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Anti-Apoptotic Effects of Catalpol on Preimplantaion Porcine Embryos

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ABSTRACT

Catalpol, an iridoid glucoside, isolated from the root of *Rehmannia glutinosa* Libosch. It possesses a broad range of biological and pharmacological activity including anti-tumor, anti-inflammation and anti-oxidant by acting as a free radical scavenger. Therefore, in this study, the effects of catalpol on blastocyst development, expression levels of reactive oxygen species (ROS) and apoptotic index were investigated in porcine embryos. After *in vitro* maturation and fertilization, porcine embryos were cultured for 6 days in porcine zygote medium 3 (PZM-3) supplemented with catalpol (0, 100, 200 and 400 μM, respectively). Blastocyst development not significantly improved in the catalpol treated group when compared with control group. Otherwise, the intracelluar levels of ROS were decreased and the numbers of apoptotic nuclei were reduced in the catalpol (100 μM) treated porcine blastocysts (P<0.05). On the other hand, blastocyst development was significantly improved in the catalpol (100 μM) treated group when compared with the untreated catalpol group under H_2O_2 (200 μM) induced oxidative stress (P<0.05). Otherwise, the intracellular levels of ROS in catalpol (100 μM) treated group were significantly decreased in the untreated catalpol group under H_2O_2 (200 μM) induced oxidative stress (P<0.05). Furthermore, the total cell numbers of blastocysts were significantly increased (P<0.05) in the catalpol (100 μM) treated group under H_2O_2 (200 μM) induced oxidative stress, whereas numbers of apoptoic nuclei were significantly reduced (P<0.05). In conclusion, our results indicate that treatment of catalpol may have important implications for improving developmental competence and preimplantation quality of porcine embryos through its anti-oxidant and anti-apoptotic effect.

(Key words : catalpol, ROS, antioxidant, apoptosis, pig)

INTRODUCTION

The *in vitro* production (IVP) of porcine preimplantation embryos is an important technique for basic and biomedical research purposes, such as animal biotechnologies, cloning and embryo transfer (Day, 2000). However, in IVP porcine embryos, there still remains the problem of low developmental competence and poor quality when compared with *in vivo*-derived embryos, which may be caused by physiological culture conditions (Mito *et al.*, 2012). Researchers have also been conducted with the purpose of improving the developmental competence and quality of IVP porcine embryos via improvement of culture conditions (Kikuchi, 2004). Especially, strong antioxidant effects for preimplantation development of mammalian embryos were showed in plant-derived flavonoids such as quercetin and hydroxyflavone (Choi *et al.*, 2013; Kang *et al.*, 2013; Su *et al.*, 2014). However, protective antioxidant effects of natural substance-derived chemicals (catalpol etc.) have not yet been investigated.

Catalpol, a major chemical constituent of *Rehmannia glutinosa* Libosch, has a broad scope of biological and pharmacological activity including purgative, analgesic, sedative, anti-tumor, anti-inflammation and anti-oxidative stress property (Liang *et al.*, 2009). Catalpol has also been displayed to protect against oxidative stress in peripheral tissues such as the kidneys (Kang *et al.*, 2005). It attenuated H_2O_2-induced ROS production and enhanced activities of glutathione reductase and glutathione peroxidase in astrocytes primary cell culture (Bi *et al.*, 2008). Also strong neuroprotective effects of catalpol were proved by increasing mitochondrial complex I and SOD activities as well as reducing lipid peroxide by loss of mitochondrial membrane potential (Mao *et al.*, 2007). However, the protective effects of catapol in the preimplantation development of porcine embryos have not been thoroughly investigated. ROS are generated during *in vitro* culture and are detrimental to embryo development (Goto *et al.*, 1993). Hydrogen peroxide

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(H_2O_2), one of the most common ROS, H_2O_2 induces apoptosis by disrupting antioxidant defense system in embryos (Takahashi, 2012). Higher levels of ROS such as H_2O_2 induce oxidative stress response have been associated with an increased likelihood of embryo of failure (Yang *et al.*, 1998) and can damage the cell membrane. Previous studies also have reported that increased ROS levels and the resulting oxidative stress are associated with poor or arrested embryo development (Guerin *et al.*, 2001). Furthermore, to protect the embryos against oxidative stress seems to be one of the keys to improve the development. To reduce the toxicity of ROS for improving embryo development, antioxidants are effective to regulate intra- and extra-embryonic environments (Takahashi, 2012). Therefore, this study was verified the protective effects of catalpol in the preimplantation of development of porcine embryos cultured under the H_2O_2-induced oxidative stress conditions.

Apoptotic cell death in preimplantation mammalian embryos has been well described. Apoptosis in response to improper culture conditions and stress is a common physiological process that occurs in embryo development *in vitro* (Nanassy *et al.*, 2008). Apoptosis is also importantly involved in the development and differentiation of embryos (Shen *et al.*, 2006). Previous studies have shown that apoptosis is important for normal embryonic development (Brill *et al.*, 1999; Lotz *et al.*, 2006; Weingaertner *et al.*, 2006) and overfull apoptosis con- tributed in early embryos by exposure to mechanistically various teratogens can cause development injury (Huang *et al.*, 2003; Shang *et al.*, 2004; Detmar *et al.*, 2006). Moreover, apoptosis is an important part of animal development and reproduction (Takahashi *et al.*, 2004) as well as an important indicator of improper culture conditions of mammalian embryos.

The aim of the present study, we demonstrated the effects of catalpol on developmental competence of preimplantation porcine embryos cultured under oxidative stress conditions. Furthermore, the expression levels of ROS and the apoptotic index in blastocyst stage embryos derived from catalpol treatment were measured under oxidative stress conditions.

MATERIALS AND METHODS

1. Chemicals

All chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

2. *In Vitro* Maturation (IVM)

Porcine ovaries were collected from at a local abattoir and transported to the laboratory at 30～35℃ in 0.9% saline supplement with 75 mg/ml potassium penicillin G. Immature cumulus-oocyte complexes (COCs) were aspirated from follicles between 3 and 6 mm through an 18-gauge needle into a disposable 10-ml syringe (Funahashi *et al.*, 1994). After, the selected using mouth pipettes and washing three times in TL-HEPES medium, approximately 50～60 COCs were matured in 500 μl of IVM medium in a four-well multi-dish (Nunc, Roskilde, Denmark) at 38.5℃ and under 5% CO_2 in air. The medium used for oocytes maturation was Carolina State University (NCSU) 23 medium with 0.57 mM cysteine, 10% follicular fluid, 10 ng/ml epidermal growth factor (EGF), 10 ng/ml β-mercaptoethanol, 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG) was used for oocyte maturation (Petters and Wells, 1993). After culturing for 22 h, COCs were washed three times and then further cultured in oocyte maturation medium without PMSG and hCG for 22 h.

3. *In Vitro* Fertilization (IVF)

In this study used medium, designated as modified IVF of porcine oocytes was performed as described by Abeydeera and Day (1997). The IVF medium, modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA. Fresh semen was kindly supplied once a week by anartificial insemination company (Darby Porcine AI Center, Anseong, Korea) and kept at 17℃ for 5 days. Semen was then washed three times by centrifugation with Dulbecco's phosphate buffered saline (DBPS, Gibco BRL, Grand Island, NY) supplemented with 1 mg/ml BSA (Fraction V, Sigma), 75 mg/ml streptomycin sulfate, and 100 mg/ml penicillin G. At the end of washing, the spermatozoa were resuspended in mTBM at pH 7.8, for 15 min. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA (fatty acid free), after which they were placed into 48 μl of mTBM under paraffin oil. Then, 2 μl of diluted spermatozoa were added to a 48 μl drop of medium containing 15～20 oocytes to give a final concentration of 1.5×10^5 sperms/ml. Lastly, the oocytes were co-incubated with spermatozoa for 6 h at 38.5℃ and under 5% CO_2 in air (Abeydeera and Day, 1997).

4. *In Vitro* Culture (IVC) and Chemical Treatment

For all experiments, the groups of 25～30 embryos were cultured in 50-μl drops of PZM-3 medium with 3 mg/ml BSA at 38.5℃ and under 5% CO_2 in air. After 2 days of culture, cleaved embryos were further cultured in a 50-μl drop of PZM-3 medium supplemented with 3 mg/ml BSA at 38.5 ℃ and under 5% CO_2 in air for 4 days. To modulate oxidative stress, fertilized embryos were treated with oxidative inducer H_2O_2 (200 μM; Lee *et al.*, In press) or antioxidant catalpol (100, 200, 400 μM) by direct addition to the culture medium. In addition, porcine embryos were cultured for 6 days in IVC medium supplemented with or without catalpol (100 μM) under oxidative stress condition (200 μM H_2O_2). Blastocyst formation was evaluated under a stereomicroscope at 6 days after insemination.

5. Measurement of ROS Levels

The level of H_2O_2 in each embryo was measured using the difluorodihydrofluorescein diacetate method (H_2DCFDA; Invitrogen, Molecular Probes, Willow, USA) described previously (Choi *et al.*, 2008). H_2DCFDA produced an intermediate difluorodihydrofluorescein (DCF) after reaction with ROS, DCF upon oxidation produced difluorofluorescein which can be monitored in fluorescence microscope. At day 6, IVP blastocysts were recovered and used for the experiment. After three washes in IVC medium, blastocysts were transferred into IVC medium containing 5 μM H_2DCFDA for 20 min at 38.5℃. A stock solution of H_2DCFDA dissolved in dimethylsulfoxide (DMSO) was then diluted in IVC medium, after which the permeabilized blastocysts in H_2DCFDA were washed three times with 0.1% PVA-dPBS and placed in to a 50-μl drop covered with mineral oil. The fluorescent emissions from the embryos were recorded as TIFF files using a cooled CCD camera attached to a fluorescent microscope (IX 51, Olympus, Tokyo, Japan) with filters at 488 nm for excitation and 520 nm for emission. The recorded fluorescent images were processed (subtract background) and analyzed (measure integrated density) was measured using Image J software version 1.38 (National Institutes of Health, Bethesda, MD). A total of 20 blastocysts were examined in each treatment group.

6. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) Assay

The number of apoptotic nuclei in the blastocysts was detected using an *In Situ* Cell Death Detection Kit, Fluoresce (Roche Diagnostics GmbH, Mannheim, Germany). Blastocysts were recovered from IVC after 6 days, washed three times with 0.1% PVA-PBS and then fixed in 4% (v/v) paraformaldehyde/PBS solution for 1 h at 4℃. For membrane permeabilization, the fixed embryos were incubated in PBS containing 0.1% (v/v) Triton X-100 for 30 min at 4℃, after which they were incubated in TUNEL reaction medium for 1 h at 38.5℃ in the dark and then washed and transferred to 2 mg/ml of DAPI and mounted on glass slides. Whole-mount embryos were examined under an epifluorescence microscope (Olympus) using the TUNEL assay and DAPI and the numbers of apoptotic nuclei and total number of nuclei were determined.

7. Statistical Analysis

All experiments were repeated at least three times. All percentage data were subjected to arcsine transformation. All percentage data and datasets obtained in the present study are presented as the mean ± standard deviation (S.D.). Rates of preimplantation embryos development, ROS levels, cell numbers and apoptotic proportions of blastocysts were analyzed by ANOVA and Student's *t*-tests. A probability of $P<0.05$ was considered significant.

RESULTS

1. Effects of Catalpol Treatment on Developmental Competence of Porcine Embryos

There are no previous reports about the concentration and effects of catalpol to treatment in porcine culture experiments. In this study, we investigated the optimal catalpol concentration of culture medium in preimplantation development of porcine embryos. After completion of IVF, porcine embryos were cultured in IVC medium supplemented with 100, 200 and 400 μM catalpol for 6 days at 38.5℃ under 5% CO_2 in air. As shown in Table 1, developmental competence of blastocysts formation significantly not increased between control groups and groups treated group with 100 μM catalpol. However, developmental competence of blastocysts formation in the presence of 200 and 400 μM catalpol was significantly decreased when compared with the untreated control group ($P<0.05$).

2. Effects of Catalpol Treatment on Expression Levels of ROS and Apoptotic Index in Porcine Blastocysts

Table 1. Effect of various concentrations of catalpol during *in vitro* culture on development of porcine embryos

Concentrations (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts produced
0	235	210 (89.1 ± 2.4)[a]	72 (31.7 ± 4.5)[a]
100	235	213 (91.8 ± 1.9)[a]	75 (31.4 ± 1.8)[a]
200	236	174 (79.7 ± 7.1)[b]	53 (23.7 ± 2.6)[b]
400	236	164 (75.0 ± 5.3)[b]	42 (16.0 ± 3.1)[c]

This experiment was replicated five times. Data are the mean ± S.D.
Different superscripts denote a significant difference compared with other groups (P<0.05).

We next investigated the intracellular levels of ROS and the apoptotic index in catalpol treated blastocysts. Intracellular levels of ROS were significantly reduced in 100 μM catalpol treated group compared with other groups (P<0.05). However, intracellular levels of ROS were significantly increased in blastocysts derived from 200 and 400 μM catalpol treated embryos when compared with untreated controls (Fig. 1A, 1B). Moreover, the number of TUNEL-positive nuclei was significantly reduced in blastocyst stage embryos derived from the 100 μM catalpol treated group when compared with the control group (Fig. 1C, 1E; P<0.05). However, the total cell number did not differ significantly between the control and

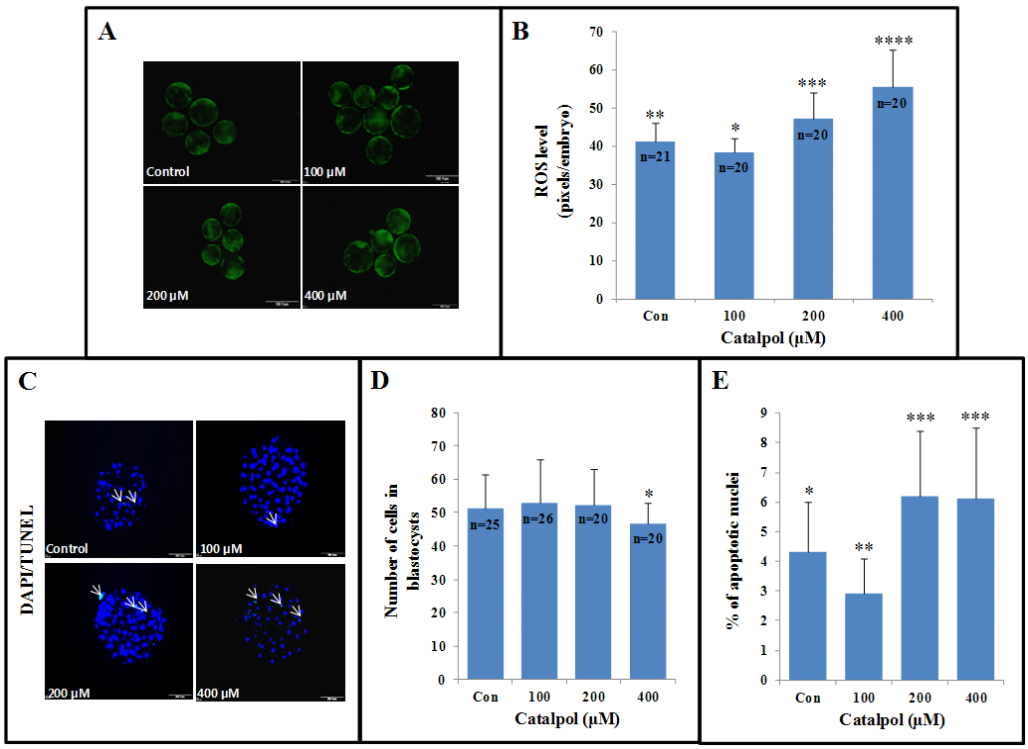

Fig. 1. Comparison of antioxidant and apoptotic characteristics in porcine blastocysts derived from catalpol treatment groups. Fluorescence microscopy imaging of intracellular ROS expression (A) and level of hydrogen peroxide (B) in blastocysts. Epifluorescent images of porcine blastocysts derived from various concentration of catalpol undergoing apoptosis *in vitro* (C). The chromatin content is stained by DAPI (blue), fragmented DNA is labeled by the TUNEL reaction (green and white arrow), and colocalization with DAPI appears sky-blue. Total cell number (D) and percentage of apoptotic nuclei (E) in porcine blastocyst stage embryos derived from catalpol treatment. Scale bars=200 μm. Total cells number. Data are the mean ± S.D. Statistically significant differences are indicated by asterisks (P<0.05).

catalpol treated (Fig. 1D). Therefore, all further experiments were performed using 100 μM catalpol.

3. Effects of Catalpol on Preimplantation Development of Porcine Embryos Cultured under Oxidative Stress Condition

Presumptive zygotes were cultured in the presence or absence of catalpol and/or H_2O_2 for 6 days at 38.5℃ and under 5% CO_2 in air. As shown in Table 2, the rates of cleavage and blastocyst formation under the in duced H_2O_2 oxidative stress were significantly improved in the presence of catalpol group when compared with absence of catalpol group (P<0.05).

4. Protective Effect of Catalpol on Intracellular Levels of ROS and Apoptotic Index in Blastocyst Stage Embryos Cultured under Oxidative Stress Condition

As shown in Fig. 2A and 2B, the intracellular levels of ROS in blastocysts under the induced H_2O_2 oxidative stress that developed from embryos cultured in the presence of catalpol was lower than that of blastocysts from embryos cultured in the absence of catalpol. Moreover, the number of apoptotic nuclei was significantly lower in blastocyst stage embryos derived from the catalpol treated group when compared with the group not treated with catalpol (Fig. 2C, 2E; P<0.05). In addition, the total cell number was significantly increased in blastocyst stage embryos cultured in the presence of catalpol when compared with those cultured in the absence of catalpol under H_2O_2 oxidative stress (Fig. 2D; P<0.05).

DISCUSSION

In the present study, we demonstrated that the catalpol significantly increased the embryo development and embryo quality such as increasing blastocyst cell number, reduction of ROS and apoptosis in the pig embryos under oxidative stress culture condition *in vitro*. Finally, catalpol induced blastocyst formation was shown to be associated with increased embryo quality in terms of blastocyst nuclei numbers and reduced apoptosis in porcine embryos.

Oxidative stress can damage oocytes and cause mitochondrial alterations, embryo block, adenosine triphosphatase depletion and apoptosis (Nasr-Esfahani *et al.*, 1990). Many previous studies have demonstrated oxygen toxicity and its harmful effects in mammalian preimplantation embryos *in vitro*, and the importance of protecting embryos from ROS is increasingly being recognized as a key factor in improving *in vitro* culture conditions for cow, human embryo (Favetta *et al.*, 2007; Takahashi, 2012). The embryos cultured in lower (5%) O_2 reduced the glucose metabolism converted to lactate when compared with embryos cultured in 20% O_2 condition (Du and Wales, 1993). These reports indicate that *in vitro* culture condition including oxygen concentration highly affect the embryonic metabolism with increase in the ROS production. Oxygen concentration in *in vitro* culture condition also affect DNA damage (Takahashi *et al.*, 2000) associated with ROS generation (Kitagawa *et al.*, 2004). Therefore, this study was conducted to monitor the levels of ROS within porcine embryos to indirectly assess H_2O_2 toxicity. Similarly, previous study demonstrated that preimplantation porcine embryos were significantly decreased developmental competence and poor quality following exposure to 200 μM H_2O_2 (Lee *et al.*, In press). Therefore, antioxidant supplementation of the medium may inhibit or reduce ROS generation, thereby suppressing ROS damage and improving the developmental ability of the embryos (Olson and Seidel, 2000).

Catalpol dependently attenuated H_2O_2-induced ROS production (Jing *et al.*, 2008). In our experiment, catalpol (100 μM) showed that, blastocyst development increased when compared

Table 2. Effect of catalpol on development of porcine embryos cultured under oxidative stress conditions

Treatment H_2O_2 (μM)	Treatment catalpol (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts produced
0	0	211	186 (89.6 ± 2.7)[a]	60 (30.5 ± 1.4)[a]
200	0	221	178 (80.6 ± 2.7)[b]	34 (18.8 ± 1.9)[b]
0	100	211	190 (91.7 ± 3.3)[a]	67 (31.1 ± 1.2)[a]
200	100	226	199 (88.2 ± 2.9)[a]	58 (28.3 ± 1.7)[a]

This experiment was replicated five times. Data are the mean ± S.D.
Different superscripts denote a significant difference compared with other groups (P<0.05).

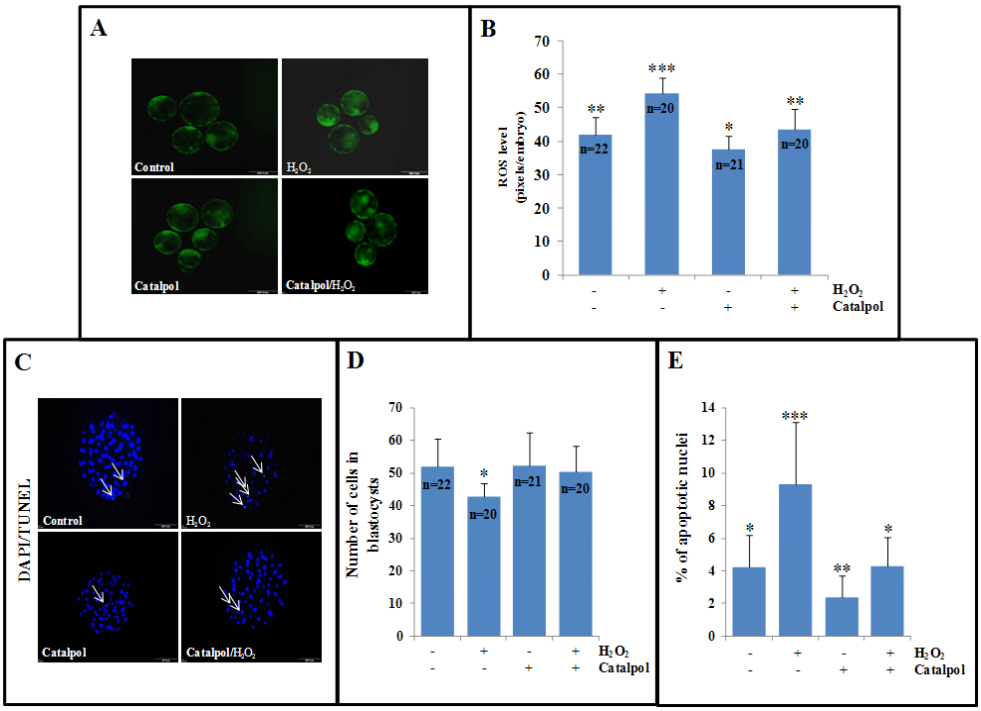

Fig. 2. Comparison of antioxidant and anti-apoptotic characteristics in porcine blastocysts derived from H_2O_2 and/or catalpol treatment groups. Fluorescence microscopy imaging of intracellular ROS expression (A) and level of hydrogen peroxide (B) in blastocysts. Epifluorescent images of porcine blastocysts stage embryos cultured with H_2O_2 and/or catalpol under going apoptos is *in vitro* (C). The chromatin content is stained by DAPI (blue), fragmented DNA is labeled by the TUNEL reaction (green and white arrow), and colocalization with DAPI appears sky-blue. Total cell number (D) and percentage of apoptotic nuclei (E) in porcine blastocyst stage embryos derived from H_2O_2 and catalpol treatment. Scale bars=200 μm. Data are the mean ± S.D. Statistically significant differences are indicated by asterisks (P<0.05).

with 200, 400 μM and untreated control. Additionally, high concentration (200, 400 μM) of catalpol reduced blastocysts development as well as quality confirms its toxicity on embryo. Thus, level of ROS was increased in porcine embryos treated with high concentration of catalpol. Following, we found significant differences in the development rate of blastocyst stages for embryos cultured with or without catalpol under oxidative stress. These results suggest that catalpol might act as an oxygen radical scavenger to protect porcine embryos against oxidative stress and improve development of blastocysts. Thus, our results indicate that catalpol treatment under H_2O_2 induced oxidative stress leads to enhancement of blastocysts formation, including improved blastocyst quality. Although the exact mechanisms mediating catalpol actions are unknown in porcine embryo, previous work reported that catalpol protects rat pheochromocytoma (PC12) cell line from H_2O_2-induced oxidative stress and apoptosis (Jiang*et al.*, 2004). Thus, the present study suggests that catalpol is may play a role for extracellular reducing agent and neutralizes superoxide radicals.

Previous studies have shown that apoptosis plays an important role in embryonic development (Huppertz *et al.*, 2005) and during analysis of developmental competence of embryos the apoptotic index of the blastocysts can be a good indicator of the quality of produced blastocysts (Gupta *et al.*, 2007). During normal embryogenesis, apoptosis functions to clear abnormal or redundant cells in preimplantation embryos (Hardy, 1997; Hardy *et al.*, 2003). Notably, H_2O_2 leads to mitochondrial dysfunction that resulting in apoptosis, which is possibly related to porcine early embryo development (Xu *et al.*, 2011). Therefore, increased incidence of cell death is an important indicator of improper *in vitro* environments for mammalian embryos. The TUNEL assay revealed that the number of apoptotic nuclei in blastocysts derived from embryos cultured with catalpol was lower than that of cultured without catalpol following the H_2O_2 induction of oxidative stress. Recent evidence suggested that the developmental potential of embryo could be related to its

apoptosis rate (Lee *et al.*, In press). Additionally, the total cell number of embryos is available indicator of the development ability of preimplantation embryos (Papaioannou and Ebert, 1988). The total numbers of cells in blastocysts derived from embryos cultured with catalpol was significantly higher than that of cultured without catalpol. These results suggest that the reduction of oxidative stress by catalpol treatment may improve the quality of porcine blastocysts.

In this study, these results suggest that decreased developmental competence caused by H_2O_2 induced oxidative stress, resulting in an increased number of apoptotic nuclei and decreased cell number in blastocysts. In contrast, the addition of catalpol under H_2O_2 induced oxidative stress improved developmental competence to the blastocyst stage, reduced the number of apoptotic nuclei an decreased the cell number in porcine IVP embryos by preventing oxidative stress. Therefore, we suggest that catalpol may be improved the development and quality of porcine embryos by preventing oxidative stress.

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