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Effects of Quercetin and Genistein on Boar Sperm Characteristics and Porcine IVF Embyo Developments

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ABSTRACT

Quercetin and genistein, plentifully present in fruits and vegetables, are flavonoid family members that have antioxidative function and plant-derived phytoestrogen activity. The antioxidative effects of quercetin and genistein on boar sperm characteristics and *in vitro* development of IVF embryo were investigated. The sperm motility was increased by addition of genistein 50 μM for 6 hr incubation compared to control (p<0.05). The sperm viability was increased by addition of quercetin 1 and 50 μM and genestein 1 and 50 μM for 3 hr incubation. In addition, the sperm viability seemed to be increased dose-dependantly by addition of quercetin or genistein 1 and 50 μM, respectively (p<0.05). The membrane integrities were not increased by quercetin or genistein treatments for 3 hr or 6 hr incubation period except for quercetin 1 μM for 3 hr incubation. In mitochondrial activities, addition of quercetin 50 μM for 6 hr incubation increased mitochondrial activity but decreased at 100 μM concentration compared with control (p<0.05).

When porcine IVF embryos were cultured in PZM-3 medium supplemented with low concentrations of quercetin (1～10 μM), the developmental rates to morula and blastocyst increased but significantly decreased at high concentrations of quercetin (25～50 μM). The highest developmental rate to blastocysts among all concentrations of quercetin was shown at quercetin 10 μM (p<0.05). The developmental rates to morula or blastocysts at low (0.01～1 μM) and high (5～10 μM) concentrations of genistein were not significantly different among all treatment group and genistein did not affect on IVF embryo development.

These results suggest that quercetin and genistein seem to have positive effects at certain concentrations on sperm characteristics such as motility, viability and mitochondrial activity. In addition, low concentrations of quercetin (1, 5 and 10 μM) in this experiment, seem to have beneficial effect on porcine IVF embryo development but genistein did not affect on it at all given concentrations (0.01～10 μM).

(Key words: quercetin, genistein, phytoestrogen, sperm characteristics, porcine IVF embryos)

INTRODUCTION

Although artificial insemination in swine industry has increased almost threefold during the past two decade, the use of long-term preservation or cryopreservation of semen and embryos in swine are still lower than those of other domestic animals. The exact reason which caused these results has not been clearly elucidated.

When boar semen and embryos are stored at low temperature for several days, they undergo the risk of generating of reactive oxygen species (ROS) production in media and are exposed to ROS. The generation of free radicals during *in vitro* storage appears to be one of the main mechanisms responsible for the reduction of sperm characteristics and embryos development. Particularly, boar semen is definitely sensitive to low temperature and is very difficult to preserve below 10℃ at liquid stage or cryopreservation due to high content of unsaturated fatty acids in the spermatozoal plasma membrane of boar sperm and low concentration of scavenging enzymes in the cytosol, resulting in the induction of lipid peroxidation and decreasing of motility and viability (Alvarez and Storey, 1995; Brezezinska-Slebodzinska *et al.*, 1995; Cerolini *et al.*, 2000; Jeong *et al.*, 2009). The long term preservation at ambient temperature or ultra-low temperature produced free radical by ROS, which

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cause the peroxidation of spermatozoal plasma membrane and DNA damage that lead to cell injury and trigger apoptosis (Aitken, 1994). The oxidative damage by ROS can cause detrimental effects and damage to all components of the cell resulting in DNA mutation, lipid peroxidation and apoptosis which drive in the decline of motility and viability, and concomitant loss of fertility (Alvarez and Storey, 1995; Cerolini *et al.*, 2000; Sierens *et al.*, 2001; Bain *et al.*, 2011).

The useful scavenging strategy of free radicals during *in vitro* culture of cell is to control the culture conditions and to supply the antioxidants in culture media. Antioxidants of *in vivo* and those of supplement in culture media are the effective defense systems against oxidative stress induced by ROS. There are two kinds of antioxidants having enzymatic and nonenzymatic property. The former are known as natural antioxidant while the latter are synthetic antioxidant or dietary supplements. They neutralize, scavenge and inhibit the surplusing ROS and prevent it from damaging the cellular component (Takahashi *et al.*, 2000; Ali *et al.*, 2003).

Phytoestrogens that having a chemically flavonoid structure are various groups of plant-derived compounds that mimic structurally and functionally mammalian natured estrogens. Phytoestrogens having flavonoid structure were included genistein, quercetin, curcumin, catechin and so on, which derived from food and medicine plants. Genistein and quercetin which are abundantly present in soybeans products, vegetable and fruits, have the antioxidative function and metal chelating abilities and protect against lipid peroxidation (Sierens *et al.*, 2001; Liu *et al.*, 2005). Quercetin, mostly in onion, has strong antioxidant properties with anti-proliferative, anti-inflammatory and immunosuppressive activities (Laughton *et al.*, 1991; Khanduja *et al.*, 2001). Quercetin is a specific inhibitor of the plasma membrane calcium ATPase that induce capacitation (Fraser *et al.*, 1995; Cordaba *et al.*, 1997). Genistein, a phytoestrogen known to as environmental estrogen, and a natural isoflavone compound present in soy products, has weak estrogenic activity and cellular antioxidant activity as well as inhibitory action of tyrosine kinase (Wei *et al.*, 1993; Coskun *et al.*, 2005; Khaki *et al.*, 2010).

The objective of present study was to evaluate whether supplementation of genistein or quercetin in media can improve the boar sperm characteristics and development of porcine IVF embryos or not.

MATERIAL AND METHODS

1. Sperm Preparation

Sperm-rich fractions were collected from three pure breeds (Duruc, Yorkshire and Landrace) with 85% motile sperm by the glove hand method at the Wonju AI and transported to the laboratory within 2 hr of collection at 17℃. Semens were washed with BTS extender and treated with H_2O_2 (100 μM, negative control), pyruvate (1 mM, positive control), genistein (1～100 μM) and Quercetin (1～100 μM), respectively. For evaluation of semen characteristics, the treated semen were incubated for 3 and 6 hours at 37℃ and 5% CO_2 in high humidified conditions. All experiments were repeated at least three times with semen samples from different boars. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (USA) and were analytical grade.

2. Sperm Evaluation

1) Motility

Sperm motility was subjectively evaluated by visual estimation using inverted phase contrast microscope at 400 × magnification and measured by determining the percentage of spermatozoa showing from wave to progressive motion (Jang *et al.*, 2009).

2) Hypoosmotic Swelling Test (HOST)

The HOST was based on methods described by Jang *et al.* (2009), modified as indicated below. The semen sample was incubated for 30 min at 37℃ and followed by mixing a 50 μl semen sample with 1 ml of hypoosmotic solution (25 mM Na-citrate and 75 mM fructose). Viable spermatozoa (positive) had coiled or swollen tails whereas non-viable spermatozoa (negative) had not damaged tails.

3) Viability

Sperm MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay that depend on the ability of metabolically active cells to reduce the tetrazolium salt to formazan was used to evaluate sperm viability (Jang *et al.*, 2009). The semen samples were washed twice with HEPES-BSA sol. and adjusted to 30×10^6 spermatozoa/ml. The 100 μl of semen samples plus 10 μl of MTT stock sol. (5 mg MTT/ml) was transferred in each well of 96-well microplate and incubated at 37℃ for 1 hr. After incubation, sperm MTT reduction rates was immediately measured in 550 nm wavelength in a microtiter plate reader

(Packard, USA).

4) Fluorescent Assay of Mitochondrial Activity

The percentage of live spermatozoa with functional mitochondria was evaluated by a dual fluorescence stain as a combination of rodamine123 (R123) and propidium iodide (PI) (Jang *et al.*, 2009). For this evaluation, 3 μl of R123 sol. was added to 1 ml of semen sample (20×10^6 spermatozoa/ml) and incubated for 15 min at 37℃ in the dark. Subsequently, the semen sample was stained with 10 μl of PI for 10 min at same conditions. Mitochondrial activity was examined under epifluorescence microscopy (Ziess, Germany) equipped with an excitation of 490/515 nm for R123 and an excitation of 545/590 nm for PI. Sperm cells displaying only green fluorescence at the mid-piece region were considered viable spermatozoa with functional mitochondria.

3. Oocyte Collection, *In Vitro* Maturation (IVM) of Oocytes, *In Vitro* Fertilization (IVF) and *In Vitro* Culture of Embryos

Cumulus oocyte complexes were aspirated from small follicles and 10～15 oocytes were matured in 100 μl of *IVM*-Ⅰ medium (TCM-199 containing of 10% porcine follicular fluid, 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 IU/ml hCG and 10 ng/ml EGF) for 22 h at 38.5℃ under 5% CO_2 in air, followed by additional culture in IVM-Ⅱ (TCM-199 containing of 10% pFF) for 20～22 hr under same condition described above. The basic culture medium for IVF embryos was PZM-3 medium. The spermatozoa (1×10^5 spermatozoa/ml) and maturated oocytes (10～15 oocytes) were transferred to 50 μl of fertilization drops and coincubated for 6 h under same condition. At 40～44 hr post IVF, 2～8 cell embryos were allotted in each 100 μl drop of culture medium (PZM-3) containing different concentration of genistein (low at 0, 0.01, 0.1 and 1 μM; high at 0, 1, 5 and 10 μM) or quercetin (low at 0, 1, 5 and 10 μM; high at 0, 10, 25 and 50 μM) and cultured for 6～8 days in 5% CO_2 in air at 38.5℃.

4. Statistical Analysis

Statistical analysis of experimental samples was performed with one-way analysis of variance using SAS program (SAS Institute Inc. USA). Duncan's multiple range test was used to compare the mean value of individual treatments. A *p*-value less than 0.05 were considered to be significant.

RESULTS

To evaluate how genistein or quercetin affects the pig sperm characteristics and IVF embryo developments, we examined sperm motility, viability, membrane integrity and mitochondrial activity treating with genistein (1～100 μM) and quercetin (1～100 μM) at 3 and 6 hr incubation periods and subsequently *in vitro* development of IVF embryos under genistein (0.01～10 μM) and quercetin (1～50 μM). The effects of genistein and quercetin on the sperm motility, viability, membrane integrity and mitochondrial activity are indicated from Table 1 to Table 4.

Both of quercetin and genistein treatment groups at various concentrations almost did not affect on the sperm motilities compared with control for 3 and 6 hr incubation periods but addition of 100 μM of H_2O_2 was significantly decreased sperm motilities compared to control (Table 1). The sperm motilities in quercetin for 3 hr treatment showed no statistical differences among different concentrations of genistein (genistein 1 μM, 91.1 ± 0.9%; 50 μM, 91.2 ± 0.6%; 100 μ1, 91.1 ± 1.1%) ($p>0.05$), however, 50 μM of genistein (88.5 ± 0.9%) showed significantly higher motility than that of control (73.9 ± 1.0) for 6 hr incubation period ($p<0.05$).

The sperm viabilities of quercetin and genistein for 3 and 6 hr incubation periods were measured by MTT assay (Table 2). Addition of H_2O_2 significantly decreased sperm viabilities upto 61.7% and 55.2% to those control for 3 and 6 hr incubation periods ($p<0.05$). The sperm viabilities in quercetin and genistein

Table 1. Effects of quercetin and genistein on boar sperm motility

Treatments (μM)	Motility (%)	
	3 h	6 h
Control	81.3 ± 0.9[a]	73.9 ± 1.0[b]
Pyruvate 1000	90.1 ± 2.7[a]	81.1 ± 3.9[ab]
H_2O_2 100	72.2 ± 3.6[b]	50.4 ± 3.5[c]
Quercetin 1	90.7 ± 2.0[a]	81.7 ± 2.9[ab]
Quercetin 50	87.5 ± 1.7[a]	82.9 ± 2.2[ab]
Quercetin 100	83.0 ± 1.7[a]	76.5 ± 4.1[ab]
Genistein 1	91.1 ± 0.9[a]	87.4 ± 1.4[ab]
Genistein 50	91.2 ± 0.6[a]	88.5 ± 0.9[a]
Genistein 100	91.1 ± 1.1[a]	85.9 ± 1.4[ab]

[a～c] Different superscripts within same column significantly differ, $p<0.05$.

Values presented here are the mean ± S.E.M of three experiments.

Table 2. Effects of quercetin and genistein on boar sperm viability

Treatments (μM)	Viability (%)	
	3 h	6 h
Control	100.0 ± 0.0[b]	100.0 ± 0.0[d]
Pyruvate 1000	134.3 ± 2.1[a]	114.5 ± 0.8[ab]
H_2O_2 100	61.7 ± 1.2[c]	55.2 ± 0.7[e]
Quercetin 1	131.1 ± 2.5[a]	114.4 ± 0.7[ab]
Quercetin 50	136.7 ± 2.4[a]	117.8 ± 0.7[a]
Quercetin 100	109.6 ± 2.3[b]	105.6 ± 0.8[c]
Genistein 1	130.8 ± 2.7[a]	111.6 ± 0.8[b]
Genistein 50	138.9 ± 2.6[a]	117.8 ± 1.0[a]
Genistein 100	115.3 ± 2.5[b]	105.2 ± 0.5[c]

[a~e] Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

at 1 μM and 50 μM concentrations were similar to that of 1 mM pyruvate treatment but significantly higher than those of control for 3 and 6 hr incubation periods ($p<0.05$). The sperm viabilities of 1 μM and 50 μM quercetin were higher than that of 100 μM for 3 and 6 hr incubation periods ($p<0.05$). The sperm viabilities of 1 μM and 50 μM genistein for 3 h incubation periods were 130.8 ± 2.7% and 138.9 ± 2.6% and these effects were higher than that of 100 μM genistein (115.3 ± 2.5 %, $p<0.05$). Otherwise, the sperm viability of 50 μM genistein (117.8 ± 1.0%) was significantly higher than those of 1 μM and 100 μM genistein for 6 hr incubation period (111.6 ± 0.8% and 105.2 ± 0.5%, $p<0.05$).

For 3 hr incubation period, the overall mean percentages of spermatozoal membrane integrities were 28.3 ± 1.3% in control, 32.1 ± 0.7% in pyruvate, 35.1 ± 0.8% in quercetin group (1 μM, 50 μM, 100 μM) and 32.7 ± 0.5% in genistein (1 μM, 50 μM, 100 μM). There were no statistical differences were shown at the given concentrations of quercetin and genestein except 1 μM quercetin treatment compared with control. For 6 hr incubation period, there were no significant differences among quercetin and genestein treatment groups ($p>0.05$).

The mitochondrial activities for 3 hr incubation period among all experimental groups (46.3～58.0%) were not significantly different ($p>0.05$). But for 6 hr incubation period, the mitochondrial activities in 1 μM and 50 μM quercetin groups were 40.6 ± 0.9% and 43.5 ± 1.2%, however, H_2O_2 and 100 μM genistein were 17.7 ± 1.0% and 27.1 ± 0.9%. The mitochondrial activity of H_2O_2 group for 6 hr incubation period sharply drops compared to that of 3 hr incubation period. The overall

Table 3. Effects of quercetin and genistein on boar sperm membrane integrity

Treatments (μM)	Membrane integrity (%)	
	3 h	6 h
Control	28.3 ± 1.3[b]	21.2 ± 1.0[ab]
Pyruvate 1000	32.1 ± 0.7[ab]	24.0 ± 0.9[a]
H_2O_2 100	18.2 ± 1.3[c]	11.6 ± 0.9[c]
Quercetin 1	38.4 ± 2.8[a]	22.8 ± 1.0[ab]
Quercetin 50	32.8 ± 1.7[ab]	23.5 ± 0.7[ab]
Quercetin 100	34.2 ± 2.6[ab]	21.4 ± 0.8[ab]
Genistein 1	31.2 ± 1.2[ab]	23.3 ± 1.1[ab]
Genistein 50	32.9 ± 1.5[ab]	22.8 ± 0.6[ab]
Genistein 100	33.9 ± 2.1[ab]	19.1 ± 0.9[b]

[a~c] Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

Table 4. Effects of quercetin and genistein on boar sperm mitochondrial activity

Treatments (μM)	Mitochondrial activity (%)	
	3 h	6 h
Control	54.3 ± 2.3[a]	37.0 ± 1.7[b]
Pyruvate 1000	56.6 ± 2.8[a]	39.6 ± 1.3[ab]
H_2O_2 100	51.6 ± 2.1[a]	17.7 ± 1.0[d]
Quercetin 1	54.8 ± 3.2[a]	40.6 ± 0.9[ab]
Quercetin 50	54.6 ± 1.7[a]	43.5 ± 1.2[a]
Quercetin 100	46.3 ± 2.0[a]	37.9 ± 0.7[ab]
Genistein 1	58.0 ± 2.6[a]	39.4 ± 1.1[ab]
Genistein 50	53.2 ± 1.9[a]	37.1 ± 1.5[b]
Genistein 100	47.9 ± 1.6[a]	27.1 ± 0.9[c]

[a~d] Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

mean percentages of mitochondrial activities among quercetin and genistein treatment groups were not significantly differ for 3 hr incubation period ($p>0.05$) but for 6 hr incubation period, quercetin group (40.7 ± 0.3%) seems to increase slightly mitochondrial activity compared to genistein group (34.5 ± 0.5%, $p<0.05$).

The developmental rates of porcine *IVF* embryos produced in embryo culture medium (PZM-3) supplemented with low and high concentration of quercetin (1～10 μM and 10～50 μM) were summarized in Table 5-1 to Table 5-2. The developmental rates to morula and blastocyst at low concentrations (47.7% and 16.8% in 1 μM; 45.5% and 20.4% in 5 μM; 44.7% and 24.7% in 10 μM) of quercetin showed higher when compared to control at each developmetal stage (37.8% and 16.5%, $p>0.05$). At low concentration of quercetin, the developmental rates of 10 μM quercetin at morula and morula + blastocysts stages were significantly increased compared to other treatment groups ($p<0.05$). In the development of porcine IVF embryos treated with high concentrations of quercetin, 10 μM quercetin (23.5 ± 0.3%) did not show difference in blastocyst development compared to control (20.6 ± 0.6%), however, both of 25 μM (12.3 ± 0.6%) quercetin and 50 μM quercetin (12.8 ± 0.3%) significantly decreased blastocyst development compared to control ($p<0.05$).

Effects of low (0.01～1 μM) and high (1～10 μM) concentrations of genistein on development of porcine IVF embryos were evaluated in Table 6-1 and Table 6-2. The developmental rates to morula at low concentrations of genistein were not different compared to control (30.0 ± 1.2% in control, 27.0 ± 0.6% in 0.01 μM, 25.6 ± 0.6% in 0.1 μM and 25.6 ± 1.2% in 1 μM). In addition, blastocyst developments at low concentrations of genistein also showed no significant differences compared to control (16.9 ± 0.3% in control, 15.6 ± 0.3% in 0.01 μM, 17.1 ± 0.3% in 0.1 μM and 19.4 ± 0.7% in 1 μM, $p>0.05$). At high concentrations of genistein treatments, the developmental capacity to blastocysts were 19.3% in control, 19.6% in 1 μM, 18.3% in 5 μM and 17.5% in 10 μM, respectively, and there were no significant differences among treatments ($p>0.05$). The percentages of morula plus blastocysts developments at high concentrations of genistein also showed similar results as those of blastocysts developments.

DISCUSSION

Table 5-1. Effects of low concentration of quercetin on development of IVM / IVF porcine embryos

Quercetin (μM)	No. of IVM / IVF embryos	No. of embryos developed(%);			Morulae plus blastocysts
		Premorulae	Moulae	Blastocysts	
0	278	127(45.7 ± 1.5^{a})	105(37.8 ± 1.5^{c})	46(16.5 ± 0.3^{bc})	151(54.3 ± 1.2^{c})
1	262	93(35.5 ± 0.6^{bc})	125(47.7 ± 1.2^{b})	44(16.8 ± 0.9^{c})	169(64.5 ± 0.7^{b})
5	255	87(34.1 ± 0.6^{c})	116(45.5 ± 0.9^{b})	52(20.4 ± 0.9^{b})	168(65.9 ± 1.5^{b})
10	320	98(30.6 ± 0.9^{b})	143(44.7 ± 0.3^{a})	79(24.7 ± 0.3^{a})	222(69.4 ± 0.6^{a})

$^{a\sim c}$ Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

Table 5-2. Effects of high concentration of quercetin on development of IVM / IVF porcine embryos

Quercetin (μM)	No. of IVM / IVF embryos	No. of embryos developed(%);			Morulae plus blastocysts
		Premorulae	Moulae	Blastocysts	
0	189	77(40.7 ± 0.9^{b})	73(38.6 ± 0.3^{b})	39(20.6 ± 0.6^{a})	112(59.3 ± 0.9^{b})
10	183	59(32.2 ± 0.7^{c})	81(44.3 ± 0.6^{a})	43(23.5 ± 0.3^{a})	124(67.8 ± 0.9^{a})
25	195	102(52.3 ± 0.6^{a})	69(35.4 ± 1.2^{b})	24(12.3 ± 0.6^{b})	93(47.7 ± 0.6^{c})
50	172	99(57.6 ± 0.6^{a})	51(29.7 ± 0.6^{c})	22(12.8 ± 0.3^{b})	73(42.4 ± 0.7^{d})

$^{a\sim d}$ Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

Table 6-1. Effects of low concentration of genistein on development of IVM / IVF porcine embryos

Genistein (μM)	No. of IVM / IVF embryos	No of embryos developed to(%):			Morulae plus blastocysts
		Premorulae	Morulae	Blastocysts	
0	130	69(53.1 ± 0.6[a])	39(30.0 ± 1.2[a])	22(16.9 ± 0.3[ab])	61(46.9 ± 0.9[a])
0.01	122	70(57.4 ± 0.9[a])	33(27.0 ± 0.6[a])	19(15.6 ± 0.3[b])	52(42.6 ± 0.9[a])
0.1	129	74(57.4 ± 0.7[a])	33(25.6 ± 0.6[a])	22(17.1 ± 0.3[ab])	55(42.6 ± 0.9[a])
1	129	71(55.0 ± 0.7[a])	33(25.6 ± 1.2[a])	25(19.4 ± 0.7[a])	58(45.0 ± 1.8[a])

[a,b] Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

Table 6-2. Effects of high concentration of genistein on development of IVM / IVF porcine embryos

Genistein (μM)	No. of IVM / IVF embryos	No of embryos developed to(%):			Morulae plus blastocysts
		Premorulae	Morulae	Blastocysts	
0	228	117(51.3 ± 1.2[a])	67(29.4 ± 0.3[a])	44(19.3 ± 0.3[a])	111(48.7 ± 0.0[a])
1	224	114(50.9 ± 1.2[a])	66(29.5 ± 0.6[a])	44(19.6 ± 0.7[a])	110(49.1 ± 0.9[a])
5	213	101(47.4 ± 1.3[b])	73(34.3 ± 0.3[a])	39(18.3 ± 1.2[a])	112(52.6 ± 0.9[a])
10	211	102(48.3 ± 0.6[b])	72(34.1 ± 1.5[a])	37(17.5 ± 0.7[a])	109(51.7 ± 1.5[a])

[a,b] Different superscripts within same column significantly differ, $p<0.05$.
Values presented here are the mean ± S.E.M of three experiments.

Manipulation of sperm and embryos by cooling, *in vitro* storage at room temperature and freezing can generate free radicals and toxic-oxidants in medium and deplete energy sources, resulting in lipid peroxidation, DNA damage and apoptosis (Cerolini *et al.*, 2000; Guthrie *et al.*, 2008).

Antioxidants ameliorate and/or scavenge free radicals produced by ROS, inhibit ROS formation and play a protective role against oxidative stress-damaged cell, resulting in the decrease of DNA damage and lipid peroxidation (Takahashi *et al.*, 2000; Ali *et al.*, 2003). In recent, there is a growing attention toward the use of natural food and medical plants as an antioxidants for *in vitro* culture of mammalian cell or embryos and maintenance of human health. Natural compounds, mainly belong to flavonoid family, are included astaxantin, quercetin, epigallocatechin, L-cartinine, curcumin, silymarin and genistein.

Genistein and quercetin, abundantly present in fruits and vegetables, are flavonoid antioxidants and plant-derived phytoestrogen extracted from plants (Johnson and Loo, 2000; Coskun *et al.*, 2005; Liu *et al.*, 2005; Khaki *et al.*, 2010). Phytoestrogen resembles structurally 17-β estradiol and can bind to the estrogen receptor (Bingham *et al.*, 1998; Sierens *et al.*, 2001). In addition, high concentrations of phytoestrogen are indicated to have harmful effects on fertility due to severe reproductive tract disorders and temporary infertility syndrome in domestic animals and DNA fragmentation in human spermatozoa (Tou *et al.*, 1998; Johnson and Loo, 2000; Mitchell *et al.*, 2001; Bennetts *et al.*, 2008). The objective of this study was to elucidate the antioxidative effects of genistein and quercetin on boar sperm characteristics and porcine IVF embryo development.

Sperm motility and viability were significantly decreased by addition of H_2O_2 compared to control ($p<0.05$), however, these sperm characteristics were increased or maintained normal level to control by addition of 1, 50 and 100 μM of quercetin or genistein for 3 or 6 hr incubation periods. The membrane integrity increased in the presence of 1 μM of quercetin for 3 hr incubation compared to control and the mitochondrial activity also increased in the presence of 50 μM of quercetin for 6 hr incubation compared to control, however, addition of H_2O_2 as ROS significantly decreased sperm membrane integrity and mitochondril activity comapred to control for 3 and 6 hr incubation periods ($p<0.05$). Addition of genistein 100 μM (high concentration) decreased sperm mitochondrial activity compared to control for 6 hr incubation period. These results indicate that quercetin and genistein have favorable effects on

sperm viability at approximate 1～50 μM ranges for 6 h incubation but addition of 100 μM of genistein (high concentration) for 6 h incubation have detrimental effect on mitochondrial activity. These result indicates that quercetin and genistein have antioxidative properties in sperm characteristics at 1～50 μM ranges and are able to improve some sperm characteristics at those concentrations.

These results are consistent with the finding of other researches that both genistein and quercetin have antioxidative effects on red blood cell culture and ram sperm and diabetic rats sperm characteristics mediated through decreasing of oxidative stress and reducing of lipid peroxidation (Wei *et al.*, 1993; Sierens *et al.*, 2001; Liu *et al.*, 2005; Coskun *et al.*, 2005; Mi *et al.*, 2007; Khaki *et al.*, 2010).

When porcine IVF embryos were cultured in PZM-3 medium supplemented with low concentrations of quercetin (1～10 μM), the developmental rates to morula and blastocyst increased but significantly decreased at high concentrations of quercetin (25～50 μM). Thus, quercetin should be supplemented at 1～10 μM range if it is used as antioxidant compound for the porcine IVF embryo development. The developmental rates to morula or blastocysts at low (0.01～1 μM) and high (5～10 μM) concentrations of genistein were not significantly different among all genistein treatment groups and did not affect on IVF embryo development. Genistein has weak estrogenic activity and cellular antioxidant activity as well as inhibitory action of tyrosine kinase (Wei *et al.*, 1993; Coskun *et al.*, 2005; Khaki *et al.*, 2010). Thus, genistein as antioxidant should be cautiously used in the general animal cell culture system because it can cause compounding effects as estrogen agonist, antioxidant and tyrosine kinase inhibitor on growing cells under various mitogen containing medium.

As summary, quercetin and genistein have positive effects on sperm motility and viability at 1～50 μM for 3 and 6 hr incubation periods. In addition, quercetin alone (at 1, 5 or 10 μM) seems to have beneficial effect on porcine IVF embryo development but genistein did not at all given concentrations (at 0.01～10 μM).

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