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Effect of Supplementation of Vitamin E on *In Vitro* Maturation and Activation of Bovine Oocytes

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ABSTRACT

This study was carried out to assess the effect of vitamin E against the reactive oxygen species (ROS) on chemical activation of *in vitro* matured oocytes. Bovine oocytes were aspirated from slaughtered ovaries and transferred to maturation medium with or without vitamin E (100 μM). After 22 hours of culture, oocytes with polar bodies were selected and submitted to activation treatments with or without vitamin E. After activation, oocytes were cultured in mSOF medium and rate of development was monitored. For ROS (H_2O_2) detection, *in vitro* matured and activated oocytes were selected and stained with DCFDA and observed under fluorescence microscope. The ROS contents were not significant differences in IVM rate, activation process and embryonic development to blastocysts with or without vitamin E. The cell number of blastocyst showed significant difference ($p<0.05$) in embryos matured and activated with vitamin E. The results of the present study demonstrated that the exposure of vitamin E in IVM and activation process improved the quality of embryos evaluated by the cell number of blastocysts.

(Key words : ROS, vitamin E, bovine, oocyte)

INTRODUCTION

Recently, reactive oxygen species (ROS) are known to be generated physiologically in the female reproductive system and to have a functional role for developmental process, such as maturation of oocyte and embryonic development or differentiation in several species (Combelles *et al.*, 2009; Ufer *et al.*, 2010). However, ROS have been still problematic in the fields of *in vitro* systems for mammalian reproduction (Combelles *et al.*, 2009; Gajda. 2009; Ufer *et al.*, 2010). Excessive ROS has been showed deleterious effects on the *in vitro* gamete culture systems (Noda *et al.*, 1991; Goto *et al.*, 1993; Guerin, *et al.*, 2001), and known to be the main causes for the low efficiency of *in vitro* development of bovine and porcine embryos (Dalvit *et al.*, 2005; Koo *et al*,. 2008; Morado *et al*,, 2009; You *et al.*, 2010). The various antioxidants, such as taurine/hypotaurine, ascorbic acid, vitamin E (α-tocopherol), anthocyanin and glutathione, have been added to culture medium for neutralizing ROS effect. The previous studies have reported that these antioxidants have allowed improved results in *in vitro* culture of oocyte and embryos from several species (Suzuki *et al.*, 2007; Wongsrikeao *et al.*, 2007; Martín-Romero *et al.*, 2008; Mozdaran and Nazari, 2010; You *et al.*, 2010). Among these, vitamin E is known as a protective agent, which interacts directly with oxidizing radicals (Burton and Ingold, 1986; Jones *et al.*, 1995), and believed to be the primary free radical scavenger in mammalian cell membrane (Chow, 1991). However, vitamin E is remained as controversial reagent for its ambiguous action, especially in the *in vitro* culture of bovine oocyte and embryos. Vitamin E have showed detrimental effect or enhanced results in bovine oocyte during *in vitro* fertilization and cytoplasmic maturation *in vitro* (Olson and Seidel, 2000; Dalvit *et al.*, 2005; Wongsrikeao *et al.*, 2007; Marques *et al.*, 2010). The present study was conducted to evaluate whether addition of vitamin E to the maturation and activation medium of bovine oocytes could reduce the generation of ROS and could improve the further development of oocytes.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were purchased from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated otherwise.

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2. Oocyte Collection and *In Vitro* Maturation (IVM)

Bovine ovaries were collected from slaughtered cows at a local abattoir and transported to the lab. at 37℃. Cumulus-oocyte complexes (COCs) were aspirated from follicles 3～8 mm in diameter in ovaries. Only COCs with compact cumulus layers were selected and washed three times in HEPES-buffered Tyrode's medium containing 0.05%(w/v) polyvinyl alcohol (PVA). Then COCs were transferred into each well of 4-well multidish(Nunc, Roskilde, Denmark) containing 500 μl maturation medium (TCM 199; Invitrogen), supplemented with 10% (v/v) fetal calf serum (Invitrogen), 100 mg/l-cysteine, 44 mg/l sodium pyruvate, gonadotropins (each 250 IU of eCG and hCG/ml) (Intervet, Boxmeer, Holland), 10 ng/ml epidermal growth factor (EGF), and with or without vitamin E (100 μM). Oocytes were cultured in at 38.9℃ in 5% CO_2 in humidified air.

3. Activation and *In Vitro* Culture (IVC) of Oocyte

After 22 hours of culture with or without vitamin E, oocytes protruded polar bodies were selected and submitted to activation treatments. Oocytes were exposed to calcium ionomycin (5 μM for 5 min) followed by incubation with 6-DMAP (2 mM) for 3.5 hours in medium supplemented with or without vitamin E (100 μM). After activation, oocytes were cultured in mSOF medium containing 0.8% BSA at 38.9℃ in 5% CO_2, 5% O_2 in humidified air for 7～8 days. Blastocyst formation were evaluated Days 7, and the total blastocyst cell count was also performed at Day 8 using Hoechst 33342 staining under an epifluorescence microscope (Olympus, Japan).

4. Measurement of Intracellular ROS

The oocytes from IVM and activation were sampled respectively to measure ROS level. Reactive oxygen species were determined by modified methods previously described (Hashimoto *et al.*, 2000) Briefly, H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate), which emitting green fluorescence, was used to detect intracellular ROS (H_2O_2). Oocytes from each treatment group (w/o vitamin E) were incubated for 30 min in HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (PVA) supplemented with 10 μM H2DCFDA. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA, mounted on the slide glasses with small drop of washing medium, and observed under an epifluorescence microscope with UV filters (460 nm). Fluorescent images were saved as graphic files in tiff format. Levels of ROS were analyzed by counting the number of black-white pixels of TIFF images by ImageJ software (Version 1.32; National Institutes of Health, Bethesda, MD, USA) after converttsion of fluorescence image. High pixel number represented high level of ROS.

5. Statistical Analyses

Data were evaluated by Student's *t*-test, Chi-square and one-way ANOVA. Difference at $p<0.05$ was considered significant.

RESULTS

Supplementation of IVM medium with or without 100 μM vitamin E resulted in no significant difference in IVM rate and ROS level between oocytes cultured *in vitro* (Table 1, $p>0.05$). The ROS level in the oocytes activated with or without vitamin E also showed no significant difference (Table 2, $p>0.05$). The embryos which matured and activated in the medium supplemented with vitamin E showed higher developmental rate to blastocyst than other groups, however, there was no difference between groups in percentages of embryos that developed to blastocyst *in vitro* (Table 3, $p>0.05$). The total cell number of blastocysts was significant ($p<0.05$) in embryos matured and activated in the medium supplemented with vitamin E than in other embryos (Table 4).

Table 1. Levels of ROS (H_2O_2) in bovine oocyte after cultured *in vitro* for 22 hours with or without vitamin E using DCFDA

Vitamin E	No. of oocytes examined	No. of oocytes matured (%)	No. of Pixels (Mean±S.E.M.)
+	75	63(84.0)	219.25±2.74
−	72	57(79.2)	220.21±9.00

+; added / −; non-added.

Table 2. Levels of ROS(H_2O_2) in bovine oocyte after activated *in vitro* for 3.5 hours with or without vitamin E using DCFDA*

Vitamin E	No. of oocytes examined	No. of Pixels (Mean±S.E.M.)
+	125	216.85±16.16
−	94	219.51±7.04

+; added / −; non-added.
* Examined at 16～18 hrs after activation.

Table 3. Development of bovine oocyte after activated with or without vitamin E*

	IVM	Activation	No. of blastocysts* examined	No. of blastocysts (%)
Vitamin E	−	+	319	52(16.30)
	−	−	326	45(13.80)
	+	+	236	44(18.80)
	+	−	213	30(14.16)

+; added / −; non-added.
* Examined at 7~8 days after *in vitro* culture.

Table 4. Cell number of blastocysts derived from oocytes activated and matured with or without vitamin E*

	IVM	Activation	No. of blastocysts* examined	No. of cells (Mean±S.E.M.)
Vitamin E	−	+	44	95.04±24.62[a]
	−	−	44	92.04±25.74[a]
	+	+	40	153.00±24.02[b]
	+	−	41	127.63±30.25[a]

+; added / −; non-added.
* Examined at 8 days after *in vitro* culture.
Different superscripts in same columns indicate significant differences ($p<0.05$).

DISCUSSION

The role of ROS in the *in vitro* culture of oocytes and embryos remains controversial. However, excessive ROS, inevitably produced in *in vitro* culture process of mammalian cells, is a potential harmful factor resulting deleterious effects on the oocytes and early embryonic development (Nasr-Esfaani *et al.*, 1990; Noda *et al.*, 1991; Goto *et al.*, 1993; Takahashi *et al.*, 2000; Guerin *et al.*, 2001). Vitamin E (α-tocopherol) is known to act as a protective agent against polyunsaturated fatty acid peroxidation. The basic action mechanism of this antioxidant is focused on the direct scavenging of peroxyl radicals, a kind of ROS, yielding a nonradical product and the tocopheroxyl radical (Liebler 1993). Thus, vitamin E is also known to be related with cellular membrane stability and endogenous antioxidants are thought to exert a role in the mammalian gamete maturation, such as capacitation of spermatozoa (Marques *et al.*, 2010). In present study, the addition of vitamin E to the maturation and activation medium did not modify maturation and developmental rate to blastocyst of oocyte, and did not alleviate the level of ROS. These results are in agreement with those observed vitamin E did not show significant improvement in nuclear maturation of rat oocyte (Takami *et al.*, 1999), in bovine oocyte IVM (Olson and Seidel, 2000; Dalvit *et al.*, 2005). Even deleterious effects of supplement of vitamin E were reported in the study of bovine spermatozoa treatment in IVF process (Dalvit *et al.*, 1998; Marques *et al.*, 2010). It has been observed that physiologically produced ROS have regulatory function of maturation process (Takami *et al.*, 1999), such as cytoplasmic maturation and resumption of meiosis, and that moderate level of ROS is needed to alter membrane features for sperm capacitation (Marques *et al.*, 2010). It can be thought that exogenous antioxidant may exert an unneeded scavenger for functional ROS.

The result of ROS level in the chemical activation adapted in the present study was different with that of electrical activation (Koo *et al.*, 2008). The condition of electrical activation may be the cause for the elevated level of ROS. From the previous studies reporting the addition of vitamin E to culture medium have showed the advance results in other species, such as pig (Kitagawa *et al.*, 2004; Gajda *et al.*, 2008; Gajda, 2009) and sheep (Peng *et al.*, 2008), it may exist an uncertain species differences in the mechanism of ROS-antioxidants reaction.

ROS are produced as superoxide anion, hydroxyl radical, hydrogen peroxide and lipid peroxides (Morado et a., 2009). DCFDA used as ROS marker for the present study is known to react mainly with hydroxy peroxide (H_2O_2). Blondin *et al.* (1997) reported that addition of the antioxidant agents, such as beta-mercaptoethanol, ascorbic acid or superoxide dismutase, which responding various type of ROS, to the maturation medium failed to modify the percentage of bovine embryos produced *in vitro*. In present study, the embryo quality measured by cell number of blastocysts showed significant improved results in the embryos which matured and activated with vitamin E. This data is corresponding to the previous results, which reported the vitamin E did not increase the *in vitro* maturation rates, but improved embryonic status after embryo transfer (Olson and Seidel, 2000) and the blastocyst rate and quality in pig (Tao *et al.*, 2010). These studies suggest that the antioxidant activity and the function which maintain cellular membrane stability of vitamin E may be more apparent in later

stage of embryonic development. From the reports of Wongsrikeao *et al.* (2007), vitamin E is thought to operate more efficiently in damaged cells, such as recipient oocyte, donor cells and cloned embryos during the experimental process, like enucleation and electric fusion.

In summary, the present study demonstrated that the effect of vitamin E against ROS is still intriguing in the culture of bovine oocyte, but exposure of vitamin E in IVM and activation process improved the quality of embryos evaluated by the cell number of blastocysts.

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