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Antioxidant Effect of Alpha-Linolenic Acid during *In Vitro* Maturation in Porcine Oocytes

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ABSTRACT

Alpha-linolenic acid (ALA) is one of n-3 polyunsaturated fatty acids and found mainly in the chloroplasts. Many studies have been reported that intracellular reactive oxygen species (ROS) in mammalian oocytes were reduced by supplementation of ALA in *in vitro* maturation (IVM) medium. Based on these reports, we expected that ALA acts as an antioxidant during IVM of porcine oocytes. Therefore, the objective of this study was to investigate the antioxidant effect of ALA supplementation during IVM in porcine oocytes. The cumulus-oocyte complexes (COCs) were incubated in IVM medium containing 200 μM H_2O_2 or H_2O_2 with 50 μM ALA for 44 h. Nuclear maturation stage of oocytes was evaluated using aceto-orcein method. For measurement of oxidative stress state, intracellular ROS and glutathione (GSH) levels were measured using carboxy-DCFDA and cell tracker red, respectively. In results, oocytes in metaphase-II (MII) stage development was significantly reduced in H_2O_2 group compared to non-treated control group (61.84±1.42% and 80.00%, respectively; $p<0.05$) and it was slightly recovered by treatment of ALA (69.76±1.67%; $p<0.05$). The intracellular GSH levels was decreased in H_2O_2 groups compared with control groups, but it was enhanced by ALA treatment ($p<0.05$). On the contrary, H_2O_2 treatment increased intracellular ROS level in oocytes and H_2O_2-induced ROS was decreased by treatment of ALA ($p<0.05$). Our findings suggested that ALA treatment under oxidative stress condition improve oocyte maturation via elevated GSH and reduced ROS levels in oocytes. Therefore, these results suggest that ALA have an antioxidative ability and it could be used as antioxidant in *in vitro* production system of porcine embryo.

(Key words : Alpha-linolenic acid, *In vitro* maturation, Porcine oocyte, Reactive oxygen species, Glutathione)

INTRODUCTION

In *in vitro* production (IVP) system, the causes of low developmental efficiency of oocytes are very various. The major reason is that *in vitro* mature oocytes have lower developmental competence than *in vivo* mature oocytes. In IVP technique of porcine oocytes, *in vitro* maturation (IVM) is one of most important steps (Kitagawa *et al*., 2004; Sovernigo *et al*., 2017). However oocytes are exposed to oxidative stress *in vitro* than *in vivo*, they are a major cause of low developmental efficiency in *in vitro*-mature oocyte (Sovernigo *et al*., 2017). Because oocytes were exposed to high-oxygen environment during IVM, they are negatively affected by oxidative stress that is mainly caused by the production of reactive oxygen species (ROS) (Taweechaipaisankul *et al*., 2016). Excessive ROS level in cells caused inactivation of enzymes, DNA fragmentation, apoptosis and dysfunction of mitochondria (Kowaltowski and Vercesi, 1999). These ROS in oocytes was reported to be increased by various factor, such as metal cations, oxygen concentration, and visible light (Guerin *et al*., 2001). Zhang *et al*. (2006) reported that supplyment of H_2O_2 has an adverse effect on the oocytic spindle formation, during IVM of mouse oocytes as an oxidizing agent. In porcine oocyte, ROS decreased the percentage of oocytes at metaphase II stage after IVM and It has detrimental effects on cytoplasm maturation and developmental competence (Alvarez *et al*., 2015).

To prevent of ROS generation, various substances, such as vitamin, trace elements, and hormone, were

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supplied to IVM medium. In ovine and porcine oocytes, vitamin C and E enhanced embryonic development via reduction of oxidative stress (Natarajan *et al*., 2010; Hu *et al*., 2012). Sovernigo *et al*. (2017) reported that quercetin, which is one of flavonoids, decreased ROS in bovine oocytes during IVM. Similarly, treatment of L-carnitine during ovine oocytes reduced the ROS level, enhanced GSH level, and increased mRNA expression of glutathione peroxidase gene (Mishr *et al*., 2016). Also, thiols reduced levels of ROS and increased levels of GSH in bovine oocytes (Rocha-Frigoni *et al*., 2016). These studies suggested that various substances, which is not antioxidants such as glutathione, superoxide dismutase, and glutathione peroxide, could act as an antioxidant reagent.

Alpha-linolenic acid (ALA) that is mainly existed in chloroplasts is one of polyunsaturated fatty acid and it is classified as omega-3 fatty acid (Marei *et al*., 2009). This fatty acid plays an important role in storage of energy for metabolism and energy source in oocytes (McKeegan and Sturmey, 2011). These function of ALA in mammalian oocytes are known to be involved with oocyte maturation and embryonic development (Marei *et al*., 2009; Lee *et al*., 2016b; Lee *et al*., 2017). Marei *et al*. (2010) reported that the addition of linolenic acid to bovine IVM medium increased the number of mature oocytes. Similarly, supplementation of ALA in culture medium enhanced GSH content, nuclear maturation, and embryonic development after pathenogenetic activation and somatic cell nuclear transfer in pigs (Lee *et al*., 2016b). In addition, our previous study shown that treatment of ALA during oocyte maturation and embryo culture improved nuclear maturation of oocyte and embryonic development (Lee *et al*., 2017). Based on these studies, we hypothesized that ALA may enhanced meiotic competence via increasing of antioxidant capacity in oocytes. Therefore, the aim of this study was to investigate whether supplementation of ALA during IVM enhances the antioxidant capacity under H_2O_2-induced oxidative environment in porcine oocytes.

MATERIALS AND METHODS

Oocytes Collection and *In Vitro* Maturation

All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). Gilts ovaries were collected from local slaughterhouse and transferred to the laboratory in 0.9% (w/v) sterilized saline within 2 h. The cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3～6 mm diameter) using 18-gauge needle with 10 cc syringe. After aspiration, COCs with compact cumulus layer and homogeneous cytoplasm were selected and incubated in medium-199 (Invitrogen, MA, USA) containing 10% (v/v) porcine follicular fluid (pFF), 10 IU/mL human chorionic gonadotropin (hCG; Intervet), 10 ng/mL luteinizing hormone (LH; Sigma-aldrich, St. Louis, MO, USA), 10 ng/mL follicle stimulating hormone (FSH; Sigma-aldrich) and 10 ng/mL epidermal growth factor (EGF; Sigma-aldrich) with 200 μM H_2O_2 or H_2O_2 with 50 μM ALA at 38.5℃ in 5% CO_2 condition for 22 h. Then, it were subsequently incubated using hormone-free medium-199 containing H_2O_2 or H_2O_2 with ALA at 38.5℃ in 5% CO_2 condition for 22 h.

Measurement of Intracellular GSH and ROS Levels

To determine intracellular GSH and ROS levels in porcine oocytes, carboxy-DCFDA and cell tracker red were used to detect intracellular ROS and GSH level, respectively. COCs were denuded using 0.1% (v/v) hyaluronidase for 44 h after IVM and fixed by 4% paraformaldehyde in the dark for 10 min. From each treatment group, 15 oocytes were incubated with 20 μM carboxy-DCFDA and 5 μM cell tracker red in the dark room for 30 min. After incubation, oocytes were washed with PBS-PVA and the fluorescence was observed using a fluorescent microscope. The fluorescence intensity in oocytes were analyzed using Image J software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA).

Evaluation of Nuclear Maturation

Aceto-orcein stain method was used to evaluate nuclear maturation stage of porcine oocyte. After IVM, mature oocytes were denuded using 0.1% (v/v) hyaluronidase and fixed in acetic alcohol solution (acetic acid:ethanol; 1:3; v/v) for 48 h at room temperature (RT). Oocytes were stained by 1% (w/v) aceto-orecein at RT for 7 min. The morphology of nuclear was observed under light microscope. The oocytes at germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase (AI) and telophase I (TI) phase were decided as immature oocyte and MII stage oocytes were classified as mature oocyte.

Statistical Analysis

All numerical data representing each parameter were analyzed using the Statistical Analysis System Software (SAS, version 9.4). Data are presented means±SEM and comparisons among treatment groups were conducted by Duncan's multiple range tests using a generalized linear model (GLM) in the SAS package. A value of *p*<

0.05 was considered to indicate a statistically significant difference.

RESULTS

Changes of Intracellular ROS and GSH Level by ALA under Oxidative Stress

Intracellular GSH and ROS levels in oocytes were shown in Fig. 1 and Fig. 2, respectively. GSH level in oocytes was significantly reduced in H_2O_2 treatment compared to control groups ($p<0.05$). And supplementation of ALA with H_2O_2 enhanced GSH level compared to H_2O_2 treatment groups ($p<0.05$). On the contrary, treatment of H_2O_2 increased intracellular ROS level compared to control groups and ALA reduced the enhanced ROS level by H_2O_2 treatment ($p<0.05$).

Antioxidant Effect of ALA on Nuclear Maturation of Porcine Oocytes

The antioxidant Effect of ALA on nuclear maturation was shown in Fig. 3. Similar to result of GSH level, population of MII stage-reached oocytes were lower in H_2O_2 treatment groups than control groups ($p<0.05$). However, it was slightly recovered by supplementation of ALA ($p<0.05$). Interestingly, ratio of mature oocytes were not completely recovered by ALA.

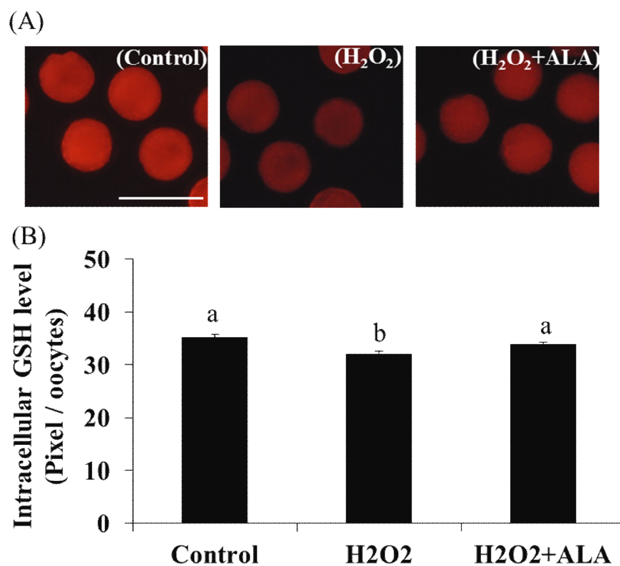

Fig. 1. Effect of alpha-linolenic acid (ALA) on intracellular glutathione (GSH) level under H_2O_2-induced oxidative stress condition during *in vitro* maturation in porcine oocytes. (A) Fluorescent microscopic images of porcine oocytes; (B) fluorescence intensity levels of GSH. [a,b] Different superscript indicate significant difference ($p<0.05$), Scale bar: 200 μm.

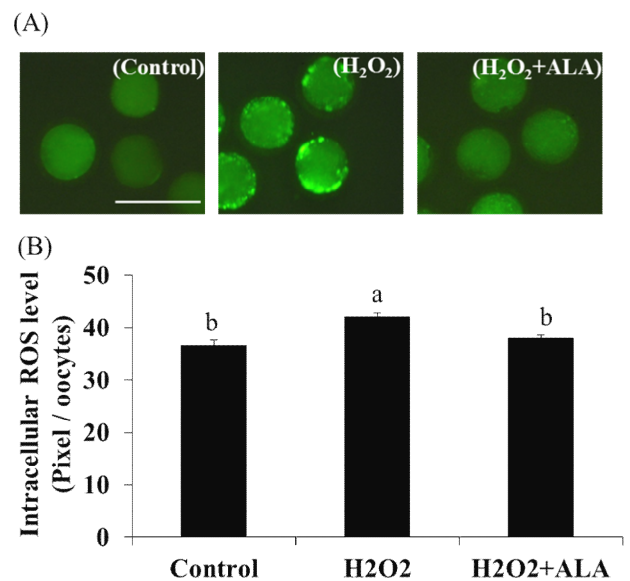

Fig. 2. Effect of alpha-linolenic acid (ALA) on intracellurar reactive oxygen species (ROS) level under H_2O_2-induced oxidative stress condition during *in vitro* maturation in porcine oocytes. (A) Fluorescent microscopic images of porcine oocytes; (B) fluorescence intensity levels of ROS. [a,b] Different superscript indicate significant difference ($p<0.05$), Scale bar: 200 μm.

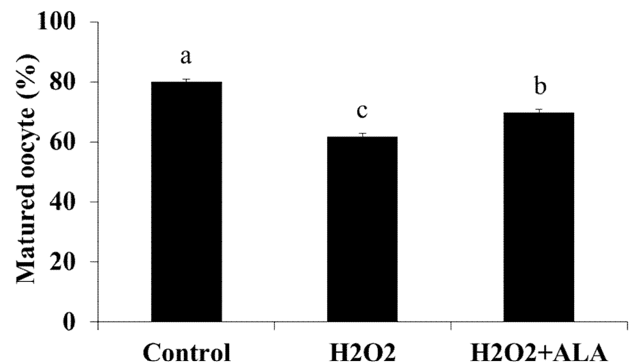

Fig. 3. Effect of alpha-linolenic acid (ALA) on nuclear maturation under H_2O_2-induced oxidative stress condition during *in vitro* maturation in porcine oocytes ($p<0.05$). [a~c] Different superscript indicate significant difference ($p<0.05$).

DISCUSSION

The balance between oxidative stress and antioxidants is one of important factor that influenced to maturation and developmental competence of mammalian oocytes. Despite of improvement of IVP technology in oocytes, oxidative stress, which is generated by *in vitro* environment, is still remained as major problem on low efficiency of IVP system. For reduction of ROS

generation during IVM process of oocytes, various supplement, such as vitamin, trace elements, and hormone as an antioxidants, were contained to culture medium of mammalian oocytes (Kere *et al*., 2013; Jeon *et al*., 2014; Li *et al*., 2015). In our previous study (Lee *et al*., 2017), supplementation of ALA during IVM and IVC of porcine oocytes enhanced the nuclear maturation and embryonic development and we expected that ALA could enhanced the maturation of oocytes through reduction of oxidative stress in oocytes. Therefore, we investigated antioxidant effect of ALA during IVM of porcine oocyte.

The intracellular GSH level is important antioxidant in animals and able to protect the cellular components from oxidative stress that is generated by ROS (Pompella *et al*., 2003; Luberda, 2005). And it participates directly or indirectly in various cellular processes, including DNA and protein synthesis, metabolism, cellular protection mechanisms, and amino acid transport (Meister and Anderson, 1983). Moreover, intracellular GSH level in mammalian oocytes is used as a molecular marker of cytoplasmic maturation (Lee *et al*., 2016a). In this present study, treatment of H_2O_2 during maturation process decreased the intracellular GSH level in porcine oocytes and it was recovered by supplementation of ALA with H_2O_2. The intracellular levels of GSH in oocytes was influenced by oxidative stress state and treatment of antioxidants, especially, supplement of antioxidants, such as antioxidants, vitamin, and hormones, in culture medium enhance GSH contents in oocytes. Supplementation of L-carnitine during IVM increased GSH level in porcine oocytes and subsequent development of embryos after parthenogenetic activation (Wu *et al*., 2011). And Li *et al*. (2015) had reported that melatonin enhanced GSH level that was reduced by heat stress-induced oxidative stress. Also, treatment of ALA increased the GSH during maturation process in porcine oocytes (Lee *et al*., 2016b). These researches indicated that supplemented ALA in oocyte maturation medium could decreased oxidative stress through enhanced GSH synthesis in porcine oocytes.

ROS-induced oxidative stress is still considered as major detrimental factor in IVP system of porcine embryos. Especially, porcine oocytes contains a large amount of lipid and fatty acid, they are sensitive to oxidative damage compared with other species (Sturmey *et al*., 2009). High oxygen concentration increased H_2O_2 level in embryo and decreased blastocyst formation of *in vitro* fertilized embryos in pigs (Kitagawa *et al*., 2004). Thus, many studies had been conducted to reduce the generation of ROS during *in vitro* culture of oocyte and embryos. Our findings shown that increased ROS level by H_2O_2 was reduced by supplementation of ALA during IVM. As an antioxidants, β-mercaptoethanol and vitamin-E reduced level of ROS in porcine embryos (Kitagawa *et al*., 2004). Moreover, supplement of resveratrol, which is one of antioxidants, decreased intracellular ROS level in porcine oocytes and enhanced developmental competence after parthenogenetic activation and *in vitro* fertilization (Kwak *et al*., 2012). Marei *et al*. (2012) had reported that H_2O_2 level in bovine oocytes were reduced by ALA treatment during IVM, whereas linoleic acid increased H_2O_2. Based on these results, we expected that ALA in porcine oocytes may have antioxidant ability during maturation process, as well as other antioxidants.

Nuclear state of oocyte in maturation process is most important parameter that influence to subsequent development and quality of embryos, and it is closely related with oxidative balance in oocytes. Immature oocytes after IVM have a lower developmental competence than mature oocytes (Bagg *et al*., 2007; Matsunaga and Funahashi, 2016). Kim *et al*. (2010) reported that porcine oocytes from small follicles (< 3 mm) had lower population of mature oocytes, which were reached metaphase-II stage, GSH content, and developmental competence than medium follicle (3～8 mm) after IVM, also, malathion-induced ROS generation suppressed nuclear maturation of porcine oocytes (Flores *et al*., 2016). These reports demonstrated that meiotic competence of oocytes was altered by intracellular ROS and GSH levels, and it influenced to subsequent development. In this present study, nuclear maturation was decreased by H_2O_2 treatment and slightly recovered by ALA supplement under oxidative environment. In porcine oocytes, supplement of L-carnitine during IVM increased GSH content and mature oocytes, decreased ROS levels (Wu *et al*., 2011). Similarly, nuclear maturation of porcine oocytes was increased in both of COCs and denuded oocytes by L-carnitine treatment (Yazaki *et al*., 2013). Therefore, we expected that enhanced nuclear maturation in oxidative environment might be caused by increased antioxidant capacity by ALA in porcine oocytes.

In conclusion, we found that supplementation of ALA in IVM medium enhanced intracellular GSH level, reduced ROS formation, and recovered nuclear maturation of porcine oocytes. Therefore, our findings suggest that treatment of ALA during IVM acts as useful antioxidant material and could improve low efficiency of porcine IVP system by *in vitro* environment-derived oxidative stress.

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