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

돼지 난자의 체외성숙과 단위발생시 7,8-Dihydroxyflavone 의 효과

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

by Ji Yei Choi

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To the required standard

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ABSTRACT

One of the factors that impair the *in vitro* produced porcine embryos is the oxidative stress that mainly caused by the imbalance between reactive oxygen species (ROS) generation and antioxidants activity, especially glutathione (GSH). Here, the effect of 7,8-dihydroxyflavone (7,8-DHF), a flavonoid antioxidant, on porcine oocyte maturation and its developmental competence was examined. Porcine oocytes were cultured in media supplemented with 0, 1, 5, and 10 μM 7,8-

DHF during both *in vitro* maturation (IVM) and *in vitro* culture (IVC) after parthenogenetic activation. Maturation of oocytes was evaluated based on the 1st polar body (PB) extrusion and intracellular GSH level and developmental competence was assessed through observing cleavage and blastocyst formation. In each step, the levels of intracellular GSH and ROS were assessed by fluorescence intensity and the apoptosis-related gene expression was examined using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Parthenogenetic mbryos treatment with 1 μM 7,8-DHF during IVM and IVC showed increased cytoplasmic maturation and reached blastocysts stage (36.1 ± 3.5%) at a higher rate than the other groups (24.7 ± 3.1, 16.0 ± 2.6, and 10.3 ± 2.2%, $P < 0.05$). In that group, intracellular GHS level was significantly increased after IVM and IVC and ROS was decreased after IVC ($P < 0.05$). Moreover, it showed high expression of anti-apoptotic gene (*BCL2L1*) and low expression of pro-apoptotic gene (*BAK1*) ($P < 0.05$). In conclusion, 1 μM 7,8-DHF treatment during IVM and IVC showed an anti-apoptotic effect by increasing intracellular GSH synthesis and scavenging ROS, therefore, it improves the developmental competence of porcine parthenogenetic embryos.

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Keywords : 7,8-Dihydorxyflavone, Porcine oocytes, Antioxidants, Glutathione (GSH), Reactive Oxygen Species (ROS)

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Ji Yei Choi, Jung Taek Kang, Sol Ji Park, Su Jin Kim, Joon Ho Moon, Islam M. Saadeldin, Goo Jang, Byeong Chun Lee. Effect of 7,8-dihydroxyflavone as an antioxidants on *in vitro* maturation of oocytes and development of parthenogenetic embryos in pigs, Journal of reproduction and development. 2012, *submitted.*

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ABSTRACTS AND PRESENTATIONS

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Jung Taek Kang, **Ji Yei Choi**, Sol Ji Park, Su Jin Kim, Joon Ho Moon, Goo Jang, Byeong Chun Lee, Effect of S-Adenosylhomocysteine, a non-toxic epigenetic modifying reagent, on porcine female donor cells and cloned embryos, International embryo transfer society, 2013

LITERATURE REVIEW

1. Redox system in oocytes and embryos

In the normal metabolism within the cells, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), superoxide anions ($O_2•^-$), and nitric oxide (NO) are generated and accumulated from the surrounding environment [1]. They are mainly produced as a by-product of electron transport chain in mitochondria for converting energy to adenosine triphosphate (ATP) via oxidative phosphorylation process. Superoxide anions are reduced form of O_2 and relatively stable, however H_2O it can release iron ions, donate a single electron to oxygen molecule (O_2) which changed highly reactive state, and dismutated to H_2O_2 which pass the cell membrane. Hydrogen peroxide generates •OH and OH^- resulting in oxidative damage because hydroxyl radical is highly reactive cell attack most cellular component [2]. Cells have intrinsic mechanisms such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) to counteract this problem. SOD which exists in the cytoplasm and mitochondria catalyzes dismutation of $O_2•^-$ to O_2 and H_2O_2. CAT supports reaction of H_2O_2 to H_2O and O_2. GPx converts H_2O_2 to H_2O. As scavenging free radicals by carrying electrons, they defense cells from oxidative damages. The balance between ROS and antioxidants is maintained in optimal conditions. However, when level of intracellular ROS over threshold, it damages to proteins, nucleic acids, lipids, and other biomolecules [3] by breaking the DNA [4], RNA, or inducing lipid peroxidation [5, 6]. Also, they disturb cellular signaling as acting like signaling messengers. Proteins located on the mitochondria detect these damages and induce cytochrome C releasing. They bind to apoptotic protease activating factor and then

apoptosomes activates caspase-9. As a result, membrane proteins are denatured and cellular apoptosis is accelerated [7].

The role of ROS in the *in vitro* maturation (IVM) of oocytes and *in vitro* culture (IVC) of embryos has been observed that high concentration of ROS have negative effect. Oxygen consumption of cumulus-oocyte complexes (COCs) increased through IVM with mitochondrial oxidative phosphorylation [8] and oxidative stress can induce aging of the oocyte [9]. Embryos are highly sensitive to these damages and it is closely associated with impaired early development and fragmented embryos [10] .

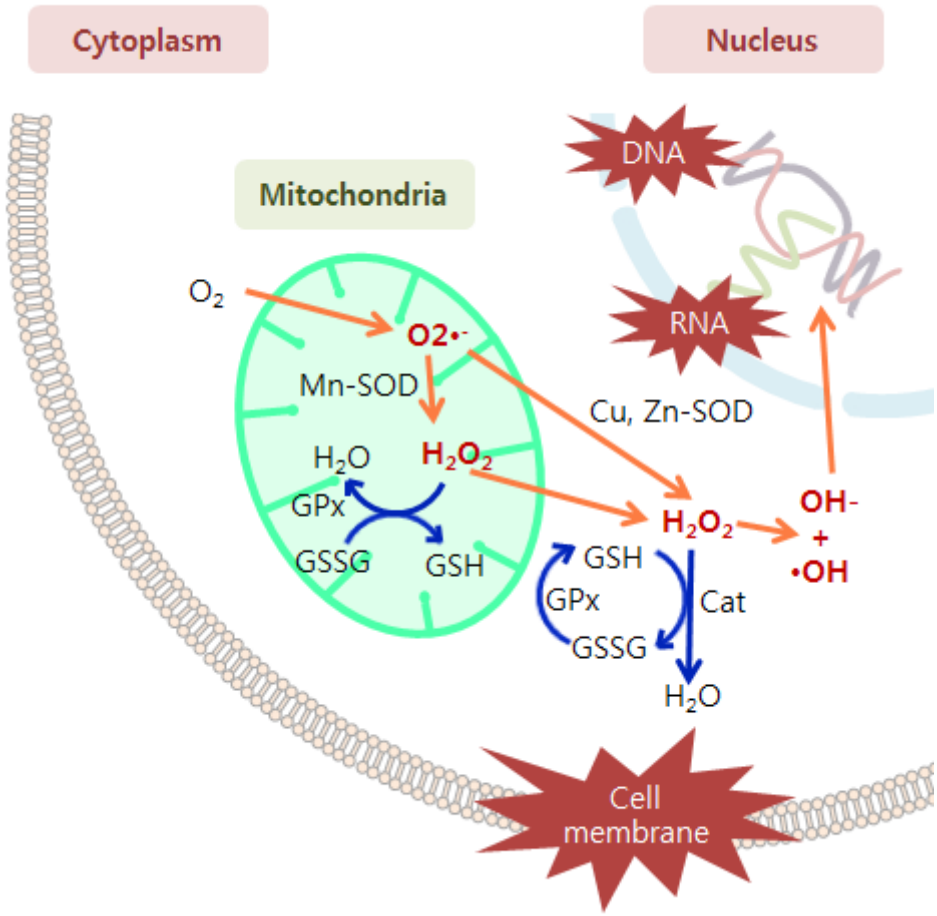


Figure 1. A schematic diagram of redox system in cells. ROS generation and intrinsic antioxidant activity interact each other and have important roles on cellular functions. (Modified from [2]).

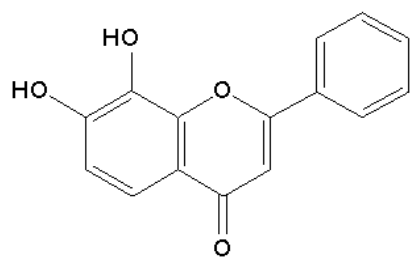
2. Antioxidants for oocytes and embryos

Antioxidants inhibit oxidation which produces free radicals as transfer electrons or hydrogen. There are many researches to overcome intracellular oxidative stress using exogenous antioxidant. Anthocyanin treatment during IVM improved developmental potential of somatic cell nuclear transfer (SCNT) embryos by increasing intracellular glutathione (GSH) synthesis, reducing ROS level, and stimulating nuclear reprogramming via increased transcription factor expression [11]. L-carnitine during IVM and IVC improved developmental competence of porcine oocytes, and also the quality of parthenogenetic embryos, probably by accelerating nuclear maturation, and preventing oxidative damage and apoptosis [12]. Presence of glutamine and hypotaurine in porcine zygote medium (PZM) -5 from day-2 to day-3 promotes the development of porcine embryos by improvement of intracellular oxidative status [13]. Vitamin C addition in PZM-3 decreases the number of apoptotic cells and increases the cell numbers in blastocysts to produce high-quality porcine embryos *in vitro* [14]. Supplementation with β-mercaptoethanol during *in vitro* fertilization (IVF) procedures has a beneficial effect in maintaining the quality of IVF embryos [15]. Selenium play important roles in reducing the accumulation of ammonia and subsequently in increasing the rate of maturation of porcine oocytes and fertilization, as well as development of the blastocyst and utilization of glucose in IVM, IVF, and IVC to blastocysts of porcine oocytes [16].

In this study, I examined the effect of 7,8-dihydroxyflavone (7,8-DHF) as an antioxidants. It is a member of the flavonoid family which is present in high concentration in fruits and vegetables. It appeared that 7,8-DHF has preventive

effect against oxidative stress [17] and inhibits glutamate-triggered apoptosis. [18, 19]. Glutamate induces GSH depletion, increases ROS production, and induces calcium influx, all of which lead to cell death.

Table 1. Physical and chemical properties of 7,8-dihydroxyflavone

Properties	7,8-Dihydroxyflavone
Molecular Fomula	$C_{15}H_{10}O_{41}/2H_2O$
Molecular Weight	263.25
CAS Number	38183-03-8
Physical Appearance	Yellow solid
Molecular Structure	

3. Parthenogenesis

Parthenogenetic activation is a way to induce embryonic development without paternal genome as mimicking events of sperm activity. In nature, ovulated porcine oocytes were arrested metaphaseII (MII) stage by high concentration of metaphase promoting factor (MPF) until fertilization. Entrance of the sperm causes calcium oscillation in oocyte and then meiosis resumes by degradation of cyclin B, a subunit of MPF. Treatment with inhibitor of 2nd polar body (PB) extrusion results in producing of parthenotes which have diploid maternal genomes. In case of pigs, electrical or chemical stimulus is used to begin the parthenogenesis. To induce influx of calcium ions to oocytes artificial stimulation such as Ca^{2+} ionophore or electrical pulse is needed. The first cloned pigs were produced by electrical activation which evokes simple rise of Ca^{2+} [20]. Chemicals which inhibit protein synthesis such as cyclohoeximide and 6-dimethylaminopurine (6-DMAP) supports activation to limit function of MPF, or combined treatment with thimerosal/dithiothreitol is suggested because thimerosal triggers Ca^{2+} spikes and dithiothreitol promote formation of pronucleus [21]. Also, addition of cytochalasin B or demecolcine blocks extrusion of 2nd PB as suppress microtubules dynamics [22].

Porcine parthenotes can't fully develop until birth because they have only maternal genomes. Twice amount of maternal imprinted genes which has to be inactivated in the chromosomes and absence of paternal genes impair normal development, especially defects in placental folding or interdigitation are observed [23]. However, parthenogenesis is a simple and time-saving method to induce early embryonic stage and they maintain day-29 post activation *in vivo*. Porcine

parhenogenetic embryos developed to limb-bud formation and cyst-like structure in heart and liver [24]. In that reason, parthenogenetic activation is widely used to examine influence of various factors.

INTRODUCTION

In vitro production (IVP) of porcine embryos has been extensively studied for improving the embryonic development and the reproductive technologies. To date, it also widens its field to biomedical research and xenotransplantation [25]. Therefore, many researchers investigate the way to optimize the condition of oocytes or embryos, including temperature [26, 27], gas tension [28, 29], and composition of medium [30-32], etc.

It is well known that one of the problems that impair the IVP of porcine embryos is the oxidative stress [33, 34] that mainly caused by the ROS generation such as H_2O_2, •OH, O2•$^-$, NO, the highly reactive molecules formed by oxygen metabolism [35]. They can damage the cell by breaking the DNA [4] and RNA or inducing lipid peroxidation [5, 6]. Cell generate antioxidants themselves such as SOD, CAT, and GPx [15] to reduce ROS levels by scavenging free radicals. However, when level of intracellular ROS over threshold, intrinsic antioxidants can't scavenge free radicals and cells are under the oxidative stress condition. Especially, early stage embryos are more vulnerable to oxidative stress [36] and further developmental competence of embryos are impaired by that damage. In addition, oxidative stresses accelerate cellular apoptosis, resulting in a decrease of total cell number [7].

Therefore, there are many researches to reduce ROS using antioxidant treatment such as anthocyanine [11], L-carnitine [12], hypotaurine [13] , vitamin C [14], β-mercaptoethanol [37, 38], and selenium [16]. The 7,8- DHF is a flavonoid which is present in high concentrations in fruits and vegetables and a brain-derived neurotrophic factor (BDNF), belongs to the brain protecting drugs [39, 40]. It inhibits glutamate-triggered apoptosis which is induced by GSH depletion and

ROS production and has antioxidant activity in neurons by acting as a selective tyrosine kinase receptor B agonist [18, 19]. In addition, 3,4-DHF support bovine embryo development *in vitro* as an antioxidant and anti-apoptotic agent [41] and 7,8-DHF appeared that has preventive effect against oxidative stress [17]. However, the effects of 7,8-DHF for porcine oocytes and embryos are not well investigated.

The purpose of this study is to determine the effect of 7,8-DHF treatment on the oocytes maturation and the embryos development in pig. Also, intracellular levels of GSH and ROS and gene expression in oocytes and parthenogenetic embryos were examined.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used for this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

Collection of oocytes and IVM

Porcine ovaries were obtained at a local slaughterhouse and transported to the laboratory in 0.9% NaCl within 3 h. COCs were collected by slicing the 3-6 mm follicles and washed 3 times in washing media containing 9.5 g/L Medium 199 powder (Invitrogen, Carlsbad, CA, USA), 5 mM sodium hydroxide, 2 mM bicarbonate, 10 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.3% polyvinyl alcohol (PVA), 1%, Pen-Strep (Invitrogen). Based on the morphological features, COCs which have which have compact, multilayered cells and homogeneous cytoplasm were selected. COCs were then transferred to IVM medium containing tissue culture medium (TCM)-199 supplemented with 10 ng/mL epidermal growth factor (EGF), 0.57 mM cysteine, 5 μL/mL insulin-transferrin-selenium sodium pyruvate solution (ITS-A) 100X (Invitrogen), 1% (v/v) Pen-Strep, 0.5 μg/mL porcine follicle stimulating hormone, 0.5 μg/mL human luteinizing hormone, 10% porcine follicular fluid (pFF) and 5 nM retinoic acid for 22 h at 38 °C in a humidified atmosphere of 5% CO_2. Subsequently, the COCs cultured further 22 h without hormones and retinoic acid. The COCs were untreated or treated with 1, 5, and 10 μM 7,8-DHF (Tocris Bioscience, Ellisville, MO, USA) during IVM.

Evaluation of porcine maturation

After 44 h of IVM, cultured oocytes were denuded by pipetting with 0.1% hyaluronidase in Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen) supplemented with 0.1% polyvinyl alcohol and then denuded oocytes were stained with 5 μg/mL of bisbenzimide (Hoechst 33342) in DPBS. Extrusion of 1st PB MII was used as an indicator for assessment of nuclear maturation with a fluorescence microscope using a 340 to 380 nm excitation filter. For examination of the cytoplasmic maturation, intracellular GSH level was measured using 10 μM CellTracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin) (Invitrogen). The staining method for observing GSH levels was fully explained in the part of assessment of oocytes and parthenogenetic embryos quality in materials and methods.

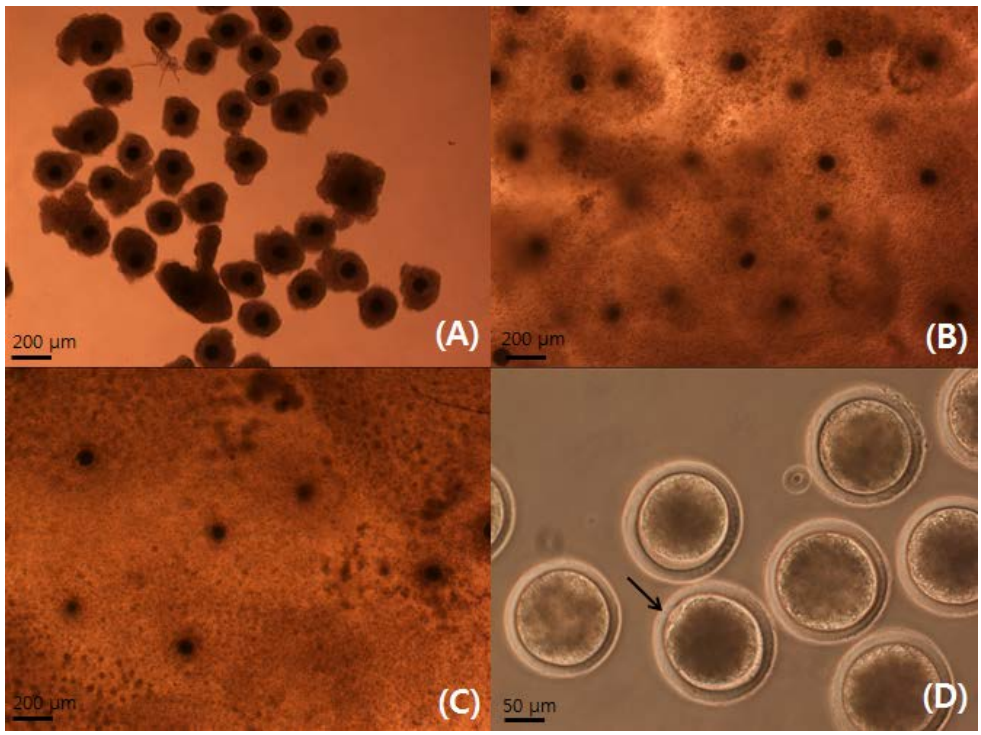


Figure 2. ***In vitro*** **maturation of porcine oocytes.** Cumulus-oocyte complexes were collected from porcine ovaries which have 3-6 mm follicles (A). Cumulus cells around oocytes expanded in process of time after 22 h (B) and 44 h (C). Extrusion of 1st polar body (black arrow) was observed in denuded oocytes (D).

Parthenogenetic activation of oocytes and IVC

Mature oocytes were denuded in the same way as above after 44 h IVM. Denuded oocytes which had homogeneous cytoplasm were selected and then gradually equilibrated in activation solution containing 0.26 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. The oocytes were activated in a chamber which has two electrode 3.2 mm apart and filled with activation medium using a single direct current pulse of 1.5 kV/cm for 60 μsec utilizing BTX electro-cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA). After washing 3 times in PZM-5 (Funakoshi co., Tokyo, Japan), parthenogenetic embryos were cultured in PZM -5 supplemented with 1, 5, and 10 μM 7,8-DHF during IVC and covered with mineral oil under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ℃ for 7 days. The rate of cleavage and blastocyst formation was checked at day-2 and day-7 of IVC, respectively. Percentage of blastocyst formation was measured using all of parthenotes. To count the total cell number, blastocysts were fixed in absolute alcohol and stained with 5 μg/mL Hoechst 33342 overnight at 4 ℃. The number of nuclei was determined with a fluorescence microscope using a 340 to 380 nm excitation filter.

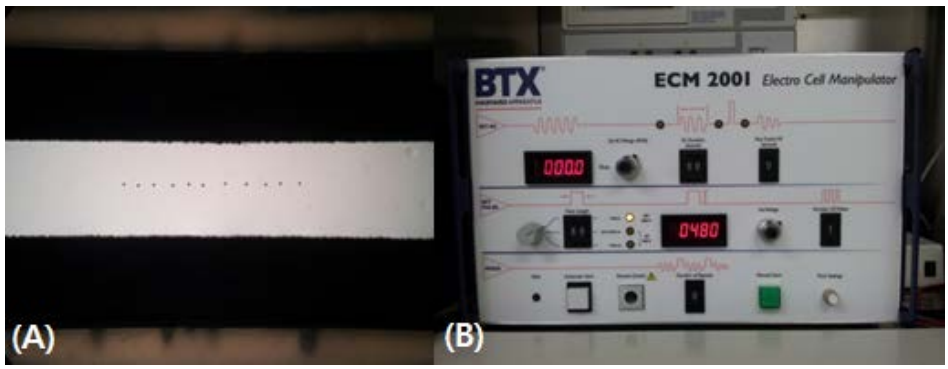


Figure 3. Parthenogenetic activation by electrical stimulation. Denuded oocytes were arranged in a row between two electrodes in a chamber which apart 3.2 mm (A) and filled with activation medium. Influx of Ca^{2+} into the oocytes was induced using a BTX electro-cell Manipulator (B).

Assessment of oocytes and embryos quality

Intracellular GSH and ROS levels were determined after oocytes IVM and day-2 parthenotes, respectively. Embryonic day-2 is decisive period that occur first cell cycle which is directly correlated with developmental potential [42]. Before that treatment, 0.01 mM H_2O_2 was added to the maturation and culture media to increase oxidative damage [43]. Oocytes were incubated for 20 min in HEPES-buffered Tyrode's albumin lactate pyruvate (TALP) medium with 10 μM carboxy-H_2DFFDA (2'7'-diclorodihydrofluorescein diacetate) and 10 μM CellTracker Blue CMF_2HC. After washing 3 times in HEPES-buffered TALP medium, they transferred into 20 μL droplets and fluorescence was observed with ultraviolet filters (370 nm for GSH and 460 nm for ROS). The fluorescence intensities were analyzed using ImageJ 1.42q software (National Institutes of Health, Bethesda, MD, USA).

RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from mature oocytes and day-2 parthenotes in each group by the easy-spin™ (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea) following the manufacturer's instructions and quantified using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). From these samples, cDNA was produced using a amfiRivertII™ cDNA synthesis Master Mix (Dawinbio Inc., Gyeonggi-do, Korea) in a 20 μL reaction volume. Briefly, 5–8 μL of total RNAs were placed in 0.5 mL PCR tubes, and then 10 μL of amfiRiverII cDNA reaction buffer with oligodT and 2 μL of amfiRiverII cDNA emzyme mix buffer (2X) mix were added. diethypyrocarbonate (DEPC)-treated water was added to the tubes to make 20 μL

total volume, which were then incubated at 70 °C for 5 min, and then hold on ice for 5 min. Reverse transcription was carried out one at 25 °C for 5 min, 50 °C for 60 min, and then 70 °C for 15 min.

PCR was done according to the manufacturer's instructions. Primers that used for gene expression analysis sequence and product sizes are listed in Table 2. Aliquots of PCR reaction were performed based on optimization curves of PCR cycles which denatured at 94 °C for 30 sec, annealed at 59 °C for 30 sec, and then extended at 72 °C for 30 sec. Amplified PCR products were analyzed gel electrophoresis (Mupid-exu; Takara Korea Biomedical Inc., Seoul, Korea) at 100 V for 25 min in 1% agarose gel with RedSafe (iNtRON Biotechnology Inc.). After gel electrophoresis, the intensities of the bands under ultraviolet were quantified using ImageJ 1.42q and results were normalized to those of the control (*GAPDH*) to express arbitrary units of relative expression.

Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's test using Prism version 5.0 (Graphpad Software, San Diego, CA, USA) to determine differences among experimental groups. Statistical significance was determined when P value was less than 0.05.

Table 2. Primer information for semi-quantitative RT-PCR

Gene	Sequence (5'-3')	Product size (bp)	Accession no.
**BCL2L1*	F : CGTCCCAGCTCCACATCACC R : AGTGCCCCACCGAAGGAGAA	130	AF216205
***BAK1*	F : ATGACATCAACCGGCGATAC R : GGAGGCGATCTTGGTGAAGT	107	AJ001204
****GAPDH*	F : ACCTGCCGTCTGGAGAAACC R : GACCATGAGGTCCACCACCCTG	252	AF017079.1

**BCL2L1*: B-cell lymphoma 2 like 1

***BAK1*: *BCL2* homologue antagonist/killer1

****GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase

RESULTS

Effects of 7,8-DHF on IVM of porcine oocytes

Nuclear maturation of porcine oocytes was evaluated measuring the rate of 1st PB extrusion. Although total 684 oocytes were assessed in four replicates, there were no significant differences among the experimental groups (Table 3). However, intracellular GSH level of each group showed that cytoplasmic maturation of 1 μM 7,8-DHF treatment group was increased than the other groups (control, 5, and 10 μM treatment groups) in Fig. 4 (A).

Table 3. Effects of 7,8-dihydroxyflavone treatment during *in vitro* maturation of porcine oocytes on nuclear maturation

Treatment concentration (μM)	No. of oocytes cultured*	No. oocytes of with 1st polar body extrusion	Metaphase II (% ± S.E.M.)
control	171	148	82.2 ± 5.4
1	171	152	84.4 ± 5.0
5	171	143	79.4 ± 5.8
10	171	134	74.4 ± 6.4

Nuclear maturation of porcine oocytes didn't showed significant difference between control and 7,8-DHF treated groups ($P < 0.05$). *It was replicated at least five times.

Developmental competence of porcine parthenogenetic embryos after 7,8-DHF treatment

The effect of 7,8-DHF treatment according to the concentration (0, 1, 5, and 10 μM 7,8-DHF) was examined. Total 776 embryos were parthenogenetically activated in five replicates. Table 3 showed that cleavage rate of parthenogenetic embryos were similar in control, 1, and 5 μM 7,8-DHF treated groups (58.8 ± 3.5%, 67.0 ± 3.4%, and 56.2 ± 3.6%, respectively.) and blastocysts formation was significantly increased in 1 μM 7,8-DHF treated group (36.1 ± 3.5%). Parthenotes cultured in a high (5 and 10 μM) concentration 7,8-DHF showed lower developmental competence based on the rate of cleavage and blastocyst formation compare to 1 μM treatment group. Also, there was no significant difference in the total cell numbers of blastocysts in all groups (Table 4).

Table 4. Effects of 7,8-dihydroxyflavone during *in vitro* development of parthenogenetic embryos

Treatment concentration (μM)	No. of embryos cultured*	% of embryos developed		No. of cells in blastocyst
		≥ 2 cell	Blastocyst	
control	194	58.8 ± 3.5[a]	24.7 ± 3.1[a]	62.0 ± 8.4
1	194	67.0 ± 3.4[a]	36.1 ± 3.5[b]	66.2 ± 3.1
5	194	56.2 ± 3.6[a,b]	16.0 ± 2.6[a,c]	60.9 ± 11.1
10	194	44.9 ± 3.6[b]	10.3 ± 2.2[c]	62.5 ± 11.5

Cleavage rate of parthenogenetic embryos were similar in control, 1, and 5 μM 7,8-DHF treated groups and blastocysts formation was significantly increased in 1 μM 7,8-DHF treated group. There was no significant difference in the total cell numbers of blastocysts in all groups. Percentage of cleaved embryos and blastocyst formation was measured based on the total number of embryos. *It was replicated at least five times.

[a-c] Within a column, values with different superscripts are significantly different ($P<0.05$).

Intracellular levels of GSH and ROS in matured oocytes and parthenotes

One μM 7,8-DHF treatment significantly altered the level of intracellular GSH and ROS during IVM and IVC. Total 419 oocytes and 544 day-2 parthenotes were examined in three and four replicates, respectively. The GSH level was increased both matured oocytes and parthenotes in 1 μM 7,8-DHF treated group and the other groups express similar level (Fig. 4.). Expression of ROS was significantly reduced in 1 μM 7,8-DHF treated group and there were no difference between control and 5 μM 7,8-DHF treat group after IVM, but 10 μM 7,8-DHF treated group showed increased expression level among experimental groups (Fig. 5). After IVC, 1 μM 7,8-DHF treated group showed lower level of ROS than any other group (Fig. 5). Fig. 6 showed oocytes and parthenotes in each experiment which was stained with CellTracker Blue (A-H) and carboxy-H_2DFFDA (I-P) according to the concentration of 7,8-DHF.

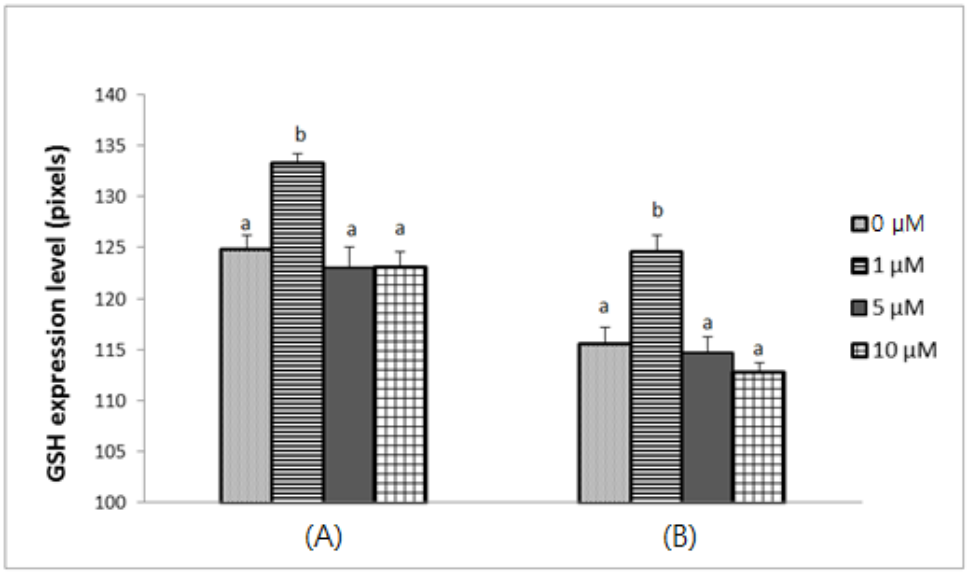


Figure 4. Relative intracellular GSH levels in matured oocytes (A) and day-2 parthenotes (B). Addition of 1 µM 7,8-DHF significantly increased level of GSH in both matured oocytes and parthenotes.

[a,b] Within a column, values with different superscripts are significantly different ($P < 0.05$).

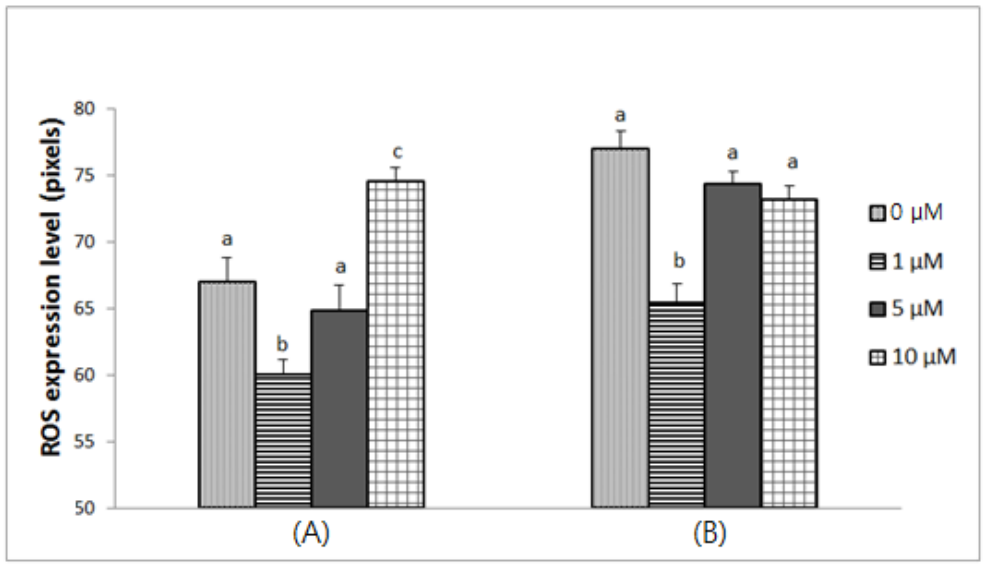


Figure 5. Relative intracellular ROS levels in matured oocytes (A) and day-2 parthenotes (B). Addition of 1 µM 7,8-DHF significantly decreased level of ROS in both matured oocytes and parthenotes.

[a-c] Within a column, values with different superscripts are significantly different ($P < 0.05$).

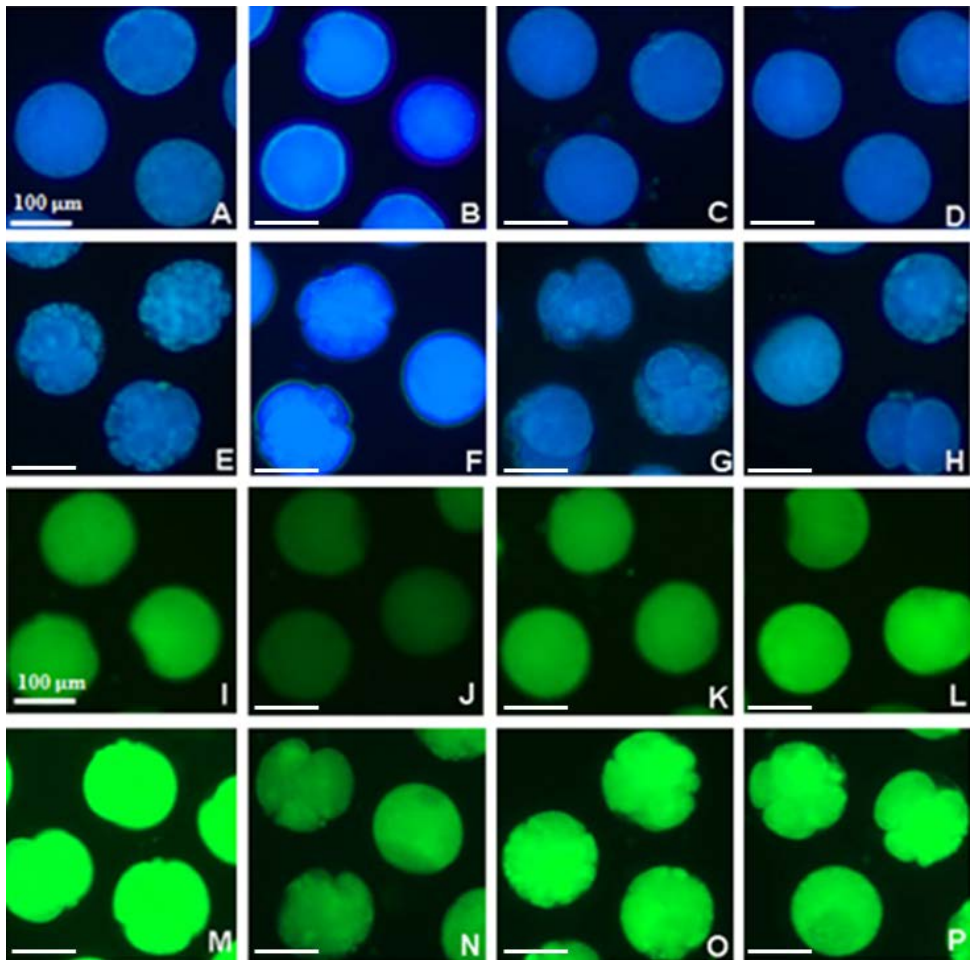


Figure 6. Photographic images of oocytes and parthenotes after GSH and ROS staining. *In vitro* matured oocytes (A-D, I-L) and day-2 parthenotes (E-H, M-P) were arranged in concentration (0, 1, 5, and 10 μM) of 7,8-DHF in order from left to right-hand side . They were stained with CellTracker Blue (A-H) and carboxy-H_2DFFDA (I–P) to evaluate intracellular GSH and ROS level, respectively.

Apoptosis-related gene expression in matured oocytes and porcine embryos

Anti-apoptotic effect of 7,8-DHF was evaluated using analysis of apoptosis-related gene and the result was shown in Fig. 7 and 8. Each sample was collected after measuring intracellular GSH and ROS level. After IVM, *BCL2L1* gene expression was significantly increased in 1 μM 7,8-DHF treated group and *BAK1* gene expression was significantly decreased in that group and 5 μM 7,8-DHF treated group (Fig 7). This pattern also appeared after IVC. Relative expression level of *BCL2L1* gene was increased and *BAK1* gene was decreased in 1 μM 7,8-DHF treated group (Fig 8). Group of 5 μM 7,8-DHF treatment reduced the *BAK1* gene expression, however, 10 μM 7,8-DHF treated group did not show difference with control group .

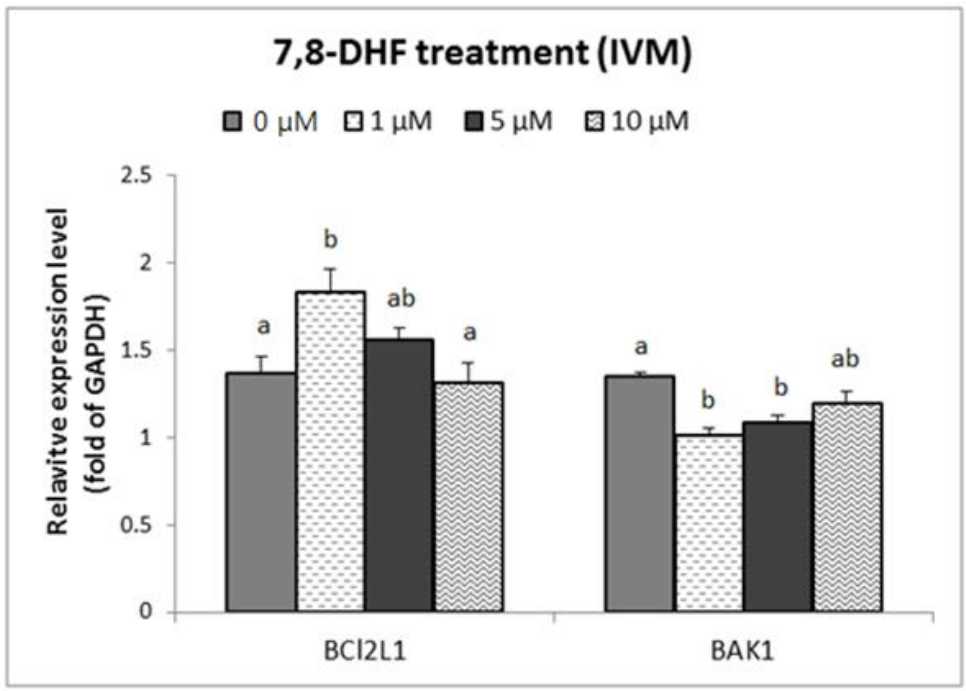


Figure 7. Relative expression level of *BCL2L1* and *BAK1* in mature oocytes. Treatment of 1 μM 7,8-DHF significantly increased *BCL2L1* and decreased *BAK1* after *in vitro* maturation (IVM). The relative gene abundance was normalized to *GAPDH* level. *BCL2L1* = B-cell lymphoma 2 like 1, *BAK1* = *BCL2* homologue antagonist/killer1, and *GAPDH* = Glyceraldehyde 3-phosphate dehydrogenase.

[a,b] Within a column, values with different superscripts are significantly different ($P < 0.05$).

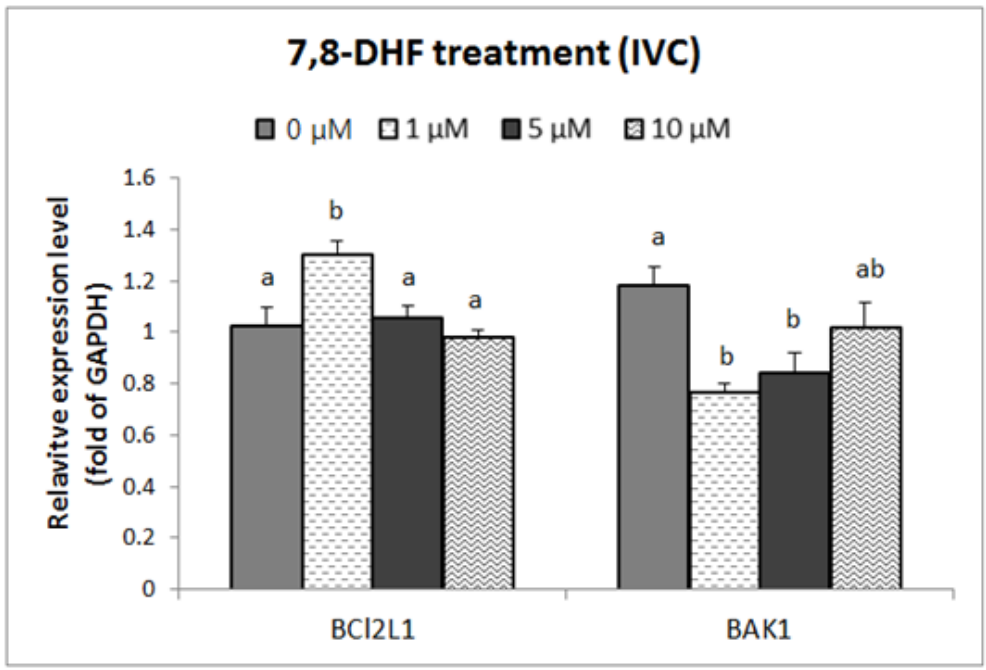


Figure 8. Relative expression level of *BCL2L1* and *BAK1* in day-2 parthenotes.

Treatment of 1 µM 7,8-DHF significantly increased *BCL2L1* and decreased *BAK1* at day-2 parthenote during *in vitro* culture (IVC). The relative gene abundance was normalized to *GAPDH* levels. *BCL2L1* = B-cell lymphoma 2 like 1, *BAK1* = *BCL2* homologue antagonist/killer1, and *GAPDH* = Glyceraldehyde 3-phosphate dehydrogenase.

[a,b] Within a column, values with different superscripts are significantly different ($P < 0.05$).

DISCUSSION

The ROS produced by normal metabolism during IVC damages oocytes and embryos constantly [13]. Cysteine, the precursor of glutathione, is already added in the IVM media to protect cells from oxidative stress, the beneficial effects of that is in controversy respect to cell number, GSH level and embryonic development [44-46]. Therefore, 7,8-DHF was examined as an antioxidant and anti-apoptotic. In this study, results showed that the treatment of 1 μM 7,8-DHF into the IVM/IVC media significantly improved the competence of oocytes cytoplasmic maturation and embryos development.

At first, the effect on 7,8-DHF for oocytes maturation was investigated. Intracellular GSH is used for a molecular marker that predict cytoplasmic maturation in porcine oocytes [47] because the level of GSH rises as oocytes proceed with their cytoplasmic maturation [48, 49] and 1st PB extrusion is closely related with nuclear maturation because it takes place by nuclear maturation in MII [50]. After culturing of COCs in maturation media supplemented with 7,8-DHF, although there were no significant difference in PB extrusion, the establishment of cytoplasmic maturation was increased in 1 μM treated group compare to other groups. Nuclear and cytoplasmic maturation are normally coordinated but some processes of cytoplasmic maturation related to successful pre-implantation probably occur without coordination with nuclear maturation [51] and cytoplasmic quality in IVM oocytes played a major role in the reconstruction of embryonic development [52]. Moreover, the levels of GSH was significantly increased and ROS was decreased in 1 μM 7,8-DHF treated group and it is supposed that intracellular redox metabolism [53] was effectively regulated in that group. It is important during IVM because oxygen consumption of COCs increases through IVM with mitochondrial oxidative phosphorylation [8]. The beneficial effects of 1

μM 7,8-DHF as an antioxidant also contribute to development of embryos reflected in cleavage and blastocysts formation. The level of GSH was higher than any other group and expression of ROS level was decreased. In terms of developmental competence, although 10 μM 7,8-DHF treated group showed poor development, the values of the intracellular oxidative level were similar with control group. Total cell number of blastocysts was similar in all groups. It means that treatment of 1 μM 7,8-DHF increases production efficiency of embryos without effect on blastocysts quality.

ROS is mainly produced as a by-product of electron transport chain in mitochondria for converting energy to adenosine triphosphate (ATP). Superoxide anions are reduced form of O_2 and relatively stable, however H_2O it can release iron ions, donate a single electron to O_2 which changed highly reactive state, and dismutated to H_2O_2 which pass the cell membrane. Hydrogen peroxide generates •OH and OH^- resulting in oxidative damage because hydroxyl radical is highly reactive cell attack most cellular component [2]. it damages to proteins, nucleic acids, lipids, and other biomolecules [3] by breaking the DNA [4], RNA, or inducing lipid peroxidation [5, 6]. Also, they disturb cellular signaling as acting like signaling messengers and induce aging of the oocytes [9]. Cells have intrinsic mechanisms such SOD, catalase, and GPx to counteract this problem. SOD which exists in the cytoplasm and mitochondria catalyzes dismutation of $O_2•^-$ to O_2 and H_2O_2. Catalase supports reaction of H_2O_2 to H_2O and O_2. GPx converts H_2O_2 to H_2O. As scavenging free radicals by carrying electrons, they defense cells from oxidative damages.

Increased level of intracellular ROS was correlated with apoptosis induced by oxidative damage [54]. Mitochondrial respiratory chain is the main oxygen consuming system in the cell and it is the major source of the toxic ROS [55] in cells. ROS changes mitochondrial integrity with various effectors such as Ca^{2+}, induces release of cytochrome c, and thereby activates caspase cascade [56], as a result, which then leads to apoptosis [57]. These damages were reflected on the individual gene expression of apoptotic factors [58, 59]. In these experiments, both porcine oocytes and parthenotes supplemented with 1 μM 7,8-DHF showed higher level of *BCL2L1* and lower level of *BAK1* expression. The group had significant difference with the other groups in terms of intracellular GSH and ROS level. Oocytes and parthenotes with 1 μM 7,8-DHF appeared increased level of GSH and decreased level of ROS compared to control group. This result correlated with production of GSH which keep intracellular redox state and protect cells from harmful effects of oxidative stress by treatment of 1 μM 7,8-DHF. GSH acts as a deducing agent and electron donor and oxidize itself to GSSG form [60]. Therefore, H_2O_2, as a major factor of ROS are reduced to H_2O in mitochondria and cytoplasm [61]. Synthesis and secretion of GSH is observed in the oviductal fluid [62], which indicates that redox balance is also important for the *in vivo* embryonic development.

It has been reported that some flavonoids generate ROS by autoxidation and redox-cycling resulted in the generation of O^{-}_{2} and H_2O_2 in high concentration [63, 64]. There were no researches about concentration dependent effect of 7,8-DHF, in this study, intracellular ROS was increased in 10 μM 7,8-DHF treatment compare to control, 1, and 5 μM treatment groups after IVM. It supposed to be resulted from

activation of metabolism and respiratory chains in COCs with autoxidation of 10 μM 7,8-DHF. Although, level of ROS was not significantly increased at day-2 parthenotes, oxidative stress during maturation may affect further development base on the the decreased rate of cleavage and blastocyst formations. However, expression level of *BCL2L* and *BAK1* was similar in 10 μM 7,8-DHF treatment group. In that reason, other factors which involve inhibition of the glucose transporter GLUT2 [65] or estrogenic activity which mimic 17 beta-estradiol by virtue of their ability to bind to and activate the nuclear estrogen [66] receptor might exist related to the poor developments independently or indirectly from apoptosis pathway.

This study was performed to determine effect of 7,8-DHF using electrical parthenogenetic activation instead of IVF and nuclear transfer because parthenogenesis is simple and maintains their early embryonic development *in vivo* [24, 67]. However, it is needed to evaluate accurate assessment using porcine embryos produced by IVF and nuclear transfer in further study.

In conclusion, 1 μM 7,8-DHF treatment during IVM promotes cytoplasmic maturation rather than nuclear maturation and improve embryonic development during IVC by increasing intracellular GSH level and decreasing ROS level. Moreover, by this concentration, it has an anti-apoptotic effect, however a high concentration of 7,8-DHF seems to have detrimental effect for porcine embryos.

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국문초록

돼지 난자의 체외성숙과 단위발생시 7,8-Dihydoxyflavone 의 효과

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수의산과학 전공

활성산소종의 생산과 항산화 물질 작용의 불균형에 의한 산화적 스트레스는 돼지 난자의 성숙과 배아의 발달에 악영향을 미친다. 본 연구에서는 이러한 문제를 해결하기 위하여 플라보노이드 계열 항산화제 중 하나인 7,8-Dihydroxyflavone(7,8-DHF)이 돼지 난자의 성숙과 배아 발달에 미치는 영향에 대하여 알아보았다. 돼지 난자의 체외 성숙 44시간 동안, 7,8-DHF를 각각 1, 5, 10 μM의 농도로 물질을 처리한 후, 대조군과 함께 제1 극체의 방출 여부로 핵성숙을 평가하고 세포 내 글루타치온 레벨을 측정하여 세포질의 성숙을 평가하였다. 성숙된 난자는 전기적인

방법으로 단위 생식을 유도하였으며, 이후 7일간 대조군과 1, 5, 10 μM의 농도로 7,8-DHF를 처리한 실험군을 배양기에서 체외배양하였다. 배양 2일 째에 난할율을 측정하고 7일 째에 배반포 형성율을 측정함으로써 7,8-DHF가 배아의 발달에 미치는 영향을 관찰하였다. 또한 단위생식 후 배양 2일 째와 7일 째에 배아를 염색하여 활성산소종과 글루타치온의 발현 강도를 측정함으로써 세포 내 산화적 스트레스의 수준과 그것을 제거하는 역할을 하는 세포 내 항산화제의 일종인 글루타치온의 발현을 평가하였다. 배양 2일 째의 배아와 7일 째의 배반포를 채집하여 추출한 RNA는 역전사연쇄중합반응을 이용하여 cDNA를 합성하고 세포자멸사와 관련된 유전자의 발현을 비교하였다. 실험 결과, 돼지 난자의 체외 성숙 기간 동안 1 μM의 농도로 7,8-DHF를 처리한 군에서 세포질의 성숙이 증가했으며, 단위생식 방법으로 유도된 배아가 2세포기에 도달하는 비율과 배반포를 형성하는 비율이 다른 군보다 증가했다. 또한 1 μM 의 7,8-DHF를 처리한 군은 항세포자멸사 관련 유전자 (*BCL2L1*)의 발현은 증가하고 세포자멸사를 유도하는 유전자 (*BAK1*)의 발현은 감소했다. 5 또는 10 μM의 농도로 7,8-DHF를 처리하는 것은 대조군과 유의차를 나타내지 않았다. 결과적으로 1 μM의 7,8-DHF는 돼지 난자의 성숙 단계와 배아의 배양 단계에서 세포 내 글로타치온 합성은 증가시키고 활성산소종은 제거함으로써 항산화 효과를 나타내고 세포자멸사를 감소시켰다. 이러한 작용은 돼지 난자의 세포질 성숙을 향상시키고, 배아의 발달율을 증가시키는 긍정적인 효과를 나타냈다.

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주요어: 7,8-Dihydroxyflavone, 돼지 난자, 항산화제, 글루타치온, 활성산소종

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