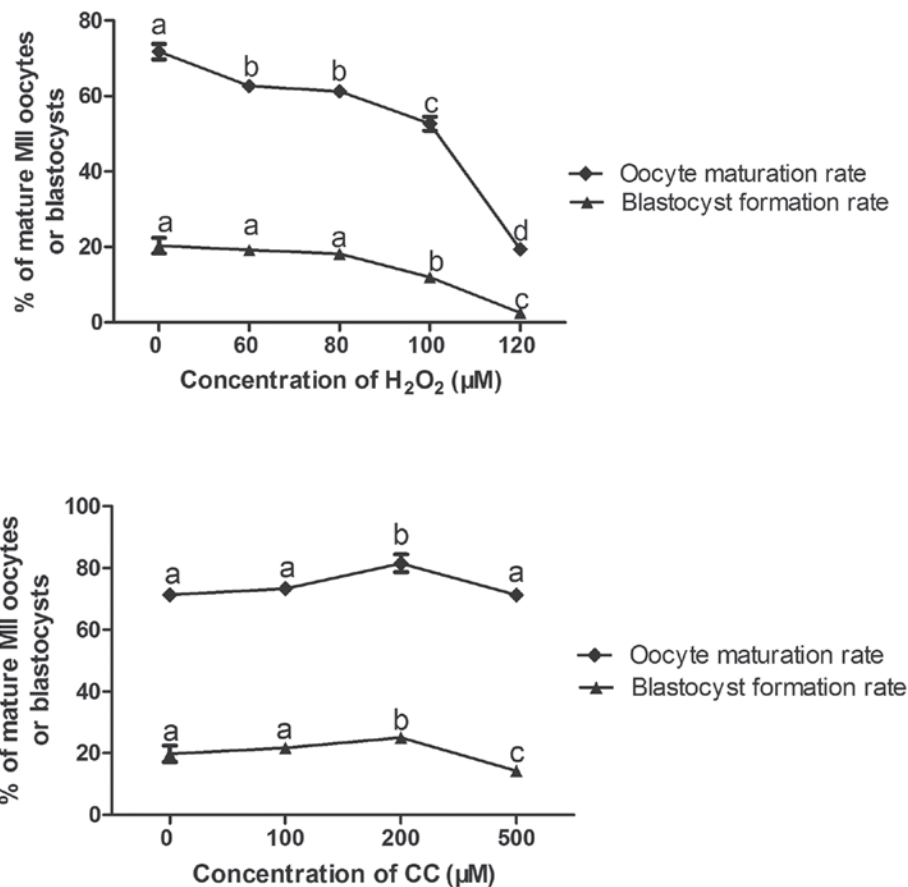


Figure 1. Optimal concentrations of H₂O₂ and CC and the parthenogenetic development of embryos. Data are expressed as the mean ± standard error of the mean. Means with different superscript letters are statistically different (P<0.05). Oocyte maturation rate: ^aP<0.05 vs. group 0; ^bP<0.05 vs. group 0, 100 µM and 120 µM; ^cP<0.05 vs. group 0, 60 µM, 80 µM and 120 µM; ^dP<0.05 vs. group 0, 60 µM, 80 µM, 100 µM and 120 µM. Blastocyst formation rate: ^aP<0.05 vs. group 0; ^bP<0.05 vs. group 0, 60 µM, 80 µM and 120 µM; ^cP<0.05 vs. group 0, 60 µM, 80 µM and 100 µM. For Optimal concentrations of CC: Oocyte maturation rate: ^aP<0.05 vs. group 0; ^bP<0.05 vs. group 0, 100 µM and 500 µM. Blastocyst formation rate: ^aP<0.05 vs. group 0; ^bP<0.05 vs. group 0, 100 µM and 200 µM; ^cP<0.05 vs. group 0, 100 µM and 500 µM. H₂O₂, hydrogen peroxide; CC cystine and cysteine.

compared with the control group (52.67±1.81, vs. 71.78±2.06 and 11.98±1.27, vs. 20.29±2.06, respectively; P<0.05; Fig. 1). However, oocytes treated with 120 µM H₂O₂ exhibited marked decrease in oocyte maturation rates and blastocyst formation rates (19.34±1.43 and 2.49±1.36, respectively), and the high concentration of H₂O₂ resulted in a low survival rate of oocytes and thus, the remaining surviving oocytes were insubstantial for the completion of subsequent experiments. Thus, 100 µM H₂O₂ was considered the optimal concentration for oxidative

stress. In addition, when 200 µM cysteine and 200 µM cystine were added to the IVM media, the oocyte maturation rate and blastocyst formation rate were higher, compared with those the control group (81.57±2.86, vs. 71.41±1.11% and 25.04±1.34, vs. 19.80±2.66%, respectively; P<0.05).

Effect of oxidative stress on goat oocyte development following parthenogenesis. Culturing the goat oocytes in M199 with H₂O₂ decreased the oocyte maturation rate and

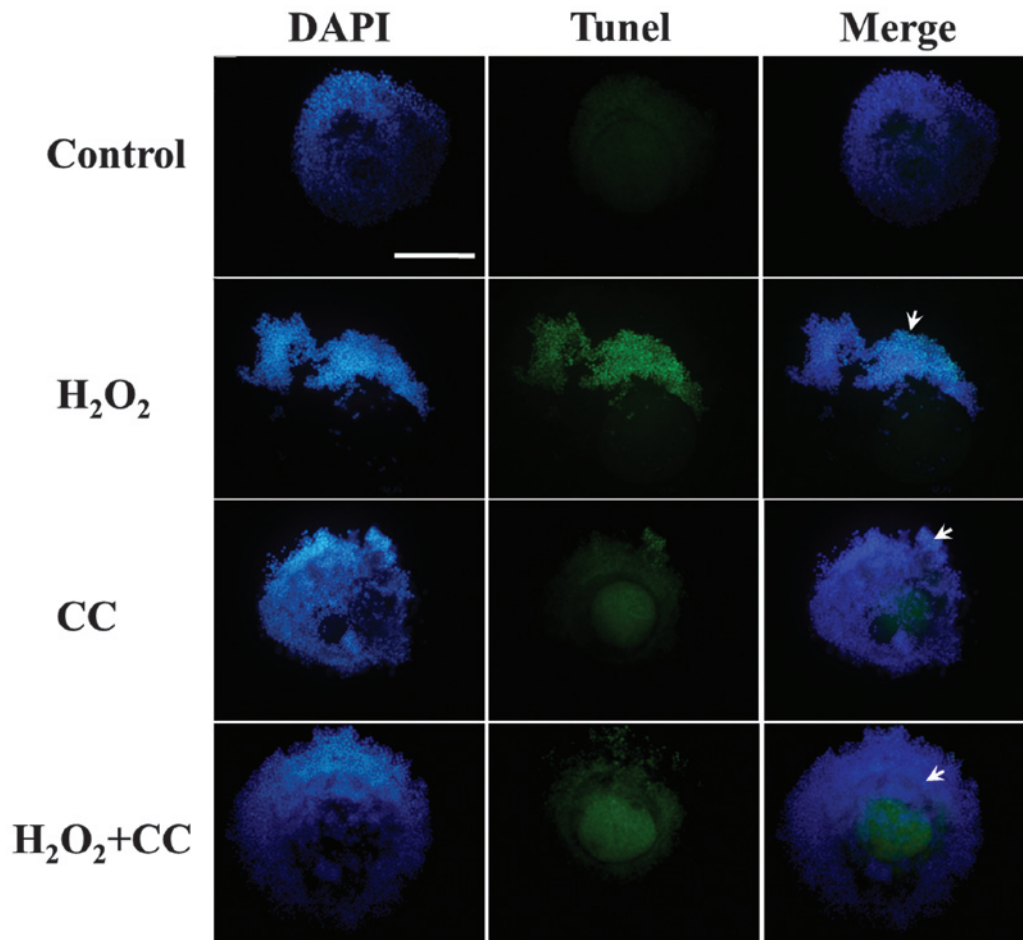


Figure 2. Representative images of the TUNEL assays of goat cumulus-oocyte complexes. The white arrowhead indicates a TUNEL-positive cumulus cell. Apoptosis occurred primarily in the cumulus cells. Blue color, DAPI-stained nuclei; green color, TUNEL-positive cells. The white arrowhead indicates a TUNEL-positive cumulus cell. The control group showed no green color; however, in the H_2O_2 group, strong immunostaining is visible primarily in the cumulus cells. The CC group also showed some positive fluorescence. In H_2O_2+CC group, the TUNEL-positive signal is reduced compared with that in the H_2O_2 group. Blue color: DAPI-stained nuclei. Green color: TUNEL-positive cells. Scale bar=100 μm . TUNEL, terminal deoxynucleotidyl transferase UTP nick end labeling; H_2O_2 , hydrogen peroxide; CC cystine and cysteine.

blastocyst formation rate, compared with the control group ($49.47 \pm 2.65\%$ vs. 65.71 ± 2.74 and 13.47 ± 1.11 vs. $20.57 \pm 0.61\%$ respectively; $P < 0.05$; Table II). The oocyte maturation and blastocyst formation rates in the $CC+H_2O_2$ group were higher, compared with those in the H_2O_2 group (69.96 ± 3.51 , vs. $49.47 \pm 2.65\%$ and 19.19 ± 0.53 , vs. $13.47 \pm 1.11\%$, respectively; $P < 0.05$). The oocyte maturation rate and the blastocyst rate in the CC group were also higher, compared with those in the H_2O_2 group (78.36 ± 2.83 , vs. $49.47 \pm 2.65\%$ and 23.06 ± 0.59 , vs. $13.47 \pm 1.11\%$, respectively; $P < 0.05$). Furthermore, the mean number of cells per blastocyst in the H_2O_2 group was significantly lower, compared with those in the other three groups ($P < 0.05$), although the embryonic cleavage rates did not differ among the four groups ($P > 0.05$).

Effect of oxidative stress on apoptosis of COCs following maturation. A TUNEL assay was used to evaluate the quality and viability of the goat oocytes cultured in M199 with stress inducers. The goat oocytes treated with H_2O_2 exhibited significantly increased DNA fragmentation, compared with the control group (121 ± 1.89 cells/COC, vs. 7.8 ± 1.32 cells/COC, respectively; $P < 0.05$), as shown in Figs. 2 and 3A). The H_2O_2

media containing CC further reduced the number of apoptotic cells (36 ± 1.13 cells/COC), similar with the CC group (25 ± 2.10 cells/COC). These findings indicated that oxidative stress induced apoptosis during goat oocyte IVM.

GSH concentrations of goat oocytes cultured in oxidative maturation media with and without CC. The mean intracellular GSH concentrations in the MII oocytes treated with H_2O_2 were significantly lower, compared with those in the control groups (0.31 ± 0.18 , vs. 3.18 ± 0.38 pmol/oocyte; $P < 0.05$; Fig. 3B). The addition of CC to the media significantly increased the GSH concentrations, compared with the control and H_2O_2 groups (7.53 ± 0.41 vs. 0.31 ± 0.18 and 3.18 ± 0.38 pmol/oocyte, respectively; $P < 0.05$). In addition, the GSH concentration of the MII oocytes in the CC group was higher, compared with those of the control and H_2O_2 groups (8.47 ± 0.46 , vs. 0.31 ± 0.18 and 3.18 ± 0.38 pmol/oocyte, respectively; $P < 0.05$).

Activity of caspase-3 activity in goat oocytes cultured in oxidative maturation media with and without CC. To confirm whether caspase-3 is involved in the IVM of goat

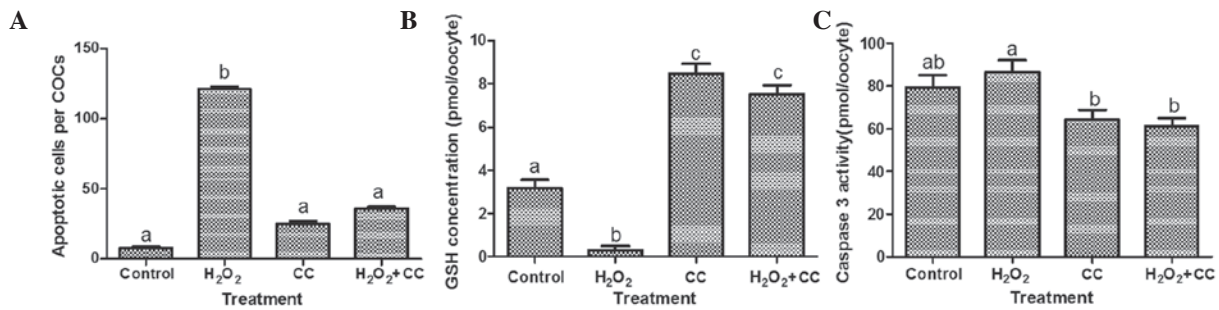


Figure 3. Effects of oxidative stress on levels of apoptosis, intracellular GSH concentrations and caspase-3 activity in goat oocytes following maturation. (A) Number of TUNEL-positive cells in goat cumulus-oocyte complexes, presented as the number of TUNEL-positive nuclei. (B) Intracellular GSH concentrations in the goat oocytes. (C) Caspase-3 activity in the goat oocytes. Data are expressed as the mean \pm standard error of the mean. Means with different superscript letters are statistically different ($P < 0.05$). In (A) ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group, CC group, and H₂O₂+CC group. In (B) ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group, CC group, and H₂O₂+CC group; ^c $P < 0.05$ vs. control group and H₂O₂ group. In (C) ^{a,b} $P < 0.05$ vs. H₂O₂ group. GSH, glutathione; TUNEL, terminal deoxynucleotidyl transferase UTP nick end labeling; H₂O₂, hydrogen peroxide.

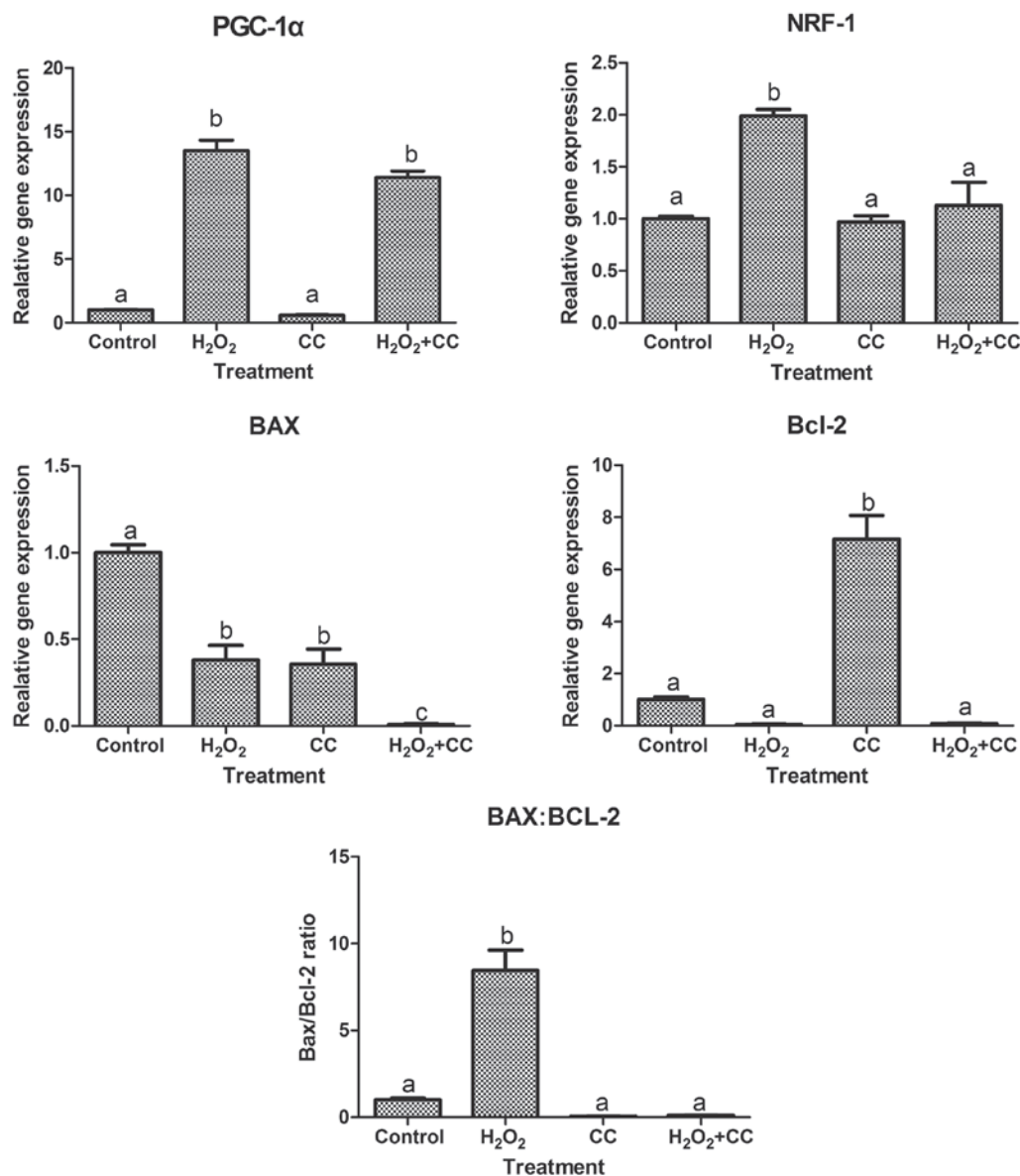


Figure 4. Gene expression levels in goat oocytes, determined using reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean \pm standard error of the mean. Means with different superscript letters are statistically different ($P < 0.05$). For PGC-1, ^a $P < 0.05$ vs. group control; ^b $P < 0.05$ vs. group control and group CC. NRF-1, ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group, CC group and H₂O₂+CC group. BAX, ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group and H₂O₂+CC group, ^c $P < 0.05$ vs. control group, H₂O₂ group and CC group. Bcl-2, ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group, H₂O₂ group, and group H₂O₂+CC. BAX:Bcl-2, ^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. control group, CC group and H₂O₂+CC group. H₂O₂, hydrogen peroxide; CC, cystine and cysteine; PGC-1 α , proliferator-activated receptor γ coactivator-1 α , NRF-1, nuclear respiratory factor-1; Bcl-2, B cell lymphoma 2; BAX, Bcl-2-associated X factor.

oocytes in H₂O₂-induced oxidative media, caspase-3 activity was measured using its specific substrate, Ac-DEVD-MCA. The activity of caspase-3 in the MII oocytes with H₂O₂ was 86.4±5.71 pmol/oocyte, which was significantly higher than the caspase-3 activities of the oocytes in the CC group and the H₂O₂+CC group (64.3±4.54 and 15±2.46 pmol/oocyte, respectively; P<0.05; Fig. 3C).

Gene expression in goat oocytes cultured in oxidative maturation media with and without CC. The expression levels of selected genes were determined in MII oocytes during IVM, following treatment with H₂O₂ and/or CC. The transcription levels were highly variable in all treatment groups. A comparable pattern of mRNA expression was observed for all transcripts analyzed, although certain differences were noted (Fig. 4). The expression levels of PGC-1 α and NRF-1 were significantly higher in the H₂O₂ group, compared with the control group (P<0.05). Adding CC to the oxidative media significantly downregulated the expression of PGC-1 α and the expression of NRF-1 (P<0.05). The gene transcription levels of BAX were significantly lower in the H₂O₂ group, compared with the control group (P<0.05). The gene expression of BCL-2 in the oocytes of the CC group was upregulated, compared with the other three groups (P<0.05). Furthermore, the BAX:BCL-2 ratio in the H₂O₂ group was higher, compared with the other three groups (P<0.05).

Discussion

Mammalian embryonic development *in vitro* is affected by culture conditions. In particular, ROS are predominantly generated from the environment surrounding embryos. Therefore, several studies have attempted to reduce levels of oxidative stress, which causes defects in embryonic development. The present study demonstrated that the oocytes exposed to 200 μ M cysteine and 200 μ M cystine exhibited improved development, compared with the oocytes in the other treatment groups. By contrast, increasing the concentrations of cysteine and cystine to 500 μ M significantly decreased blastocyst formation. This result indicated that higher concentrations of cysteine and cystine do not improve embryonic development, possibly due to high cystine concentrations disrupting the redox equilibrium in cells (18). However, a previous bovine study suggested that adding 1.2 mM of cysteine to IVM media inhibits the production of ROS in oocytes and the apoptosis of cumulus cells (19). Different culture media, and the combination of cysteine and cystine may affect the concentrations used. Salmen *et al* confirmed that supplementing cysteine to oocyte culture media increases the intracellular concentration of GSH in oocytes, and promotes pronuclear formation and embryonic development in mice (20). Yoshida *et al* found that adding cysteine to the maturation medium of pig oocytes increases GSH synthesis, producing GSH concentrations comparable to those found in oocytes matured *in vivo* (21). These data suggest that culturing early embryos *in vitro* with cysteine and cystine provides more suitable conditions for embryonic growth and development. In the present study, 100 μ M H₂O₂ was used to induce oocyte oxidative stress. Adding cysteine and cystine to the oocytes under oxidative conditions improved oocyte maturation and the parthenogenesis of embryonic development, and

also demonstrated that the addition of cysteine and cystine at appropriate concentrations during oocyte *in vitro* culture reduced oxygen tension and improved blastocyst development.

Cysteine, is arises in mammals through the metabolism of pantetheine, a component of coenzyme A (22). Cystine is essential for GSH synthesis, and it directly increases the GSH content of cells and tissues. The GSH content is increased through increased synthesis by adaptive mechanisms to oxidative stress. However, severe oxidative stress suppresses GSH levels through deactivation of these adaptive mechanisms (23). GSH functions as an antioxidant through two mechanisms: i) it functions as an ROS scavenger by reacting with certain species; and ii) it acts as an inhibitor of transcription factor nuclear factor- κ B activation, thereby controlling the synthesis of various enzymes (24,25). Acute increases in levels of oxidative stress in cells significantly increases cellular apoptosis, which can be attenuated by pretreatment with GSH-ethyl ester (26). In the present study, supplementation with cysteine and cystine under oxidative conditions decreased the number of apoptotic cells and decreased caspase-3 activity, which suggested that increasing GSH prevented apoptosis of the goat oocytes. Kizhakkayil *et al* further demonstrated that GSH depletion induces caspase-dependent and caspase-independent apoptosis in human leukemic cells (27).

The mitochondria reportedly have anti-apoptotic and antioxidant functions in germ cells and granulosa cells (28,29). The mitochondrial regulators, NRF-1 and PGC-1 α , interact to regulate oxidative metabolism (30,31). By contrast, endotoxin-induced oxidative stress causes the mitochondrial translocation of the pro-apoptotic protein, Bax, increases the total expression of Bax and downregulates the expression of the antiapoptotic protein, BCL-2 (32). In the present study, PGC-1 α and NRF-1 were expressed at higher levels under oxidative conditions, compared with the other groups, possibly due to the oocytes requiring mitochondrial biogenesis and a high metabolism when exposed to oxidative stress (33). In addition, the BAX/BCL-2 ratio was highest in the oxidative stress group, which suggested that the mitochondrial pathway may be involved in the apoptosis mediated by H₂O₂-induced oxidative stress (34).

In conclusion, the results of the present study suggested that the addition of appropriate concentrations of cysteine and cystine reduces the oxygen tension triggered by H₂O₂ and improves parthenogenetic blastocyst development. Apoptosis is involved in this process via the mitochondrial pathway. These results may improve current understanding of the apoptotic mechanisms involved in oxidative stress, and provide novel insights into optimizing embryonic *in vitro* development systems.

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