pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 4, pp. 289~298
http://dx.doi.org/10.12750/JET.2015.30.4.289

Antioxidant Effect of Edaravone on the Development of Preimplantation Porcine Embryos against Hydrogen Peroxide-Induced Oxidative Stress

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ABSTRACT

Edaravone (Eda) is a potent scavenger of inhibiting free radicals including hydroxyl radicals (H_2O_2). Reactive oxygen species (ROS) such as H_2O_2 can alter most kinds of cellular molecules such as lipids, proteins and nucleic acids, cellular apoptosis. In addition, oxidative stress from over-production of ROS is involved in the defective embryo development of porcine. Previous study reported that Eda has protective effects against oxidative stress-like cellular damage. However, the effect of Eda on the preimplantation porcine embryos development under oxidative stress is unclear. Therefore, in this study, the effects of Eda on blastocyst development, expression levels of ROS, and apoptotic index were first investigated in preimplantation porcine embryos. After *in vitro* fertilization, porcine embryos were cultured for 6 days in PZM medium with Eda (10 μM), H_2O_2 (200 μM), and Eda+H_2O_2 treated group, respectively. Rate of blastocyst development was significantly increased (P<0.05) in the Eda treated group compared with only H_2O_2 treated group. And, we measured intracellular levels of ROS by DCF-DA staining methods and investigated numbers of apoptotic nuclei by TUNEL assay analysis is in porcine blastocyst, respectively. Both intracellular ROS levels and the numbers of apoptotic nucleic were significantly decreased (P<0.05) in porcine blastocysts cultured with Eda (10 μM). More over, the total cell number of blastocysts were significantly increased (P<0.05) in the Eda-treated group compared with untreated group and the only H_2O_2 treated group. Based on the results, Eda was related to regulate as antioxidant-like function according to the reducing ROS levels during preimplantation periods. Also, Eda is beneficial for developmental competence and preimplantation quality of porcine embryos. Therefore, we concluded that Eda has protective effect to ROS derived apoptotic stress in preimplantation porcine embryos.

(Key words: edaravone (Eda), antioxidants, porcine embryo, reactive oxygen species (ROS), apoptosis)

INTRODUCTION

In vitro production (IVP) of porcine embryos has been extensively studied for improving embryonic development and reproductive technologies (Choi *et al.*, 2013). IVP embryos are very susceptible to oxidative damage because their defense mechanisms are insufficient to protect their delicate cellular structure (Rocha-Frigoni *et al.*, 2015). Therefore, many researchers are investigating ways to optimize the condition of *in vitro* maturation (IVM) of oocytes or *in vitro* culture (IVC) of embryos, including temperature, gas tension, composition of media, etc. (Booth *et al.*, 2005; Jin *et al.*, 2007). It is well known that one of the problems that impair IVP of porcine embryos is the oxidative stress that is mainly caused by reactive oxygen species (ROS) production from highly reactive molecules such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), superoxide anions ($O_2 \cdot^-$) and nitric oxide (NO). In particular, oocytes and early stage embryos are more vulnerable to oxidative stress, and the developmental competence of embryos is impaired by the resulting damage (Harvey *et al.*, 2002).

Edaravone (Eda) is a potent free radical scavenger which has been shown to provide neuroprotection against cerebral ischemia-reperfusion injuries in experimental animal models (Yan *et al.*, 2012). Eda is a potent and novel synthetic scavenger of free radicals inhibiting not only hydroxyl radicals but also iron-induced peroxidative injuries. In addition, Eda has been prescribed clinically in Japan, since 2001 to treat

This work was supported by grants from the Next-Generation BioGreen 21 Program (PJ01117604) and the Bio-Industry Technology Development Program (112130031HD030) through the Rural Development Administration and the Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.
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patients with cerebral ischemia (Tsuji *et al.*, 2005). Eda has been used as a free novel radical scavenger to inhibit oxidative stress and apoptosis. Also, Eda has antioxidant effects and decreases hydroxyl radicals and superoxide radical production (Cheng *et al.*, 2014). Eda has been reported to exert antioxidant effects because it can quench hydroxyl radicals and hydroxyl radical-dependent lipid peroxidation (Kikuchi *et al.*, 2013).

Most of all, the generation and production of intracellular level ROS are involved in several signaling mechanisms. ROS can alter most kinds of cellular molecules such as lipids, proteins and nucleic acids, which results in mitochondrial damage, embryo cell block, ATP depletion, and apoptosis (Guerin *et al.*, 2001; Gualtieri *et al.*, 2014). The level of ROS production in mammalian embryos is particularly important during various stages of preimplantation development *in vitro*. Furthermore, during *in vitro* culture periods, embryos are exposed to relatively high oxidative stress compared to the environment of *in vivo,* thus the production of ROS within embryos is increased (Choi *et al.*, 2008). Therefore, low rate of preimplantation embryo development is one of the main problems due to elevation of ROS during *in vitro* procedures (Dehghani-Mohammadabadi *et al.*, 2014). Oxidative stress, depending on its severity, can lead to either cell necrosis or apoptosis. Also, apoptosis is characterized by cell shrinkage and chromatin condensation (Liu *et al.*, 2000). Then, apoptosis, in response to inappropriate cultural conditions and stress, is a common physiological process in *in vitro* embryo development (Choi *et al.*, 2008). And, cellular apoptosis has been assayed in mammalian blastocysts stage using TUNEL staining, which is influenced by suboptimal developmental conditions and stress, but mainly in *in vitro* fertilized (IVF) and parthenogenetic embryos (Isom *et al.*, 2007; Neuber *et al.*, 2002).

Although an antioxidant effect of Eda has been reported in some mammalian cell models, there is currently no research regarding the effect of Eda on porcine embryo development. Therefore, in this study, we evaluated the effects of Eda on the developmental competence of preimplantation porcine embryos cultured under oxidative-stress conditions. In addition, the expression levels of ROS and the apoptotic index in blastocyst stage embryos derived from Eda treatment were measured.

MATERIALS AND METHODS

1. Chemicals

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

2. *In Vitro* Maturation

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 30~35℃ in 0.9% saline supplemented with 75 μg/ml potassium penicillin G. Cumulus oocyte complexes (COCs) were aspirated through an 18 gauge needle into a disposable 10 ml syringe from follicles of 3 to 6 mm in diameter (Funahashi *et al.*, 1994). After washing three times with Tyrode's lactate (TL)-N-2-hydroxyenthylpiperazine-N'-2-ethanesulfoic acid (HEPES) medium, approximately immature 50 COCs were matured in 500 μl of IVM medium in a four-well multidish (Nunc, Roskilde, Denmark) at 38.5℃, 5% CO_2 in air. The medium used for oocyte maturation was North Carolina State University (NCSU) 23 medium (Petters and Wells, 1993) supplemented with 10% follicular fluid, 0.57 mM cysteine,10 ng/ml β-mercaptoethanol, 10 ng/ml epidermal growth factor, 10 IU/ml pregnant mare's serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG). After 22 h of culture, oocytes were washed three times and then further cultured in maturation medium without hormones supplement (PMSG and/or hCG) for 22 h.

3. *In Vitro* Fertilization

After IVM stage, the oocytes were subjected to IVF as described by Abeydeera and Day (1997). This medium was designated as modified Tris-buffered medium (mTBM). Fresh semen was kindly supplied twice a week by artificial insemination company (Darby Pig AI Center, Anseong, Korea) and kept at 17℃ for 4 days. Semen was washed three times by centrifugation with Dulbecco's phosphate buffered saline (DPBS; Gibco-BRL, Grand Island, NY, USA) supplemented with 1 mg/ml bovine serum albumin (BSA; Fraction V), 100 mg/ml penicillin G and 75 mg/ml streptomycin sulfate. After washing, the spermatozoa were suspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA (fatty acid free) and placed into 48 μl of mTBM under paraffin oil. Diluted spermatozoa (2 μl) were added to 48 μl drop of the medium containing 20 oocytes to give a final concentration of 1.5×10^5 sperms/ml. The oocytes were co-incubated with the spermatozoa for 6 h at 38.5℃, 5% CO_2 in air.

4. *In Vitro* Culture and Chemical Treatment

For all experiments the embryos were cultured in 50 μl drops of porcine zygote medium 3 (PZM-3) medium with 3 mg/ml BSA at 38.5℃ under 5% CO_2 in air. After 48 h 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℃ under 5% CO_2 in air for 4 days. To investigate oxidative stress, fertilized embryos were treated with oxidative inducer H_2O_2 (200 μM) or antioxidant Eda (1, 10 or 20 μM) by direct addition to the culture medium. In addition, porcine embryos were cultured for 6 days in IVC medium with or without Eda (10 μM) under oxidative stress condition though H_2O_2 200 μM treatment. Blastocyst formation was evaluated by stereomicroscopic observation at Day 6 after insemination.

5. Measurement of Reactive Oxygen Species Levels

The level of H_2O_2 in each embryo was measured using the dichlorofluorescin diacetate method (DCF-DA; Molecular Probes, Eugene, OR, USA) described previously (Choi *et al.*, 2008). At Day 6, *in vitro* produced blastocysts were recovered and used for the experiment. After three time washes in IVC medium, blastocysts were transferred into IVC medium containing 5 μM DCF-DA for 20 min at 38.5℃, 5% CO_2 in air. A stock solution of DCF-DA dissolved in dimethylsulfoxide (DMSO) was then diluted in IVC medium, after which the permeabilised blastocysts in DCF-DA were washed three times with 0.1% polyvinylalcohol PVA in DPBS and placed in to a 50 μl drop covered with mineral oil. The fluorescent emissions from the embryos were recorded with a fluorescent microscope (Olympus) equipped with a cooled charge coupled device (CCD) camera where filters at 488 and 520 nm were used for excitation and emission, respectively. The recorded fluorescent images were analyzed by subtracting background and measuring integrated density with Image J software Version 1.38 (National Institutes of Health, Bethesda, MD, USA).

6. TUNEL Assay Analysis

Apoptotic cells in blastocysts were detected using the *in situ* Cell Death Detection Kit (Roche diagnostics GmbH, Mannheim, Germany). The blastocysts were recovered from fixed in 4% (v/v) formaldehyde diluted in DPBS for 1 h at 4℃. For membrane permeabilization, the fixed embryos were incubated in DPBS containing 0.1% (v/v) Triton X-100 for 30 min at 4℃. The fixed embryos were incubated in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction medium for 1 h at 38.5℃ in the dark and then washed and transferred into 2 mg/ml of DAPI and mounted on slides. After TUNEL and DAPI staining, whole-mount embryos were examined under an epifluorescence microscope (Olympus) to determine the number of apoptotic nuclei and total number of nuclei.

7. Statistical Analysis

All experiments were repeated more than three times. All percentage data and data sets obtained in the present study are presented as the mean±standard deviation (SD) and mean± standard error of the mean (SEM). The results were analyzed using a one-way ANOVA followed by Dunnett's multiple Comparison Test and the Student's *t*-test. All calculations were performed using the GraphPad Prism 5.0 software package (San Diego, CA). Differences were considered significant at * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

RESULTS

1. Effects of Eda on Developmental Competence and Fertilization Rate of Porcine Embryos

To investigate the effect of Eda on embryonic development, we confirmed the blastocyst development and fertilization rate in embryo of porcine after Eda treatment. First, the effect of Eda on embryonic development was investigated. We investigated the optimal Eda concentration of culture medium in development competence of porcine embryos. After IVM and IVF, porcine embryos were cultured in IVC medium supplemented with various concentrations (1, 10 or 20 μM) during full-time culture periods at 38.5℃ and under 5% CO_2. Table 1 showed that the proportion of blastocysts development was significantly increased ($P<0.05$) in culture media supplemented with Eda at a concentration of 10 μM. Based on the results, we proceeded in a following set of experiments the concentration of 10 μM. Next, we investigated the effects of Eda on development of porcine embryos against oxidative stress induced by H_2O_2. Presumptive zygotes were cultured in the presence or absence of Eda and H_2O_2 for 6 days at 38.5℃ and under 5% CO_2 in air. These results showed that the rates of cleavage and blastocyst formation under H_2O_2-induced oxidative stress were significantly improved in the presence of Eda group when compared with absence of the only Eda treatement group (Table 2), suggesting that development competence improved due to Eda treatment.

Table 1. Effect of Eda treatment on the developmental competence in porcine embryos

Groups (μM)	No. of embryos examined	No.(%) of embryos cleaved	No.(%) of blastocysts produced
0	285	257 (90.1±3.8)	79 (27.6±2.1)[a]
1	265	239 (90.2±2.8)	79 (29.8±1.1)[a]
10	265	241 (91.9±2.9)	84 (31.7±2.7)[b]
20	265	234 (88.2±2.7)	65 (24.3±2.6)[c]

Data are the mean±SD.
[a~c] Values from ten replicates with different superscripts denote a significant difference relative to other groups (P<0.05).

2. Effects of Eda on Intracellular ROS Level, Cell Number and Frequency of Apoptotic Nuclei in Porcine Blastocysts

This study was carried out to assess the effect of ROS by measuring the effects of H_2O_2 on blastocyst by Eda treatments. After determining the optimal concentration of Eda groups, an experiment was designed to analyze the effect of Eda on the ROS level of porcine blastocysts (Fig. 1A). As shown in Fig. 1B, the intracellular ROS level in blastocysts did not differ significantly between the control and groups treated with 1 μM Eda. However, the quantification analysis showed that Eda 10 μM exposure significantly decreased (P<0.05) the level of intracellular ROS in blastocysts. Next, we used merge data stained by blue and green find out for apoptosis in blastocysts. In order to determine the frequency of apoptosis in porcine blastocysts, TUNEL assay was used (Fig. 2A). As shown in Fig. 2B, the total cell number in blastocysts did not differ significantly between the control and groups treated with 1 and 10 μM Eda. Furthermore, the number of TUNEL positive cells decreased (P<0.05) significantly in blastocysts derived from concentration of 10 μM Eda treated group (Fig. 2C). These results the number of TUNEL positive cells decreased significantly in blastocysts derived from Eda treated groups as well, suggesting a protective effect of Eda against apoptosis in blastocysts.

3. Effects of Eda Treatment on Intracellular ROS Level, Cell Number and Frequency of Apoptotic Nuclei in Porcine Blastocysts against Oxidative Stress induced by H_2O_2

We next investigated the intracellular levels of ROS in blastocyst-stage embryos treated with or without Eda under oxidative stress induced by H_2O_2 (Fig. 3A). As shown in Fig. 3B, the intracellular ROS level in blastocysts formation under H_2O_2-induced oxidative stress did not differ significantly between control and Eda treated group. These results in the quantification analysis is showing that Eda exposure significantly decreased the level of intracellular ROS in blastocysts, suggesting that Eda can prevent an increase in ROS induced by H_2O_2. We also investigated the apoptotic index in blastocyststage embryos treated with or without Eda under oxidative stress induced by H_2O_2 (Fig. 4A). As shown in Fig. 4B, the total cell number in blastocyst formation under H_2O_2-induced oxidative stress did not differ significantly between the control and groups treated Eda group. However, the proportion of TUNEL positive cells decreased significantly in blastocysts derived from Eda treated group under H_2O_2-induced oxidative stress (Fig. 4C). These result indicated that Eda treatment effect on blastocyst quality and development proportion.

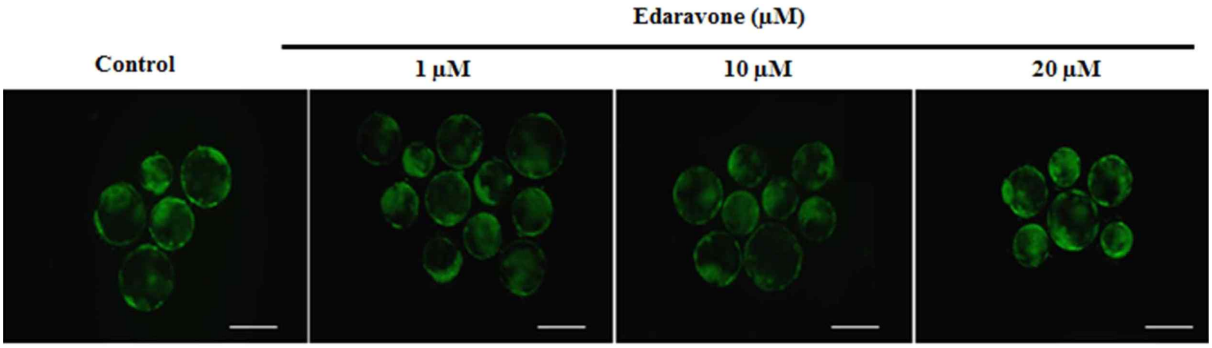
DISCUSSION

The aim of this study was to investigate the protective role

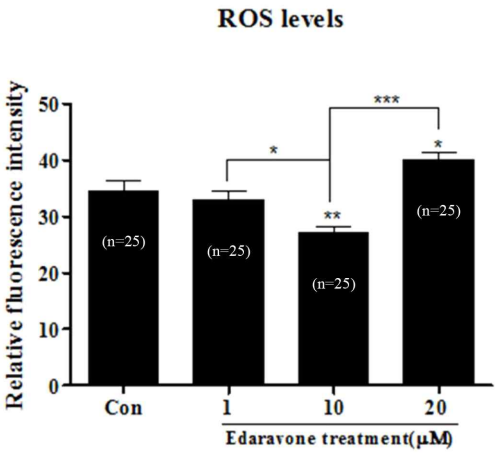
Table 2. Effect of Eda treatment on the development of porcine embryos against oxidative stress induced by H_2O_2

Treatment		No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts produced
H_2O_2 (μM)	Eda (μM)			
0	0	219	202 (92.2±2.2)[a]	64 (29.3±1.1)[a]
0	10	196	183 (93.4±2.4)[a]	49 (32.1±2.0)[b]
200	0	199	171 (85.9±3.0)[b]	38 (21.6±1.9)[c]
200	10	219	198 (90.5±2.7)[a]	48 (27.5±2.4)[a]

Data are the mean±SD.
[a~c] Values from ten replicates with different superscripts denote a significant difference relative to other groups (P<0.05).



(A)



(B)

Fig. 1. Detection of intracellular ROS level in porcine blastocyst. (A) To determine the production of ROS in porcine blastocyst, we performed the DCF-DA staining. (B) Eda of relative fluorescence intensity of ROS per blastocysts. Scale bars = 100 μm. The quantification analysis of fluorescence intensity in DCF-DA stained porcine blastocyst obtained by the image J program. Data in the bar graph represents the means±SEM of three independent experiments. Statistically differences are indicated by asterisks (* P< 0.05; ** P<0.01; *** P<0.001, compared to control group).

of the Eda from oxidative stress through the preimplantation embryo development. Oxidative stress is directly related to the quality and developmental competence of blastocyst stage embryos. Previous report documented that Eda, a potent anti-oxidant, reduced oxidative stress in cells (Zong *et al.*, 2014). However, the anti-oxidative role of Eda in development process of porcine preimplantation embryos and the effect of Eda on apoptosis mechanisms of early embryogenesis are not fully understood. Therefore, our study investigated the Eda known to have antioxidant affects in early embryo development. As expected, cell death is decreased by oxidative stress of the preimplantation embryo through the treatment of Eda. Through this reduction in apoptosis in the early embryo development, the efficiency of fertilization and the embryo development is increased.

In a healthy body, ROS and antioxidants remain in balance (Agarwal *et al.*, 2005). ROS is known as regulation of embryo metabolism, these metabolism generates ROS via several enzymatic mechanisms (Guerin *et al.*, 2001). Oxidative stress caused by excessive ROS production and/or breakdown mechanisms of the antioxidant defense has been shown to be detrimental to fertilized eggs, resulting in the impairment of early embryonic development (Ruder *et al.*, 2008). In particular, many studies reported a sustaining increase in oxidative stress by loss of balance between ROS and antioxidants in embryo of *in vitro* production (Combelles *et al.*, 2009). The development until blastocyst stage is an important period during preimplantation embryogenesis, and quality of blastocyst portends their ability to implant (Fabian *et al.*, 2005). Our result showed that developmental competence of blastocyst formation increased

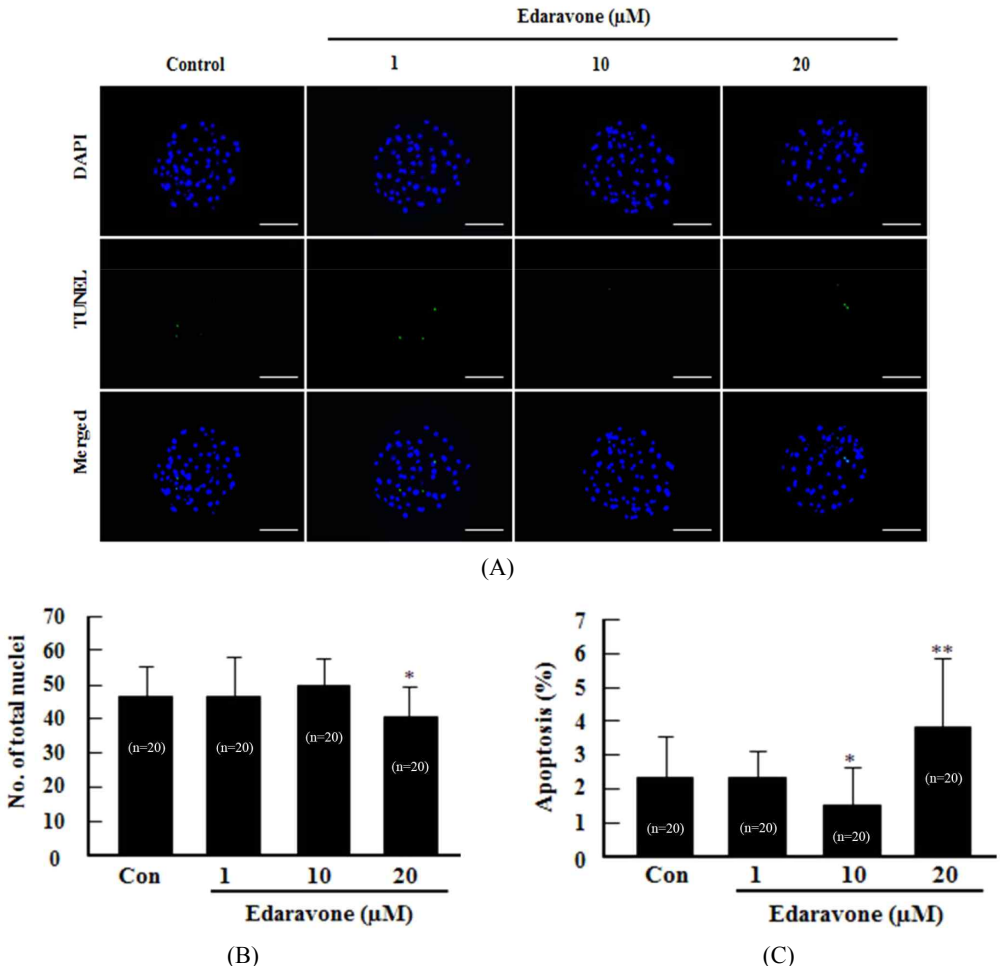
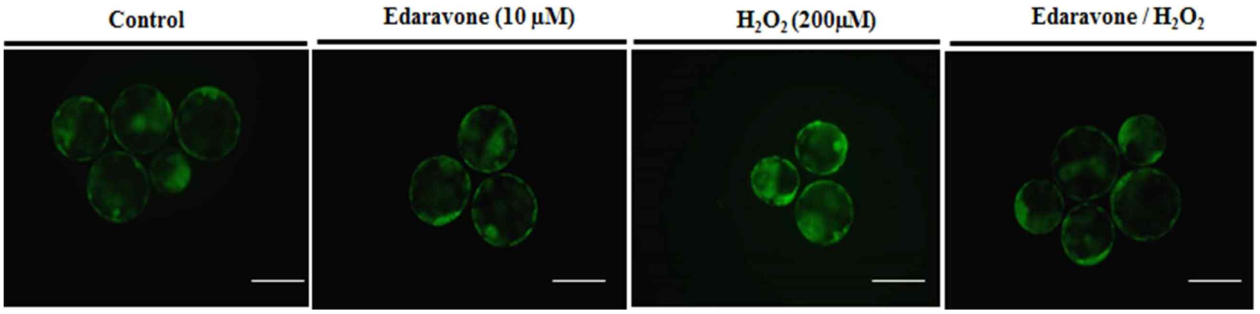


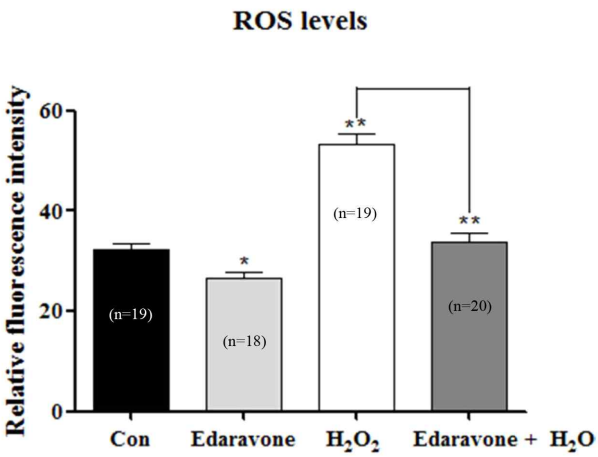
Fig. 2. Evaluation of cellular apoptosis by using TUNEL assay in porcine blastocyst. (A) Detection of apoptosis by TUNEL assay using fluorescent images in porcine blastocyst-stage embryos cultured with or without Eda undergoing apoptosis *in vitro*. Hoechst 33342 staining identifying nuclei, TUNEL staining identifying apoptotic cells and merged photograph of Hoechst 33342 and TUNEL-stained porcine blastocyst (scale bar = 100 μm). (B) Eda of the total cell numbers per blastocyst. (C) Percentage of TUNEL positive cell per blastocyst. The quantification analysis of fluorescence intensity in TUNEL stained porcine blastocyst obtained by the image J program. Data in the bar graph represents the means±SEM of three independent experiments. Statistically differences are indicated by asterisks (P<0.05).

significantly in 10 μM Eda treated groups (Table 1). We also found the antioxidant and anti-apoptotic effects of Eda confirmed in porcine early embryo development process for the first time in this field of research.

As shown in the Fig. 1, we confirmed the level of intracellular ROS in porcine embryo of preimplantation development by DCF-DA staining method via culture medium within Eda. Eda significantly reduced ROS levels in dose-dependent manner at the blastocyst stage of porcine. A similar effect of antioxidant addition such as Eda, vitamin E on development has previously been reported in porcine embryo studies (Kitagawa *et al.*, 2004). Based on the results, Eda acts as an oxygen free radical scavenger to protect porcine embryos from oxidative stress and improve blastocyst development. Moreover, ROS detected the by DCF-DA staining analysis in blastocyst of control group during *in vitro* production. ROS production by embryo has been reported on many occasions and culture environment (Guerin *et al.*, 2001). This being so, the IVP embryos by *in vitro* fertilization is known that ROS are generated in culture (Agarwal *et al.*, 2012). To investigate the detailed functional role of Eda as antioxidant, we performed dividing into four treatment groups (control, Eda, H_2O_2 and Eda+H_2O_2). H_2O_2 treatment group had



(A)



(B)

Fig. 3. Detection of H_2O_2-induced intracellular ROS level in porcine blastocyst after treatment of Eda. (A) Detection of ROS in porcine blastocysts derived from Eda treated and H_2O_2 treated groups. (B) Eda of relative fluorescence in tensity of ROS per blastocysts. Scale bars = 100 μm. The quantification analysis of fluorescence intensity in DCF-DA stained porcine blastocyst obtained by the image J program. Data in the bar graph represents the means±SEM of three independent experiments. Statistically differences are indicated by asterisks (* $P<0.05$; ** $P<0.001$, compared to control group).

decrease in blastocysts developmental rate. However, the group treated with H_2O_2 and Eda recovered as the control group. Under oxidative stress condition, the Eda may also play a significant role as antioxidant in regulating oxidative stress during porcine preimplantation embryo development.

Also, we found significant differences in the development competence of blastocyst formation embryos cultured with and without Eda under oxidative stress (Table 2). Thus, our data showed that Eda treatment greatly improved the quality of H_2O_2-induced oxidant stress by increased blastocyst formation, reducing apoptosis and fewer incidences of fragmentation and developmental retardation. We believe that the Eda may also play a significant role as an antioxidant in regulating apoptosis and quality of embryo in preimplantation development until blastocyst.

The regulation of ROS production and oxidative stress in the early embryonic development of the porcine affects apoptosis (Choi *et al.*, 2008). Moreover, the regulation of apoptosis *in vitro* fertilization (IVF) since the early embryo development process is very important for embryo development efficiency and determining of quality of blastocyst (Lichnovsky *et al.*, 2000). In addition, apoptosis during early embryonic development supports embryo survival under healthy conditions by selectively eliminating abnormal cells with nuclear and chromosomal abnormalities and by preventing massive cell death, which may lead to the loss of the whole embryo (Han *et al.*, 2008). Previously studies observed elevated increasing of the apoptotic factors of the mRNA expression levels and apoptotic cell in embryo development via ROS increasing condition (Cui and Kim, 2005). These studies are supported that regulating of

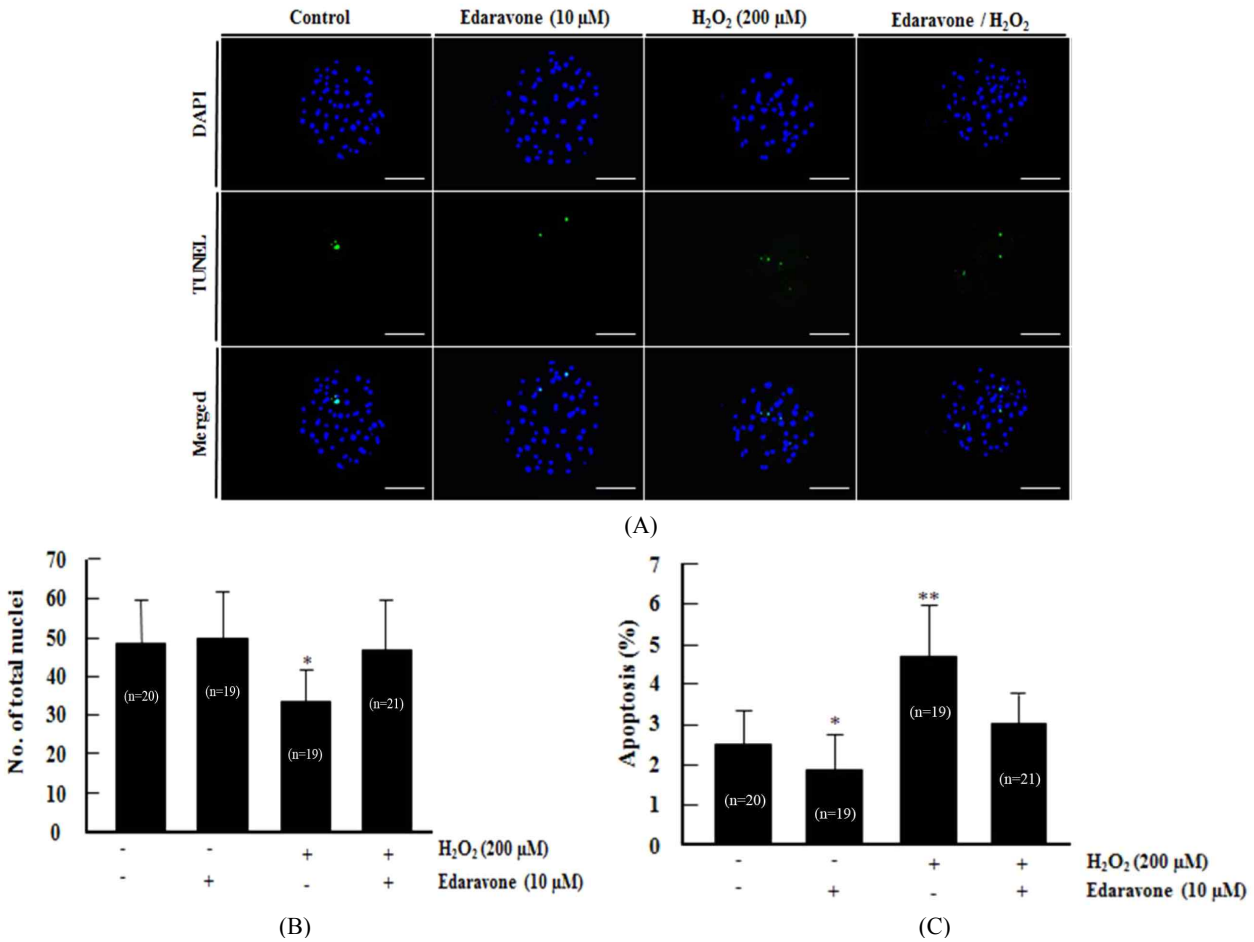


Fig. 4. Evaluation of H_2O_2-induced apoptosis by using TUNEL assay in porcine blastocysts cultured with Eda. (A) Apoptosis scanning images and anti-apoptotic characteristics in porcine blastocysts derived from H_2O_2 and/or Eda treatment groups. The chromatin content is stained by DAPI (blue), fragmented DNA is labeled by the TUNEL reaction (green), and colocalization with DAP lappears sky-blue. (B) Eda of the total cell numbers per blastocyst. (C) Percentage of TUNEL positive cell per blastocyst. The quantification analysis of fluorescence intensity in TUNEL stained porcine blastocyst obtained by the image J program. Data in the bar graph represents the means±SEM of three independent experiments. Statistically differences are indicated by asterisks (P<0.05).

apoptosis and apoptotic gene expression is critical role in preimplantation embryo development. So, we performed the analysis of TUNEL assay that revealed the number of apoptotic nuclei in blastocysts derived from embryos cultured with Eda was lower than that in those cultured without Eda following the H_2O_2 induction of oxidative stress. The total number of cells in blastocysts derived from embryos cultured with Eda was significantly higher than that of those cultured with out Eda (Fig. 4). Overall, these finding suggest that reduction of oxidative stress by Eda treatment may improve porcine blastocyst quality.

In conclusions, the results of the present study suggest that H_2O_2-induced oxidative stress results in decreased developmental competence as shown by an increased number of apoptotic nuclei and decreased cell numbers in blastocysts. Moreover, the addition of Eda under H_2O_2-induced oxidative stress improved developmental competence to the blastocyst stage, reducing the number of apoptotic nuclei and increasing the cell number in porcine IVP embryos by preventing oxidative stress. Therefore, ours study has provided the first evidence of Eda functional role associated with antioxidant and/or blocking of apoptosis in early embryo development stage until blastocyst of porcine *in vitro* production. Also, our results suggest that Eda may improve the development and quality of porcine embryos by preventing oxidative stress. Furthermore, these data will be beneficial for better understanding the antioxidant mechanisms

of *in vitro* culture condition in early embryo development stage of porcine *in vitro* production.

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Received November 25, 2015, Revised December 18, 2015, Accepted December 21, 2015