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3-Hyroxyflavone in Maturation Medium Supports *In Vitro* Development of Fertilized Bovine Follicular Oocytes

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ABSTRACT

Antioxidants, as reactive oxygen species scavengers, are one of the beneficial additives in serum-free defined culture medium. In this study, three separate experiments were performed to determine the effects of 3-hyroxyflavone added to the culture medium on the developmental competence of follicular bovine oocytes during *in vitro* maturation (IVM) and/or *in vitro* culture (IVC). The rate of blastocyst developed from oocytes cultured in IVM medium with 3-hyroxyflavone was significantly higher than that from control oocytes (39.0% vs. 26.3%, $p<0.001$), respectively. However, oocytes cultured in the medium with addition of 3-hyroxyflavone only at IVC period did not show significance in the blastocyst development when compared with control. When 3-hyroxyflavone was added to both IVM and IVC media, the rate of blastocyst formation was even significantly lower (21.1%) than control (26.5%; $p<0.05$). The present findings suggested that antioxidative activity of 3-hydroxyflavone added to only IVM medium beneficially affected the developmental competence of follicular bovine

(Key words : 3-Hyroxyflavone, *In vitro* maturation, *In vitro* culture, Bovine, Oocyte, Embyo)

INTRODUCTION

In vitro production (IVP) of bovine embryos involves various processes such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC). For IVM and IVC of bovine embryos, an environment consisting of 5% CO_2 and 95% air (20% O_2), and 5% CO_2 and 90% N_2 (5% O_2), respectively, is widely used. O_2 concentration in *in vivo* is known to decline in growing follicles (3～13 %) (McNatty, 1978). In addition, it has been reported that oviductal oxygen concentration showed around 7% or less at period of embryonic development just after fertilization, and uterine environment showed even lower oxygen concentration than that of the oviduct (Fischer and Bavister, 1993).

Cellular reactive oxygen species (ROS) are generated when there is a deviation of electrons to oxygen during electron transfer reactions in the mitochondrial respiratory chain and in other intracellular electron transfer systems (Guille and Joenje, 1991; Ho *et al*., 1996). The ROS are highly reactive with intracellular macromolecules including proteins, lipids and DNA, and may cause significant dysfunction such as enzyme inactivation, mitochondrial abnormalities or DNA fragmentation. Thus, protecting the embryos against these oxidative stresses seems to be important for embryonic development *in vitro* (Guérin *et al*., 2001). The harmful effects of oxygen radicals *in vivo* are usually prevented or limited by endogenous antioxidants (or scavengers of free radicals) such as superoxide dismutase (SOD), catalase and selenium-dependent glutathione peroxidase as well as lipid- and water-soluble antioxidants such as vitamin C and E, and uric acid (Knapen *et al*., 1999). To reduce the toxicity of ROS during IVM and IVC, antioxidants may be effective to regulate intra- and extra-embryonic environments. However, addition of antioxidant agents such as mercaptoethanol, ascorbic acid or SOD to IVM medium have shown no beneficial effect on IVP of bovine embryos (Blondin *et al*., 1997).

The flavonoids have evoked considerable interest because of their antioxidant property. Structure of flavo-

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noid is shared by the monomeric flavonoids, flavones and flavanones. Some flavonoids exhibit potent antitumor property and can modulate apoptosis, differentiation and cell cycle, probably by virtue of their antioxidant function (Dong *et al*., 2013). Flavonoids may inhibit the generation of primary oxygen radicals and subsequent oxidation chains as they are effective chelators of transitional metal ions (Lee *et al*., 2007). However, flavonoids have biphasic effect such as embryonic toxicity depending on their types and concentrations used (Wu *et al*., 2005). In pig, 10 μM 3-hydroxyflavone (a flavonoid having hydroxyl group at 3 carbon position) improves blastocyst development and quality while 100 μM 3-hydroxyflavone is detrimental to the embryos (Uhm *et al*., 2011).

The present study was performed to demonstrate the effect of 3-hyroxyflavone, an antioxidant agent, on IVM and/or IVC of bovine oocytes and embryos, respectively.

MATERIALS AND METHODS

Chemicals

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

Oocyte Recovery and IVM

Korean native, HanWoo cattle ovaries were collected at a local slaughterhouse and transported to the laboratory within 2~3 h in saline at 25~35℃. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 8 mm follicles using an 18 gauge hypodermic needle attached to a 10 ml disposable syringe.

After washing three times in HEPES-buffered Tyrode's solution, the COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in Nunc™ 4-well culture dishes (Nunc, Roskilde, Denmark) containing 500 μl of IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 10 μg /ml FSH-P (Folltrophin-V; Vertrepharm, London, UK) and 10% FBS (Gibco-BRL, NY, USA) under warmed and gas-equilibrated mineral oil for 20~22 h at 38.5℃, 5% CO_2.

Sperm Preparation and *In Vitro* Fertilization (IVF)

After IVM, the matured oocytes were washed three times in IVF100 medium (Research Institute for the Functional Peptides, Yamagata, Japan), and placed into 45 μl drops of IVF100 medium under mineral oil. A frozen semen straw from the HanWoo cattle was rapidly thawed in a 38℃ water bath and the semen was diluted with Tyrode's albumin lactate pyruvate (TALP) solution and washed twice in a same medium by centrifugation at 503 x*g* for 5 min. The final sperm pellet was resuspended in IVF100 medium and the number of spermatozoa was counted using a hemocytometer then adjusted to 1.0×10^7/ml by further dilution. A 5 μl aliquot of the sperm suspension were introduced to a 45 μl droplet of IVF100 medium containing matured oocytes. Incubation was carried out at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 6 h.

IVC

At the end of the insemination period, groups of 10 oocytes were stripped free from cumulus cells, and transferred into 50 μl drops of modified potassium simplex optimized medium containing 70.2 μM myo-inositol and 1 mM GlcNAc supplemented with 20% RD (RPMI1640 + DMEM, 1:1 v/v) which was described by Momozawa and Fukuda (2011). Following 24 h of culture, the presumptive zygotes which did not undergo cleavage were removed and at this time, the IVC medium was replaced with fresh medium according to each experimental group. The incubation was conducted at 38.5℃ under the 5% CO_2, 5% O_2, and 90% N_2 humidified atmosphere for 7 days, and blastocyst formation rate from 2-cells were evaluated.

Experimental Design

To determine the effect of 3-hyroxyflavone on IVM and/or IVC, 10 μM 3-hyroxyflavone was supplemented to IVM (FV-IVM), IVC (FV-IVC) or both media (FV-IVMC).

Statistical Analysis

All the experiments of treated group were repeated 3 times. Results subjected to statistical analyses were expressed as mean±SD. Data were subjected to one-way ANOVA (PRISM software version 4.0; GraphPad, San Diego, USA). Difference at $p<0.05$ was considered significant.

RESULTS

As shown in Fig. 1, FV-IVM group (39.0±6.7%, 97/250) showed significantly higher blastocyst formation rate than 3-hyroxyflavone-free control (26.1±3.7%, 144/550, $p<0.001$) whereas other experimental groups (FV-IVC: 26.2 ±3.7%, 63/240 and FV-IVMC: 21.1±2.1%, 55/260) did not

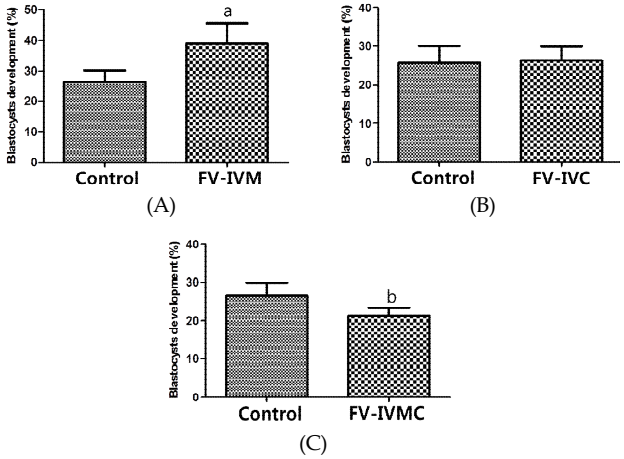

Fig. 1. Effect of 3-hyroxyflavone supplementation on blastocyst formation of bovine embryos cultured *in vitro*. To determine the effect of 3-hyroxyflavone on *in vitro* maturation (IVM) and/or *in vitro* culture (IVC), 10 μM 3-hyroxyflavone was supplemented to IVM (FV-IVM; A), IVC (FV-IVC; B) or both media (FV-IVMC; C). The letters indicate significant differences than control ($^{a}p<0.001$, $^{b}p<0.05$). Data were expressed as mean±SD from 3 replicates.

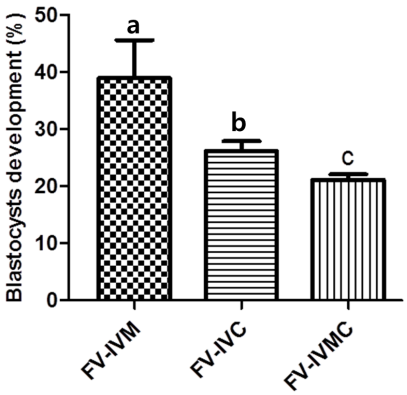

Fig. 2. Comparison of the effect of 3-hyroxyflavone on bovine blastocyst development when supplemented to the different stage of *in vitro* production procedures. 10 μM 3-hyroxyflavone was supplemented to IVM (FV-IVM), IVC (FV-IVC) or both media (FV-IVMC). Different letters indicate significant differences among groups ($^{ab}p<0.001$, $^{bc}p<0.05$, $^{ac}p<0.001$). Data were expressed as mean ±SD from 3 replicates.

show significant difference to FV-free control group. Interestingly, FV-IVM group showed remarkably higher blastocyst development than FV-IVC and FV-IVMC groups as shown in Fig. 2 ($p<0.001$). FV-IVMC group embryos also showed lower blastocyst rate than control and FV-IVC ones ($p<0.05$, Fig 1 and 2, respectively).

DISCUSSION

The present study demonstrates that 3-hyroxyflavone, an antioxidant, has beneficial effect when supplemented only to IVM medium not to IVC medium. Supplementation to both IVM and IVC media even has detrimental effect on the formation of blastocysts.

Recent reports suggested that 10 μM 3-hyroxyflavone supplemented to IVC medium was effective on embryo development in pig (Uhm *et al*., 2001) and cattle (Lee *et al*., 2011). However in the present study, addition of same concentration of 3-hyroxyflavone to IVC medium did not improve blastocyst development. Although the factor which supplemented only to IVM medium significantly improved embryonic development *in vitro*, long-term treatment of 3-hyroxyflavone from IVM to IVC (except IVF) even tended to be detrimental to bovine embryos. Although the concentration of 3-hyroxyflavone in previous studies is known to be effective, long-term treatment with same concentration from IVM to IVC in present study may be similar to high dose treatment for embryos, and may be toxic to *in vitro* development.

Cytotoxic effect of flavonoids includes the inhibition of topoisomerases (Bandele *et al*., 2008) and kinases (Hou and Kumamoto, 2010), and their prooxidant action (Miyoshi *et al*., 2007). Dietary intake (Zhuang *et al*., 2010) or *in vitro* exposure to genistein, a natural flavonoid, in mouse oocytes (Chan, 2009) and embryos (Chan *et al*., 2007), causes severe apoptosis in blastocysts and drastically inhibit early embryonic development leading to increased embryonic resorption after implantation. Nonetheless, the supplementation of 3-hyroxyflavone only to IVM medium in present study showed an exclusive antioxidative activity in follicular bovine oocytes and supported their maturation and developmental competence for blastocyst formation *in vitro*. Further studies would be needed to investigate the antioxidative activity of 3-hyroxyflavone in respect of embryonic stage-specific action under different O_2 concentration in each part of reproductive tract.

In conclusion, our findings suggest that addition of 3-hydroxyflavone during IVM improves developmental competence of follicular bovine oocytes following IVF and the supplementation should be limited to IVM procedure.

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