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Effect of 7,8-Dihydroxyflavone on *In Vitro* Maturation of Oocytes in Pigs

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ABSTRACT

In porcine embryo culture, one of reactive oxygen species (ROS) is harmful factors that are made during *in vitro* culture. To decrease the detrimental effect of ROS on embryo development, superoxide dismutase, catalase and glutathione peroxidase could be used in the embryo culture. Out of these antioxidants, 7,8-dihydroxyflavone (7,8-DHF) was reported its antioxidant effects to prevent the glutamine-triggered apoptosis. Therefore, this study was performed to investigate the most appropriate concentration of 7,8-DHF in porcine embryonic development. For that, 5 different concentration (0, 0.1, 0.5, 1, 2 uM) of 7,8-DHF was supplemented in the porcine IVM media and then maturation and blastocyst formation rates were compared among 5 groups. In maturation rates of porcine oocytes, significant higher maturation rates was shown in the 1.0 uM group compared with another 4 groups (83.3 ± 2.1 vs. 80.7 ± 1.4, 79.8 ± 1.4, 78.3 ± 1.2, 79.4 ± 1.6), respectively (P<0.05). In the embryo culture, 1.0 uM group also showed the significant higher cleavage rates (76.8 ± 3.1 vs. 62.1 ± 5.0, 65.7 ± 4.0, 68.6 ± 3.7, 64.6 ± 4.0%) and blastocyst formation rates - (39.6 ± 4.0% vs. 28.6 ± 3.3, 31.1 ± 3.9, 29.3 ± 2.5, 39.6 ± 4.0, 26.4 ± 3.2%), respectively (P<0.05). There was no significant difference among 5 groups in the cell number of blastocyst (P<0.05). In conclusion, supplement of 1.0 uM of 7,8-DHF was effective to increase the porcine embryonic development competence as antioxidant to ROS.

(Key words : porcine, embryo, *in vitro* production, 7,8-dihydroxyflavone)

INTRODUCTION

With the development of reproductive biotechnology, producing transgenic animals and *in vitro* embryo production have significant potential to generate biomedical animal models or to study reproductive research (Yoshioka, 2011). Especially, the pig is considered a suitable biomedical model for human study, in particular organ develop- ment and disease progression, because of its similar physiology and size (Lunney, 2007).

Many researchers have conducted to improve the efficiency of transgenic pig production. To accomplish this, there are various strategies or trials, such as modulation of methylation or acetylation levels in cloned embryos (O'Neill *et al.*, 1994). To improve oocyte quality, focus on the first step of *in vitro* embryo production, known as *in vitro* maturation (IVM). Thus, most of researchers are try to find answer to optimize *in vitro* maturation and *in vitro* culture of oocytes like temperature, gas tension, media etc. One of those, oxidant stress can reduce problem in IVP (Booth *et al.*, 2005). Reactive oxygen species (ROS) are made when *in vitro* culturing of oocytes (Guerin *et al.*, 2001). Oocyte quality significantly influences early embryonic development and survival, fetal growth and maintenance of pregnancy, and even for the health of postnatal offspring. Thus, a better understanding of IVM could improve the quality of oocytes and have positive effects on transgenic pig production (Sirard *et al.,* 2006).

Superoxide dismutase, catalase, glutathione peroxidase that produced by cells can make decreased ROS degree(Choe *et al.,* 2010). However, endogenic antioxidants cannot prohibit cell injury if ROS degree exceed threshold value (Guerin *et al.*, 2001). So research about exogenetic antioxidant is progressed sprightly. There are some researches ongoing for the effects of substances like antocyanine (You *et al.*, 2010), L-carnitine (Wu *et al.*, 2011), hypotaurine (Suzuki *et al.*, 2007), vitamin C (Hu *et al.*, 2012). 7,8-DHF is a member of the flavonoid family, which is a diverse class of secondary plant metabolites, present in fruits and vegetables. Flavonoids exhibit several biological functions. Historically, the biological actions of

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flavonoids have been attributed to their antioxidant properties, through their ability to scavenge reactive species or through their influences on the intracellular redox status (Thirupathy *et al.*, 2011). Brain derived neurotrophic factor, a member of the neurotrophin family and a ligand for the tropomyosin receptor kinase B (TrkB), mediates neuronal survival, differentiation and synaptic plasticity. However, BDNF is not used to treat neuro degenerative diseases because of its poor pharmacokinetic profile, side effects and absence of survival properties in clinical trials. Consequently, alternative approaches such as TrkB receptor agonist application are gaining importance. 7,8-Dihydroxyflavone (7,8-DHF), a member of the flavonoid family, has been described as a robust TrkB receptor agonist in hippocampal neurons. Nevertheless, the influence of 7,8-DHF on motoneurons, one of the main targets of BDNF *in vivo*, is so far unknown (Tsai *et al.*, 2013).

In this study, it was investigated for the influence of 7,8-DHF on IVM and IVC with different concentration on oocyte maturation and embryo development in pigs. Within the period of research determined the maturation, embryonic development and quality of blastocysts of porcine embryos contrast to different concentrations of 7,8-DHF.

MATERIALS AND METHODS

1. *In Vitro* Maturation of Porcine Oocytes

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Pig ovaries were collected from the slaughter house and transported to the laboratory washed with pre warmed saline water, and follicles were aspirated using 18 gauges needle syringe. Washed oocytes in the TLH-PVA three times and cultured in maturation medium consist culture media (TCM-199, Gibco) supplemented with 20 μg/ml of eCG/hCG and, 0.2 mM sodium pyruvate, and 15 μg/ml kanamycine, 15 μg/ml epidermal growth factor, 10 μg/ml insulin for 22 h (39℃, 5% CO_2). After 22 hours of incubation, oocytes were transfer to the culture media contains above formulation without hormone for another 22 h of incubation 39 ℃, 5% CO_2 conditions. The COCs were untreated or treated with 0.1, 0.5, 1.0 and 2.0 μM 7,8-DHF during IVM. 44 hours of total incubation, Oocytes stripped of cumulus cells by denuding of 0.1% (w/v) hyaluronidase and oocytes with a visible first polar body was used for assessment of nuclear maturation and used for parthenogenesis embryo production.

2. Production of Parthenogenetic Embryos

Parthenogenetic activation of oocytes accomplished by submerging oocytes in activation medium contains 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES. In between two platinum wire electrodes, follow by two successive 60 msec pulses (1.2 kV/cm) of direct current (DC) channeled by a BTX Electro-Cell Manipulator. Parthenogenetic embryos put into post activation media 7.5 μM cytochalasin B for 4 hrs in the incubator at 39℃, 5% CO_2 in air at saturating humidity immediately after exposure to the electrical activation stimulus transfer to post activation media and keep in the incubator. After 4 hours of incubation in post activation media, parthenogenetic embryos were transfer to the PZM-5 media 25 μl drops embryos were cultured in PZM-5 supplemented with 0, 0.1, 0.5, 1.0 and 2.0 μM 7,8-DHF during IVC and covered by mineral oil. Parthenogenetic embryos conduct at 39℃, 5% CO_2, 5% O_2 at saturating humidity. Cleavage rate and blastocyst formation rate were checked at day 2 and day 7 of IVC, respectively. Percentage of blastocyst formation was measured using all of the embryos.

3. Evaluation of Blastocyst Quality

The quality of blastocysts was assessed by differential staining of the inner cell mass (ICM) and the trophectoderm (TE) according to the (Thouas *et al.,* 2001). Briefly, trophectoderm cells of blastocysts at 7 days were stained with the fluorochrome propidium iodide after treatment with permeabilizing solution containing the ionic detergent Triton X-100. Blastocysts were then incubated in a second solution containing 100% ethanol and bisbenzimide. Fixed and stained whole blastocysts were mounted and assessed for cell number using ultraviolet fluorescent microscopy.

4. Experimental Design

In this study compared four different concentrations of untreated or treated with 0.1, 0.5, 1.0 and 2.0 μM 7,8-DHF during IVM in porcine embryos. Maturation rate were evaluate after denuding oocytes with a visible first polar body was used for assessment and mature oocytes were taken for the parthenogenetic activation. Then activated embryos were post activated for 4 hrs and cultured in PZM-5 media for further development. Controls were cultured in PZM-5 media after post activation without giving any treatment. After 2 and 7 days of incubation, embryos were examined, counted cleavage and blastocysts rates

respectively. Blastocysts were stained and count total cell numbers.

5. Statistical Analysis

Data were analyzed by one-way ANOVA with a general linear model procedure using the SPSS System (version 21.0; IBM, USA), followed by the least significant difference mean separation procedure when treatments differed at $P<0.05$. Cell number was expressed as mean ± S.E.M.

RESULTS

1. Effects of 7,8-DHF on *In Vitro* Maturation of Porcine Oocytes

Table 1 shows the maturation of oocytes in IVM was evaluated by determining first polar body extrusion and calculate the maturation rate of the oocytes in separately in replicates. From four treatment groups and control group, 1.0 μM 7,8-DHF added oocytes maturation were significantly higher ($p<0.05$) than rest of other groups.

2. Developmental of Porcine Parthenogenetic Embryos

Examined the development competence of parthenog enetic embryos with different concentrations of 7,8-DHF in IVM and IVC. Total 1,400 embryos were parthenogenetically activated in 14 replicates. Table 2 shows that the cleavage rates and blastocyst formation was significantly increased in the group treated with 1.0 μM 7,8-DHF.

3. Blastocysts Cell Number of Porcine Parthenogenetic Embryos

Examined the total cells in the parthenogenetic blastocysts with different concentrations of 7,8-DHF. Table 3 shows that the cells in blastocyst was significantly increased in the group treated with 1.0 μM 7,8-DHF.

DISCUSSION

In the first experiment, nuclear maturation of porcine oocytes was evaluated by measuring the rate of first polar body extrusion. With the intracellular GSH is an increase cytoplasmic maturation progresses in oocytes and first PB extrusion is closely related to nuclear maturation. Because this process takes place during nuclear maturation in MII, the cytoplasmic maturation was increased in 1.0 μM treated group compared with the other groups nuclear and cytoplasmic maturations are normally coordinated and cytoplasmic quality in IVM oocytes plays a major role in the reconstruction of embryonic develop-

Table 1. Effects of 7,8-dihydroxyflavone treatment during *in vitro* maturation on nuclear maturation

Concentration of 7,8-DHF(μM)	No. of embryos (%)	
	Cultured	Matured (%)
Control	793	639(80.7 ± 1.4)^{ab}
0.1	603	483(79.8 ± 1.4)^{ab}
0.5	610	479(78.3 ± 1.2)^{a}
1	608	509(83.3 ± 2.1)^{b}
2	621	495(79.4 ± 1.6)^{ab}

11 replicates were performed.
^{a,b} Values in the same column with different superscripts are different($p<0.05$).

Table 2. Effects of 7,8-dihydroxyflavone during *in vitro* development of parthenogenetic embryos

Concentration of 7,8-DHF (μM)	No. of embryos (%)		
	Cultured	Cleaved	Develop to blastocyst
Control	280	174(62.1 ± 5.0)^{a}	80(28.6 ± 3.3)^{a}
0.1	280	184(65.7 ± 4.0)^{ab}	87(31.1 ± 3.9)^{ab}
0.5	280	192(68.6 ± 3.7)^{ab}	82(29.3 ± 2.5)^{a}
1	280	215(76.8 ± 3.1)^{b}	111(39.6 ± 4.0)^{b}
2	280	181(64.6 ± 4.0)^{a}	74(26.4 ± 3.2)^{a}

14 replicates were performed.
^{a,b} Values in the same column with different superscripts are different($p<0.05$).

Table 3. Blastocysts cell number of porcine parthenogenetic embryos after culture with different concentration of 7,8-DHF during the *in vitro* maturation and culture

Concentration(μM)	Control	0.1	0.5	1	2
Cell number(AVG ± SE)	57.5 ± 5.5	62.0 ± 4.0	77.0 ± 4.0	80.0 ± 13.0	70.0 ± 8.0

ment.

Considering cleavages and blastocysts rates, the effect of 7,8-DHF treatment according to the concentration the cleavage rates of parthenogenetic embryos and blastocyst formation was significantly increased in the group treated with 1.0 μM 7,8-DHF. High (2.0 μM) concentration of 7,8-DHF showed lower developmental competence based on the cleavage rate and blastocyst formation compared with the 1.0 μM treatment group. ROS production is a normal process occurring within cells when electron transfer reactions to the oxygen occur in the mitochondrial respiratory chain and endoplasmic reticulum (Nabenishi *et al.*, 2012). *In vitro* environments usually increase ROS production in the cell (Luvoni *et al.*, 1996).

There was significant difference in the total cell numbers of blastocysts with compared control group. The blastocysts total cell number directly correlated with the quality of blastocysts. With the increase of 7,8-DHF, the total number of cells in the blastocysts significantly ($p<0.05$) increased and at 2.0 μM 7,8-DHF concentration was toxic to the embryonic development and reduced the development of blastocysts. Oocytes and parthenotes with 1.0 μM 7,8-DHF appeared to have an increased level of GSH and decreased level of ROS compared with the control group. This result correlated with production of glutathione, which maintained the intracellular redox state and protected cells from the harmful effects of oxidative stress, resulting from treatment with 1.0 μM 7,8-DHF. This clearly implies the 7,8-DHF directly effect to the development of blastocysts in the IVC and increases the quality of porcine blastocysts.

In conclusion, within the research duration clearly can identify the direct effect of 7,8-DHF to embryonic maturation and development and the activity of an enzymatic antioxidant system in the porcine oocyte, which would partly control ROS levels during IVM, shown to participate in meiotic and/or cytoplasmic maturation regulation. After maturation, oocytes could be capable of controlling the increase in ROS to their own enzymatic antioxidant system. However the influence of 7,8-DHF greatly effect to the development of embryos up to blastocysts stage. Further studies on the identification of pertinent ROS and their biochemical mechanisms involved in oocyte maturation should therefore be conducted.

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