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Relationship Between the Level of Reactive Oxygen Species and Apoptosis Pathways Through Activation of Caspase Family in Mouse Preimplantation Embryos

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DXM, ZSL and NZF Perceived and design the study. DXM, YYF,HZJ and QGJ performed the experiments. HXL, HS. OU wrote the manuscript. IA prepared the draft version for submission.

Key words
Reactive oxygen species, mouse embryo, apoptosis, Caspase genes

ABSTRACT

In mammals, the embryonic developmental block phenomena mostly occur in the early stages, which negatively affect the quality of embryos and finally lead to apoptosis. Reactive oxygen species (ROS) is one of the basic causes of cell apoptosis. Caspase family activates the apoptosis induced by different pathways, but the relation of the caspase family and the level of ROS stress is unclear. The purpose of this study was to evaluate the effects of ROS on the activation of the caspase family in embryo development. Our finding shows that the ROS level in H_2O_2 treated group was significantly higher ($P<0.05$) compared to *in vivo* and *in vitro* derived embryos. The RNA expression level of caspase family related genes (*caspase-3, 8, 9 and 12*) was significantly higher ($P<0.05$) in H_2O_2 treated group compared to *in vivo* and *in vitro* derived embryos, where there is no significant change in the RNA expression of caspase-9. Similarly, the RNA expression level of pre-apoptotic gene BAX was high ($P<0.05$) and BcL2 was low ($P<0.05$) in H_2O_2 treated group as compare to other groups. Our result demonstrates that induced H_2O_2 enhance the RNA expression level of caspase genes, apoptosis related genes by increasing the intracellular ROS level in mouse preimplantation embryo.

INTRODUCTION

In the embryo transfer technology methods, the *in vitro* fertilization (IVF) and embryo culture is an important process. Although in IVF, all the steps have to be done precisely to get a positive result, the embryo culture stage is more vital to establish the success rate of IVF (McElroy *et al*., 2008). The success rate of the preimplantation embryo from fertilization to the blastocyst stage has not yet been successfully accomplished in most species during *in vitro* culture condition (Nasr-Esfahani et al., 1992). Various studies revealed the mischievous effect of embryo culture *in vitro* conditions, almost 47.9% in human (Lim *et al*., 2007) and 50% in bovine (Hardy *et al*., 2001) embryos culture *in vitro* has been arrested and shown to have chromosomal abnormalities. Intracellular reactive oxygen species (ROS) play a critical role in embryo development, such as maintain genomic stability, but detrimental environmental factors (such as drugs or various pathological conditions) can lead to increased activity of ROS thus it leads to DNA damage, follicular atresia, embryo aging, gene mutation and even apoptosis (Fruehauf and Meyskens, 2007).

During *in vivo* environment, the cell has endogenous factors to protect themselves from ROS by activating antioxidant enzymes such as superoxide dismutase and catalase to protect the cell from apoptosis (Pourova *et al*., 2010). Moreover, Pandya *et al*. (2013) reported that an increased level of antioxidant can protect the cell from apoptosis. It is eminent that ROS enhance the cell

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apoptosis, however but the interaction between the ROS and apoptosis molecular mechanisms remain indistinct (Scherz-Shouvaland Elazar, 2011). There are 3 distinguish apoptotic pathway that is (a) the first apoptosis signal (FAS) receptor or tumor necrosis factor (TNF) receptor are present on the surface of the cell that causes apoptosis by activation of caspase 8, (b) mitochondrial apoptosis pathway: mitochondrial signaling pathway are activated by BCL2 family, When the *BAX* gene is activated its lead to the cytochrome C release from cytoplasm. The cytochrome C combined with apoptotic enzyme activation factor 1 (APAF-1), ATP and pro-caspase 9. (c) The endoplasmic reticulum (ER) stress caused by an imbalance of the Ca^{2+} and proteins, result in the accumulation of the unfolded protein in ER lumen, which leads to unfolded response pathway activation. Moreover, prolonged ER stress can cause the activation of caspase-12, thus activated caspase-9-dependent cycles and to perform apoptosis (Rao *et al*., 2004).

In most cell types caspase family is responsible for the initiation of apoptosis and is precisely regulated by a series of phosphorylation kinase. The intracellular level of ROS may depend on caspase family (Peng and Jou, 2010). The most important member in the caspase family is caspase 3, due to its core status in the process of apoptosis (Pan and Berk, 2007) and due to its versatility and ability with many different bases (Fifre *et al*., 2006). In the exogenous apoptosis pathway caspase 8 play the main role in the initiation of apoptosis due to the signals from proximal death receptor such as FAS receptor and activities of caspase family or mitochondrial apoptosis pathway (Tentori *et al*., 2002). In the mitochondrial-mediated apoptosis signaling, caspase-9 is thought to play a central role (Rao *et al*., 2004). It was found that caspase-12 as an apoptotic protein of the caspase family plays a vital role in ER stress-mediated apoptosis pathway, and reported that caspase-12 initiates apoptosis induced by ER stress (Di Sano *et al*., 2006). Caspase-12 is a proximal cysteine enzyme and an important mediator of cell apoptosis caused by ER stress and is an apoptosis-inducing gene as well as an apoptotic initiation factor (Song *et al*., 2008).

In vitro the level of ROS is high as compared to *in vivo* and ultimately it negatively effects the embryo development and enhances apoptosis, so this study was designed to confirm that these negative effects of ROS is due to caspase family activation.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used in this study were purchased from Sigma Aldrich chemical company (St. Louis, MO, USA) unless otherwise indicated. The entire experimental procedure was approved by the institutional animal care and use committee of University.

Super ovulation and embryo collection

Kunming female mouse 8-10 weeks old were housed under conditions of 12-12 h light and dark at approximately 24°C. Female mouse was injected with intraperitoneal 10 IU of pregnant mare serum gonadotropin (PMSG; Ninbo hormone Co., Ltd., Ninbo China), followed by 10 IU human chorionic gonadotropin (hCG; Ninbo Hormone Co., Ltd.) 48 h later. Immediately after the administration of hCG, each female mouse was mated with a male mouse. After 22 h after the administration of hCG, the mouse with vaginal plugs was sacrificed. Their oviducts were collected and placed in the M_2 medium. Embryos were released from the oviducts under a stereo microscope. Two group of the embryo (1st group with H_2O_2 and 2nd group without H_2O_2) were used for further analysis, in the first group embryo were cultured in 50 µl M_{16} medium containing 30µmol/L H_2O_2 for 30 min. Then the pretreated embryos was washed with fresh M_2 medium to remove H_2O_2 and transferred into M_{16} medium covered with mineral oil. In the second group, embryos were cultured in the M_{16} medium. Then entire embryos were incubated at 37°C under water-saturated 5% CO_2 in the air. Embryonic development was observed every 24 h. Each experiment was repeated at least three times.

Measurement of the ROS level

Mouse embryos at 2-cell, 4-cell and blastocyst stage from *in vivo*, *in vitro* and H_2O_2 treated groups were washed twice in polyvinyl alcohol (PVA) (1 mg/ml) and then placed in a 50 µl drop of 2', 7'-dichlorodihydro-fluoroscein diacetate (DCHFDA) (10 mol/L). The embryos were incubated for 15 min in dark at 37°C in a humidified atmosphere of 5% CO_2. Embryos were then washed twice with phosphate-buffered saline (PBS) and examined under an epifluorescent microscope (Leica DM IRM, Leica, Wetzlar, Germany) equipped with blue-light (535 nm) excitation. The images were analyzed by ImageJ 1.49 software-(Pro plus 6.0) (National Institutes of Health, Bethesda, MD, USA). The experiment was performed three times.

RNA isolation and cDNA synthesis

For PCR analysis, *in vivo* and *in vitro* embryos at the 2-cell, 4-cell and blastocyst stage were collected. Total RNA was isolated from whole embryos using the (Qiagen RNeasy mini kit, Germany) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription using the prime script™ RT reagent kit with gDNA Eraser (Takara BiotechnologyCo.,

Ltd., Dalian, China) according to the manufacturer's instructions. PCR was carried out in 25 µl reaction volumes containing 13 µl of 2× Taq PCR master mix (Tiangen Biotech Co., Ltd., Beijing, China), 2 µl of cDNA, 0.5 µl of forward and reverse primers (5 mM) (Table I), and 9 µl of sterile H_2O (Tiangen Biotech Co., Ltd.). Following the initial preincubation step at 95°C for 3 min, the reaction consisted of 35 cycles of denaturation at 95°C for 30 sec., annealing at 56–62°C for 30 sec, and extension at 72°C for 30 sec. The final extension was performed at 72°C for 5 min. The PCR products were separated by electrophoresis on 2% agarose gels. The band intensities were analyzed by lane 1D analysis software (Beijing sage creation science Co., Ltd., Beijing, China). Three biological replicates were performed.

Statistical analysis

Statistical analysis was conducted with the Statistical Package for Social Science (Windows version 15, SPSS Inc., Chicago, IL, USA). The data was implanted as mean ± SD. The data was analyzed by using one-way analysis of variance (one-way ANOVA). The differences were considered significant at $P < 0.05$.

RESULTS

Internal ROS content in mouse embryos

The intracellular ROS level plays a key role in the development of preimplantation embryos (Ali *et al.*, 2017). We assessed the ROS level at 2-cell, 4-cell and blastocyst stage. Our results showed that there is no significant difference between H_2O_2 and *in vitro* groups (*P<0.05*) but a significant difference was observed *in vivo* group (*P<0.05*). During embryo development H_2O_2 and *in vitro* group the ROS level was higher (*P<0.05*) at the 2-cell, 4-cell and blastocyst stage compared to *in vivo*. Moreover, in the *in vitro* group compared to *in vivo* group the ROS level at 4-cell stage was higher compared to 2-cell and blastocyst stage but at 2-cell ROS level was slightly higher compared to blastocyst stage as shown in Figure 1.

Morphological observation of mouse embryos

We developed mouse embryo in different reactive oxygen levels. Our result shows that 30µmol/L H_2O_2 negatively affects the morphology of blastocyst as shown in Figure 2.

Effects of exogenous H_2O_2 on the RNA expression of caspase-3, 8, 9 and 12 genes in mouse early embryo

The RNA expression level of caspase genes was observed at the 2-cell stage of the mouse embryo. Our result shows that the RNA expression level of caspase-3, 8, 9 and 12 was significantly higher ($P<0.05$) in 30µmol/L H_2O_2 treated group as compared to *in-vivo* and *in-vitro* groups, whereas there is no significant difference was observed in the RNA expression level of caspase-3,8,9 and 12 in *in-vitro* and *in-vivo* groups as shown in Figure 3.

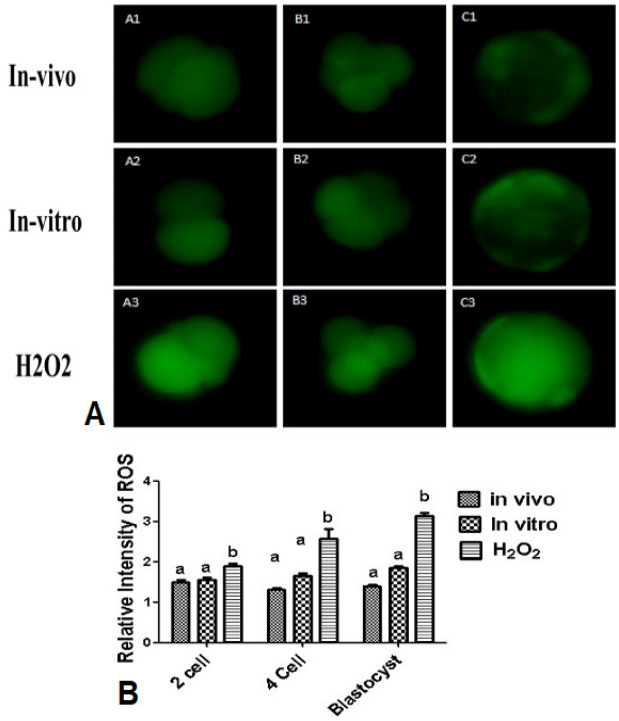

Fig. 1. Evaluation of ROS content in embryos at 2-cell, 4-cell and blastocyst stage in different H_2O_2 level by staining with DCFH-DA. (A) Fluorescence microscopy of embryos cultured in different H_2O_2 level (B). Quantification of ROS levels in the indicated groups. Fluorescence intensity analysis demonstrates a higher significance (P >0.05) embryo exposed to H_2O_2. Images are presented at 400× magnification. Data are expressed as a mean ± standard error of the mean (SEM) of three independent experiments. Asterisks represent statistically significant differences (P >0.05).

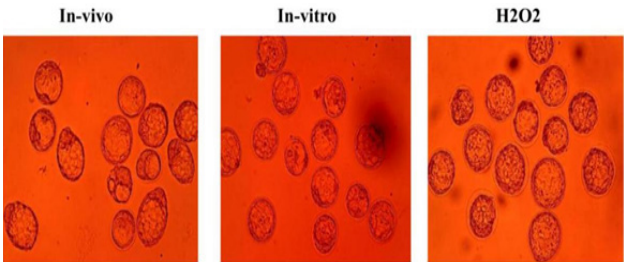

Fig. 2. Effect of different ROS level on the blastocyst morphology.

Table I. Information on the primers used for amplification.

Gene	Sequence 5`→3`	Tm °C	length(bp)
Caspase-3	F GGCCTGAAATACCAAGTCAGGAA	59	1178
	R CCATGGCTTAGAATCACACACACA		
Caspase-8	F GTCGTCTATGGAACGGATGG	59	279
	R ATCTCGGTAGGAAACGCAGT		
Caspase-9	F CAACTTGGACCGTGACAAAC	62	444
	R ATGACCACCACAAAGCAGTC		
Caspase-12	F TGGAAGGTAGGCAAGACT	59	518
	R ATAGTGGGCATCTGGGTC		
BAX	F CCAGGATGCGTCCACCAA	60	195
	R AAGTAGAAGAGGGCAACCAC		
BCL2	F ACCTCTTCAGGGATGGGG	60	144
	R GCCGGTTCAGGTACTCAG		
GAPDH	F TGTGTCCGTCGTGGATCTGA	59	357
	R TTGCTGTTGAAGTCGCAGGAG		

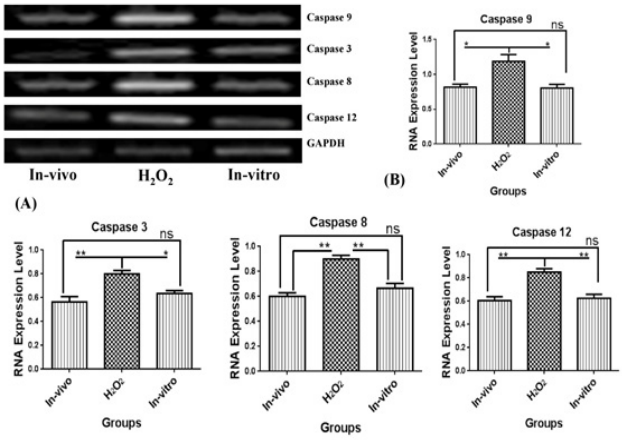

Fig. 3. The expression of caspase3,8,9 and 12 at 2-cell stage mouse embryos in different H_2O_2 condition (GAPDH) was used as an internal control. (A) PCR results of caspase3,8,9 and 12 in mouse embryos. (B) Relative expression caspase 3, 8, 9 and 12 normalized with the internal marker GAPDH (P >0.05). Data are expressed as mean ± SEM of three independent experiments. Asterisks represent statistically significant differences (P >0.05).

To further explore the effect of different reactive oxygen level we detect the RNA expression level of caspase genes at the 4-cell stage of the mouse embryo. Our result shows that the RNA expression level of *caspase-3, 8, 9 and 12* was significantly higher (*P<0.05*) in 30µmol/L H_2O_2 treated group as compare to *in-vivo*. No significant difference was observe in the RNA expression level of caspase 12 in 30µmol/L H_2O_2 treated group as compared to *in-vitro* where a significant difference was observed in the RNA expression level of *caspase-3, 8* and 12 in 30µmol/L H_2O_2 treated group as compare to *in-vitro*. No significant difference was observed in the RNA expression level of caspase-3,8 and 12 in *in-vivo* and *in-vitro* group and a significant difference (*P<0.05*) was observed in the RNA expression level of caspase-9 in *in-vitro* and *in-vivo* group as shown in Figure 4.

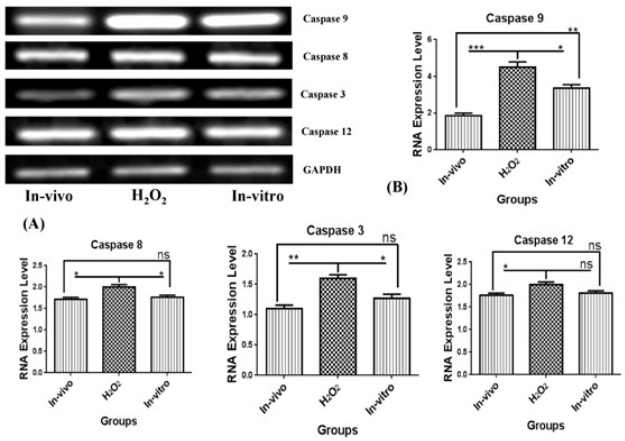

Fig. 4. The expression of caspase 3, 8, 9 and 12 at 4-cell stage mouse embryos in different H_2O_2 condition (GAPDH) was used as an internal control. (A) PCR results of caspase-3,8,9 and 12 in mouse embryos. (B) Relative expression caspase-3, 8, 9 and 12 normalized with the internal marker GAPDH (P >0.05). Data are expressed as mean ± SEM of three independent experiments. Asterisks represent statistically significant differences (P >0.05).

The RNA expression level of caspase genes at the blastocyst stage of mouse embryo was observed. Our result shows that the RNA expression level of *caspase-3, 8, 9 and 12* was significantly higher (*P<0.05*) in 30µmol/L H_2O_2 treated group as compare to *in-vivo* and *in-vitro* groups, whereas no significant difference was observed in the RNA expression level of caspase-3 and caspase-9 and highly significant difference (*P<0.05*) was observed in the RNA expression level in caspase-8 and caspase-12 between *in-vivo* and *in-vitro* groups (Fig. 5).

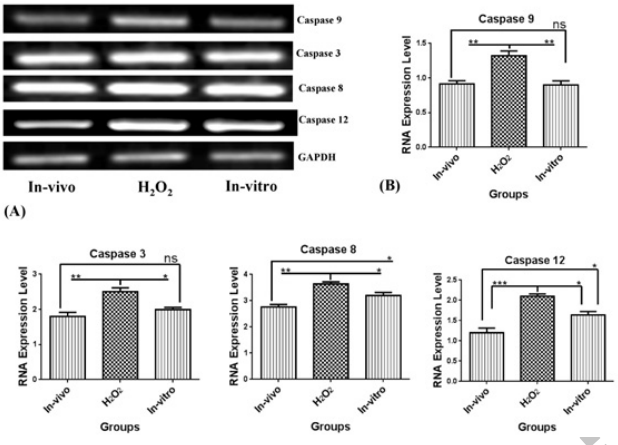

Fig. 5. The expression of caspase-3, 8, 9 and 12 at blastocyst stage mouse embryos in different H_2O_2 condition (GAPDH) was used as an internal control. (A) PCR results of caspase-3,8,9 and 12 in mouse embryos. (B) Relative expression caspase-3,8,9 and 12 normalized with the internal marker GAPDH (*P* >0.05). Data are expressed as mean ± SEM of three independent experiments. Asterisks represent statistically significant differences (*P* >0.05).

Effects of exogenous H_2O_2 on the RNA expression of pre-apoptotic and anti-apoptotic genes BAX *and* BCL2

The expression levels of anti- and pro-apoptotic genes, including B cell lymphoma 2 (*BCL2*) and B cell-associated X protein (*BAX*), at 4 cell and blastocyst stage mouse embryos were measured using RT-PCR. The RNA expression of *BCL2* significantly increased, (P<0.05) whereas that of *BAX* significantly decreased (P<0.05) in 30µmol/L H_2O_2 treated group as compare to *in-vivo* and *in-vitro* (Figs. 6 and 7).

To further explore the effect of exogenous ROS on the apototis of embryo we measured the RNA expression level of BAX and BcL2 at blastocyst stage. Our result shows that the RNA expression of BCL2 significantly increased, (P<0.05) whereas that of BAX significantly decreased (P<0.05) in 30µmol/L H_2O_2 treated group as compare to *in-vivo* and *in-vitro* (Fig. 7).

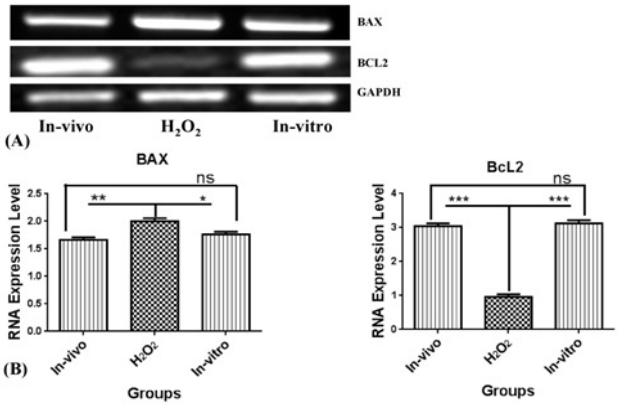

Fig. 6. The expression of BAX and Bcl2 at 4-cell stage mouse embryos in different H_2O_2 condition (GAPDH) was used as an internal control. (A) PCR results of BAX and BcL2 in mouse embryos. (B) Relative expression of BAX and BcL2 normalized with the internal marker GAPDH (*P* >0.05). Data are expressed as mean ± SEM of three independent experiments. Asterisks represent statistically significant differences (*P* >0.05).

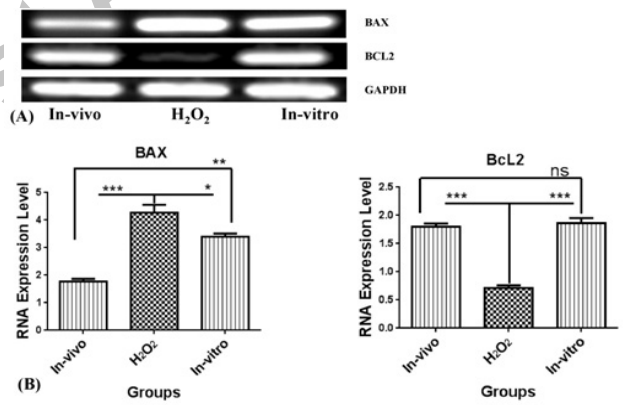

Fig. 7. The expression of BAX and Bcl2 at blastocyst stage mouse embryos in different H_2O_2 condition (GAPDH) was used as an internal control. (A) PCR results of BAX and BcL2 in mouse embryos. (B) Relative expression of BAX and BcL2 normalized with the internal marker GAPDH (*P* >0.05). Data are expressed as mean ± SEM of three independent experiments. Asterisks represent statistically significant differences (*P* >0.05).

DISCUSSION

High concentration of ROS causes oxidative damage in embryos, metabolic disorders, and oxidative stress contributing to developmental blocks in early embryos. High concentrations of ROS can inhibit normal cell division (Agarwal *et al*., 2003). A similar phenomenon was found in our study, the addition of 30µmol/L exogenous

H_2O_2 to *in-vitro* embryo culture medium increase the intercellular ROS as compared to *in-vivo* and *in-vitro* groups. Exposure of embryo to 30µmol/L exogenous H_2O_2, increase the intracellular ROS level as compared to *in vitro* and *in vivo* group however the ROS content during *in vivo* embryo development was lower compared to *in vitro* group, which are in the line of the finding of Hu and its colleagues' that revealed that embryo developmental ability decreased after H_2O_2 treatment, which confirmed that high concentration of H_2O_2 had caused oxidative damage of embryo, moreover high level of H_2O_2 level in the embryo cause in the imbalance of homeostasis and the cytoplasmic fragmentation of the embryo, thus lead to apoptosis (Hu *et al*., 2017).

As mentioned earlier, ROS levels in general causes the oxidative stress which exceed the antioxidant defenses of the cells, thus lead to changes in the structure and function of the cell. So far, several studies have highlighted the mechanism of early embryo apoptosis in the mouse. The caspase activation during *in vitro* embryo development induces apoptosis, as the detection of caspase family proteins was found during embryo development (Jurisicovaet al., 2003). Therefore, the role of the caspase family in the embryo development needs to be further evaluated. The RNA expression level of caspase-3, 8, 9, 12 was measured at 2-cell, 4-cell, and blastocyst stage. Our result shows that the RNA expression level of all the caspase genes was high in 30µmol/L H_2O_2 treated group as compare to *in-vitro* and *in-vivo* groups. Moreover, we found that caspase-12 was involved in the apoptosis of early embryos induced by reactive oxygen species, while the mRNA expression of caspase-12 gene *in vitro* was significantly higher (P<0.05) in 4-cell and blastocyst stage compared to 2-cell stage. Our result is in line with the finding of Cao et al. (2014) who revealed that there was a positive correlation between the expression level of caspase-12 mRNA and the rate of apoptosis after the stimulation of external drugs on the differentiated mouse podocytes.

Our data confirmed that the mRNA expression of apoptotic gene *caspase-3, caspase-8, caspase-9,* and *caspase-12*, causes the apoptosis of early embryo in the mouse. In the FAS receptor pathway, caspase-8 increase and activate the downstream genes, including caspase-3, caspase-6, and caspase-7, which leads to cell apoptosis (Stepien *et al*., 2007). Our result revealed that in the H_2O_2 treatment group in the blastocyst stage increase (P <0.05) the expression of caspase-8, compare to 2 cell and 4 cell, which is similar to the results of Mishra et al. (2016) who reported that the expression level of caspase-3 mRNA at the blastocyst stage was higher compared to 2 and 4-cell embryo in sheep. These results showed that caspase-8 may induce the apoptosis of mouse embryos, thus also confirmed that the apoptosis of mouse embryos induced by ROS is related to the activation of the mitochondrial apoptotic pathway. Furthermore, the caspase-3 and caspase-9 expression level showed the same trend, as the expression in each group of genes in the 4-cell and blastocyst stage significantly increase in H_2O_2 treated group compared to another group. The expression of caspase-3 and caspase-9 gene in 2-cell, 4-cell and blastocyst stage was consistent, thus indicated the activation of the mitochondrial apoptotic pathway in mouse embryo under oxidative stress. Our result is supported by the finding of Nazima *et al*. (2016) who reported that oxidative stress causes the activation of the mitochondrial apoptotic pathway (Nazima *et al*., 2016).

In order to determine whether oxidative stress induces apoptosis, we detect the RNA expression of pro and anti apoptotic gene *BAX* and *BcL2* at 4 cell and blastocyst stage. Our result shows the supplementation of 30µmol/L H_2O_2 to *in-vitro* embryo culture medium significantly increase the expression of *BAX* and decrease the expression of *BcL2* as compared to other groups. Our result is supported by the finding of Obaid et al. (2019) they reported that addition of exogenous H2O2 to mouse embryo culture increase the expression of BAX and decrease the expression of BcL2.

CONCLUSION

In conclusion, our result demonstrated that induced H_2O_2 cause the embryo arrest, enhance the RNA expression level of caspase genes, increase the RNA expression level of pro-apoptotic gene and decrease the RNA expression of anti-apoptotic gene by increasing the intracellular ROS level in mouse preimplantation embryo.

DECLARATIONS

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Disclosure statement

No potential conflict of interest was reported by the authors.

Statement of conflict of interest

The authors have declared no conflict of interest.

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