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Antioxidant Favors the Developmental Competence of Porcine Parthenogenotes by Reducing Reactive Oxygen Species

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ABSTRACT : Reactive oxygen species (ROS) generate during electrical activation of oocytes which has detrimental effects on embryo survival when overwhelmed. The present study was designed to investigate the ability of L-ascorbic acid, a novel water soluble antioxidant, to reduce the ROS level in developing embryos and their subsequent effects on embryo development *in vitro*. The compact cumulus oocyte complexes (COCs) were cultured in tissue culture medium (TCM)-199 supplemented with 10 ng/ml epidermal growth factor, 4 IU/ml pregnant mare serum gonadotropin (PMSG), and human chorionic gonadotropin (hCG) and 10% (v/v) porcine follicular fluid (pFF) for 44 h. After maturation culture, the denuded oocytes were activated with a single DC pulse of 2.0 kV/cm in 0.3 M mannitol solution containing 0.5 mM of HEPES, 0.1 mM of $CaCl_2$ and 0.1 mM of $MgCl_2$ for 30 μs using a BTX Electro-cell Manipulator. The activated oocytes were cultured in modified North Carolina State University-23 (mNSCU-23) medium for 168 h. The level of H_2O_2 in each embryo was measured by the dichlorohydrofluorescein diacetate (DCHFDA) method at 48 h after activation. The blastocyst formation rate was significantly higher when culture medium was supplemented with 50 and 100 μM L-ascorbic acid (31.2 and 38.7%, respectively) compared to non-supplemented (16.1%) group. Accordingly, significantly more cells in blastocyst were found for 50 and 100 μM L-ascorbic acid (50.0 and 56.4, respectively) compared to 0 and 200 μM L-ascorbic acid (36.5 and 39.8, respectively). L-ascorbic acid reduces the H_2O_2 level in developing embryos in a dose-dependant manner. The H_2O_2 level (pixels/embryos) was 191.5, 141.0, 124.0 and 163.3 for 0, 50, 100 and 200 μM L-ascorbic acid, respectively. So, we recommend to supplement 50 or 100 μM L-ascorbic acid in porcine *in vitro* culture medium. (**Key Words** : Antioxidant, Parthenogenitic Activation, Porcine, Embryo, Reactive Oxygen Species)

INTRODUCTION

During fertilization process, spermatozoa activate fully grown mammalian oocytes from the metaphase II arrest (Fissore et al., 2002). Following penetration into oocytes, spermatozoa trigger a number of cellular responses, most notably the intracellular calcium oscillation, which activate the oocytes (Snell and White, 1996; Evans, 1999). However, oocytes can be activated parthenogenetically by a variety of physical or chemical stimuli which can stimulate calcium oscillation (Whittingham, 1980). A physical stimulus may be mechanical (such as pricking), thermal (heating and cooling), or electrical. A chemical stimulus can be osmotic, ionic (divalent cations or calcium ionophores), or enzymatic. In porcine, electric pulse is commonly used to activate oocytes artificially for both partheogenetic study and after somatic cell nuclear transfer (Jolliff and Prather, 1997). Parthenogenetic activation is a useful tool to study effects of any supplement of interest on embryonic development. As a defined condition, it has advantages over *in vitro* fertilized (IVF) embryos because it minimizes the sources of variability that may rise from the spermatozoa.

The preimplantation embryos, especially after the morula stage (in case of porcine, around 4 days from fertilization), produce endogenous reactive oxygen species (ROS) by various enzymatic actions and metabolic pathways (Gardner and Lane, 2002; Harvey et al., 2002). *In vivo*, the damaging effects of oxygen radicals are usually prevented or limited by endogenous antioxidants. These include enzymes such as superoxide dismutase, catalase, glutathione peroxidase as well as low molecular weight

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antioxidants such as vitamin C, uric acid, glutathione and vitamin E (Guerin et al., 2001). As embryos are deprived this natural defense mechanism against ROS, various antioxidants have long been supplemented into *in vitro* maturation and *in vitro* culture media to improve the developmental competence of embryos. For example, addition of superoxide dismutase (SOD) or catalase to murine (Orsi and Leese, 2001) and bovine (Iwata, 1999) embryos and cysteine, β-mercaptoethanol (β-ME) or Vitamin-E to bovine embryos (Takahashi, 1993; Olson and Seidel, 2000) improved their development with up-regulation of glutathione (GSH) synthesis and controlling the redox environment. Kitagawa et al. (2004) reported that β-ME and vitamin E supplementation improved the developmental competence of porcine embryos after IVF.

Ascorbic acid is a water-soluble vitamin which has been considered to be the most important antioxidant in extracellular fluids (Buettner, 1993; Rose and Bode, 1993). It participates in many biological processes, such as biosynthesis of collagen and other components of the extracellular matrices (Buettner, 1993). A large amount of L-ascorbic acid is present in the preovulatory follicles in rat ovaries (Guarnaccia et al., 2000). In fact, ascorbic acid prevents follicular apoptosis in rat (Tilly and Tilly, 1995) and mouse (Eppig et al., 2000) follicles in *in vitro* condition. There is no information about the effects of L-ascorbic acid on the development of porcine parthenogenotes.

Thus, we designed the present study to evaluate the effects of L-ascorbic acid on the developmental competence of porcine oocytes. The hypothesis was that as an antioxidant L-ascorbic may reduce the ROS in embryos which may favor the embryonic development.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Oocyte collection and culture of cumulus–oocyte complexes

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in physiological saline at 30 to 35°C within 2 h. Follicular fluid and cumulus-oocyte complexes (COCs) from follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle attached to a 10-ml disposable syringe. Compact COCs were selected and cultured in tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 ng/ml epidermal growth factor, 4 IU/ml pregnant mare serum gonadotropin (PMSG) (Intervet, Boxmeer, Netherlands), and human chorionic gonadotropin (hCG) (Intervet) and 10% (v/v) porcine follicular fluid (pFF). Each group of 50 COCs was cultured in 500 ml modified TCM-199 medium in a 4-well dish (Nunc, Roskilde, Denmark) at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, the COCs were washed three times and cultured in PMSG- or hCG-free TCM-199 medium for another 22 h. The pFF was aspirated from follicles 3 to 7 mm in diameter from the prepubertal gilt ovaries. After centrifugation at 1,600×g for 30 min, supernatants were collected and filtered sequentially through 1.2 and 0.45 mm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). Prepared pFF was stored at -20°C until use.

Parthenogenic activation of oocytes

At the end of maturation culture, COCs were transferred to HEPES-buffered NCSU-23 medium (pH 7.2) containing 0.5 mg/ml hyaluronidase for 1 min and the cumulus cells were subsequently removed by gentle pipetting. Denuded oocytes were placed in a 0.3 M mannitol (pH 7.4) solution containing 0.5 mM of HEPES, 0.1 mM of $CaCl_2$ and 0.1 mM of $MgCl_2$ for 4 min (activation solution) and transferred to a chamber consisting of 2 electrodes overlaid with activation solution. Oocytes were activated with a single DC pulse of 2.0 kV/cm for 30 μs using a BTX Electro-cell Manipulator 2001 (BTX Inc.; San Diego, CA, USA).

Embryo culture

The basic *in vitro* culture (IVC) medium was modified North Carolina State University-23 (mNSCU-23) medium (Kim et al., 2004) containing 0.5 mM pyruvate and 5.0 mM lactate as energy sources instead of 5.5 mM glucose, and 0.4% w/v bovine serum albumin (BSA). Oocytes were cultured at 39°C under a 5% CO_2, 5% O_2 and 90% N_2 atmosphere for 168 h. The initiation of activation was considered as 0 h. The rates of cleavage, morula and blastocyst formation were evaluated under a stereomicroscope at 48, 120 and 168 h post activation, respectively.

Differential staining

The cell number of blastocysts was assessed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cells according to a modified staining procedure (Thouas et al., 2000). Briefly, TE cells of blastocysts at 168 h were stained with 100 μg/ml propidium iodide fluorochrome containing a permeabilizing solution of 1% (v/v) Triton X-100 ionic detergent for 15 sec. Blastocysts were carried out simultaneous staining and fixation in a second solution of 2 μg/ml bisbenzimide (stain) in 99% ethanol (fixation) then incubated overnight at 4°C. Fixed and stained whole blastocysts were mounted and assessed for cell number using epifluorescence microscopy.

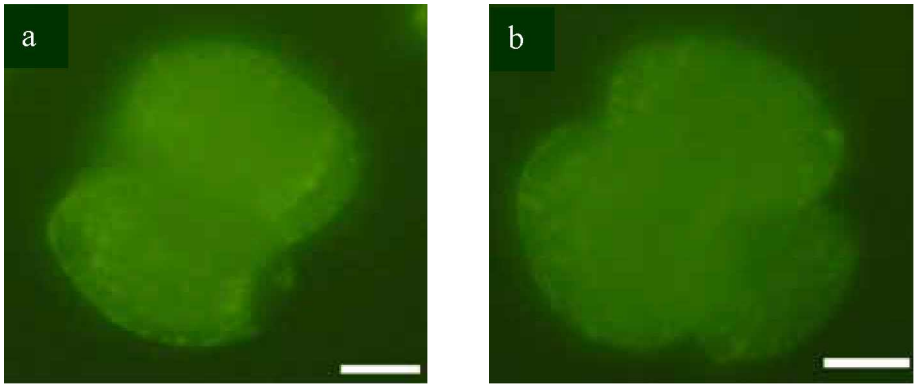

Figure 1. A 2-cell (A) and 4-cell (B) embryo at 48 h of parthenogenetic activation. Embryos were stained with 10 μM DCFHA and pictures were taken under ultraviolet ray (Bar represents 30 μM).

Table 1. Effects of various concentrations of L-ascorbic acid on the development of porcine oocytes after parthenogenic activation

L-ascorbic acid (μM)	No. of oocytes	No (%, mean[1]±SD) of embryos developed to		
		2-cell	Morula	Blastocyst
0	244	162 (66.4±4.6)	78 (31.3±4.5)[a]	39 (16.1±2.9)[a]
50	235	171 (72.7±5.4)	114 (48.5±6.5)[bc]	73 (31.2±5.8)[bc]
100	215	173 (80.4±5.3)	122 (56.8±5.0)[b]	83 (38.7±4.7)[b]
200	221	152 (69.8±12.4)	87 (39.5±5.1)[ac]	52 (23.5±7.0)[ac]

[1] Mean was calculated from the percent value of 4 replications.
[a, b, c] Within the same column, values with different superscripts were significantly different ($p<0.05$).

The blue nuclei represent ICM and pink-to-red nuclei represent TE cells.

Measurement of hydrogen peroxide (H_2O_2) content

The level of H_2O_2 in each embryo was examined by measuring the 2`, 7` dichlorohydrofluorescein diacetate (DCHFDA) method described by Yang et al. (1998) and Kim et al. (2006). Embryos at the 2- to 4- cell stages at 48 h post activation were transferred into culture medium containing 10 mM DCHFDA. After 20 min of culture, embryos were washed in HEPES buffered NCSU-23 medium, and measured for the H_2O_2 level. The fluorescent emissions from the embryos were recorded as Tagged Image File Format (TIFF) files (Figure 1) using a cooled charge-coupled device (CCD) camera attached to a fluorescence microscope (Axio Photo; Carl Zeiss Jena GmbH, Jena, Germany) with excitation filters at 405-435 nm and 515 nm for emission. The recorded fluorescent images were analyzed using NIH image software 1.55 (National Institutes of Health, Bethesda, MD, USA) by counting the number of pixels after color inversion.

Experimental design

The experiment I was designed to standardize the optimum concentration of L-ascorbic on the development of porcine parthenogenotes. Three different concentrations, 50, 100 and 200 μM L-ascorbic acid, were added to the mNCSU-23 medium for the entire 168 h culture period. L-ascorbic was dissolved in to deionized water as a 10x stock solution. The experiment II was evaluated the effect of L-ascorbic acid on the H_2O_2 level of early embryos. After parthenogenic activation the putative embryos were cultured in to mNCSU-23 for 48 h. All the 2- and 4-cell embryos at 48 h of culture were stained with DCHFDA by above mentioned procedure.

Statistical analysis

The differences in embryo development among experimental groups were analyzed using one-way ANOVA after arcsine transformation to maintain homogeneity of variance. Post hoc analyses to identify between-group differences were performed using the Tukey test. The same test was used to determine statistical significance for the cell numbers in blastocysts among experimental groups without arcsine transformation. All analyses were performed using SAS (SAS Institute, version 8.1). The significant differences among the treatments were determined, where the P value was less than 0.05.

RESULTS

Effect of L-ascorbic acid on embryo development

The effect of L-ascorbic acid on the development in porcine parthenogenotes is shown in Table 1. L-ascorbic acid showed a marked beneficial effect on embryonic development in a dose-dependant manner. The morula and blastocyst formation rate were significantly higher when culture medium was supplemented with 50 and 100 μM L-

Table 2. Effects of various concentrations of L-ascorbic acid on cell allocation to inner cell mass (ICM) and trophectoderm (TE) in blastocysts

L-ascorbic acid (μM)	No. of blastocyst	ICM (Mean±SD)	TE (Mean±SD)	Total (Mean±SD)
0	32	10.2±2.0[a]	26.3±5.6[a]	36.5±7.6[a]
50	61	12.2±1.9[bc]	37.9±6.3[b]	50.0±8.1[b]
100	69	12.4±2.1[b]	42.4±7.2[c]	56.4±8.4[c]
200	42	11.3±1.9[ac]	28.5±4.3[a]	39.8±5.4[a]

[a, b, c] Within the same column, values with different superscripts were significantly different ($p<0.05$).

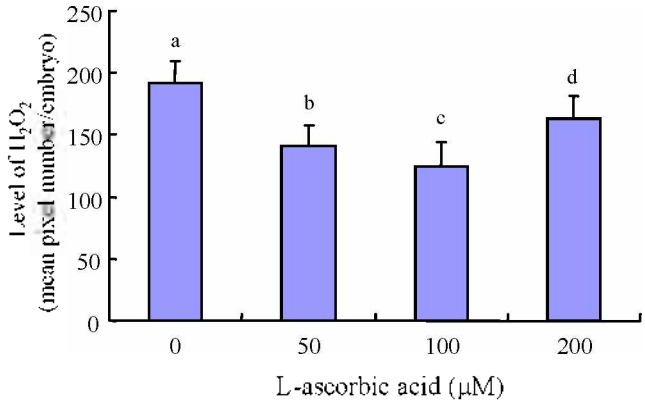

Figure 2. Levels of hydrogen peroxide (H_2O_2) in porcine pathenogenically activated embryos cultured with different concentrations of L-ascorbic acid. [a, b, c, d] Different superscripts were significantly different ($p<0.05$).

ascorbic acid compared to non-supplemented control group. There was no difference between 50 and 100 μM L-ascorbic acid. The blastocyst formation rate was 16.1±2.9, 31.2±5.8, 38.7±4.7 and 23.5±7.0 for 0, 50, 100 and 200 μM L-ascorbic acid, respectively.

Effect of L-ascorbic acid on cell number

Table 2 represents the number of total cells as well as cell allocation to inner cell mass (ICM) and trophectoderm (TE) in blastocysts. L-ascorbic acid significantly increased the total cells, ICM and TE cell number in blastocyst in a dose-dependant manner. Significantly more cells were found for 50 μM (50.0±8.1) or 100 μM (56.4±8.4) L-ascorbic acid compared to 0 (36.5±7.6) or 200 μM (39.8± 5.4) L-ascorbic acid.

Effect of L-ascorbic acid on the H_2O_2 level in embryos

The effect of L-ascorbic acid on the H_2O_2 level in embryos is shown in Figure 2. All the embryos at the 2- to 4- cell stages at 48 h post activation were collected to measure the H_2O_2 level. A total of 69, 92, 98 and 71 embryos (3 replications) for 0, 50, 100 and 200 μM L-ascorbic acid, respectively were stained with DCHFDA. The non supplemented group had significantly higher H_2O_2 level compared to supplemented groups. The H_2O_2 level (mean pixel number/embryo) was 191.5±18.5, 141.0±16.6, 124.0±20.4 and 163.3±18.3 for 0, 50, 100 and 200 μM L-ascorbic acid, respectively.

DISCUSSION

Efficient scavenging of ROS in culture media can improve the developmental competence of embryos. The present study demonstrated a higher blastocyst formation rate and improvement of blastocyst quality by the presence of L-ascorbic acid into the embryo culture media. The blastocyat quality was evaluated by the number of total cells as well as ratio of ICM and TE. L-ascorbic acid also reduces the H_2O_2 level in the developing embryos. However, the beneficial effects were found to be varied in a dose-dependant manner.

Oxidative stress seems to be responsible for numerous types of embryo damage. ROS such as super oxides are able to diffuse and pass trough cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids (Bilodeau et al., 2000). The consequences are mitochondrial alterations, embryo cell block, ATP depletion and apoptosis (Guerin et al., 2001). Therefore, ROS must be continuously inactivated in order to keep only a small amount necessary to maintain normal cell functions. L-ascorbic acid and α-tocopherol can efficiently neutralize H_2O_2 induced ROS production in spermatozoa (Donnelly et al. 1999). Indeed, the antioxidant activity of L-ascorbic acid in preventing free radical-initiated peroxidative tissue damages is well documented (Rose and Bode, 1993; Wang et al., 2001). In the present study, 50 and 100 μM L-ascorbic acid improved blastocyst formation rate and blastocyst quality. Wang et al. (2001) used ascorbic acid and α-trolox an analogue of Vitamin E in mouse embryo culture system and reported the superiority of ascorbic acid on embryo development.

L-ascorbic acid may act as a prooxidant under some condition. Like in presence of transition metal, it converts ferric into ferrous ion, thereby catalyses the Fenton reaction (Guerin et al., 2001). Furthermore, it can induce apoptotic cell death at higher concentrations (Tilly and Tilly, 1995). Wang et al. (2001) reported that any concentration more than 100 μM L-ascorbic acid reduced blastocyst production rate in mouse. Even, embryo toxicity was developed in mouse embryos at 400 μM concentration. In agreement

with these findings, our results revealed the presence of higher concentration of L-ascorbic acid i.e., 200 μM during the IVC of porcine parthenogenotes significantly reduced the number of blastocyst and blascocyst quality (Table 1 and 2). 200 μM L-ascorbic may be act as a prooxidant in porcine IVC medium resulted in retarded development and reduced cell number in blastocysts.

ROS induced oxidative damage in the cells largely depends on the balance of between its production and detoxification (Guille and Joenje, 1991). Indeed, advantageous effects of ROS at a physiological concentration have already been proven in different biological processes, especially in cell signaling pathways. ROS also participates in reproductive functions like a physiologic level of ROS is necessary for the normal development of oocyte maturation and subsequent embryo development *in vivo* (Guille and Joenje, 1991). ROS has an obligatory role in the fertilization process of sea urchin (Shapiro, 1991) and may have an important role in successful sperm-zona interaction (Miesel et al., 1993). Like wise, in the present study, lower concentration of ROS in the embryos did not block the development as ROS was detected in all 2- and 4-cell embryos. We found that higher ROS concentration in developing embryos leads to developmental block or produces inferior quality blastocyst. The blastocyst formation and cell number in blastocyst was significantly less for 0 or 200 μM L-ascorbic acid where higher ROS level was detected (Table 1 and 2, and Figure 2). However, some of the limitation of this paper should be addressed. We only measured ROS level in early stage embryos. Further experiments on the temporal pattern of ROS production in developing embryos may provide valuable information about antioxidant defense mechanism.

In conclusion, L-ascorbic acid at a concentration of 50 to 100 μM can protect porcine parthenogenotes from oxidative stress, and 200 μM L-ascorbic acid reduces blastocyst production rate. So, we recommended 50 or 100 μM L-ascorbic acid could be supplemented in porcine embryo culture media.

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