Curcumin Attenuates Hydrogen Peroxide Induced Oxidative Stress on Semen Characteristics during *In Vitro* Storage of Boar Semen

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ABSTRACT

Curcumin is a major active component of the food flovour tumeric. It has been used for the treatment of many diseases such as inflammatory and infectious diseases, cancer and other disease due to its antioxidant properties. Curcumin is a powerful scavenger of many free radicals such as superoxide anion, hydroxyl radical and nitric oxide. The objective of this study was to investigate the antioxidative effects of curcumin against hydrogen peroxide on semen quality during *in vitro* storage of boar semen. The sperm treated with different concentration of curcumin (1, 5 and 10 μM) in the presence or absence of hydrogen peroxide (250 μM H_2O_2) were incubated for 3, 6 and 9 hr at 37℃ and analyzed sperm characteristics such as motility, membrane integrity (MI), lipid peroxidation (LPO), reactive oxygen species (ROS) and DNA fragmentation (DF). The sperm motility and MI in H_2O_2 treated group (47.8 %±6.8 and 24.8%±2.2) were significantly decreased when compare to curcumin treated group (79.8 %±2.7 and 34.6 %±1.0, respectively) irrespective of incubation periods($p<0.05$). The LPO of spermatozoal plasma membrane was measured by thiobarbituric acid (TBA) reactions for malondialdehyde (MDA), MDA level in control (11.6±0.6 nmol/L $\times10^6$) and curcumin groups (10.7±0.3 nmol/L$\times10^6$) were lower than those of curcumin plus H_2O_2 (17.1±0.8 nmol/L $\times10^6$) or H_2O_2 group (22.5±1.9 nmol/L$\times10^6$) from 3 to 9 hr incubation periods. The DF by sperm chromatin dispersion (SCD) test and ROS production measured by 2′,7′-dichlorofluorescein (DCF) fluorescence intensity were no significantly difference through all experimental groups ($p>0.05$). Correlation among evaluation methods for sperm quality, motility vs MI and DF vs ROS was positively correlated while motility vs DF and ROS vs LPO were negatively correlated in all treatment groups. These results demonstrate that curcumin can effectively improve the sperm quality during *in vitro* storage of boar semen through its hydrogen peroxide scavenging mechanism as an antioxidant.

(Key words : Curcumin, Semen quality, Hydrogen peroxide, Antioxidant, Pig)

INTRODUCTION

Today, artificial insemination (AI) with extended liquid boar semen is extensively used in the swine industry in the world. Extended liquid boar semen is often used within the same day of collection, but may be stored at a temperature of 15～20℃ for up to 5 days before AI (Johnson *et al.*, 2000). This semen storage technique has several adventages, however, distrimental effects on farrowing rate and litter size may be detected when storage exceeds 48 or 72 hrs in *in vitro*. This distrimental effects is closely related to reactive oxygen species (ROS) production during *in vitro* storage (Christensen *et al.*, 2004). ROS appears to be one of the major factors of impaired semen quality during *in vitro* storage and also associated with decline in fertility. ROS can not only alter most types of cellular molecules, but also induce the decline of the sperm motility and viability (Pena *et al.*, 2003).

DNA and membrane phospholipids are the two main molecular target for ROS. Therefore, the motility and viability of semen stored *in vitro* are limited by ROS. Exposure of sperm to ROS is associated with the formation of lipid peroxidation and DNA damage, resulting the decline of fertilizing ability (Aitken *et al.*, 1989; Chen *et al.*, 1997). Lipid peroxidation in sperm cell may be one of the mechanisms responsible for the negative biochemical and physiological changes during sperm storage (Stubbs and Smith, 1984). Extensive DNA damage, with the loss of sperm motility and ability to fuse with egg, has been observed in conditions of high oxidative stress (Aitken *et al.*, 1998).

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene- 3,5-dione, 1) is a naturally occurring phenolic compound isolated as a yellow pigment from turmeric (dry rhizomes of Curcumin longa) which is commonly used as a spice and food colorant (Buescher

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and Yang; 2000). This compound has attracted considerable attention due to its various biological and pharmacological activities, including antioxidant (Rukkumani *et al*., 2003; Venkatesan and Rao., 2000) and anticancer activities (Shukla *et al*., 2002; Aggarwal *et al*., 2003). Curcumin exhibits protective effects against oxidative damage and it is considered to be a powerful scavenger against free radicals (Duvoix *et al*., 2005; Ruby *et al*., 1995).

However, whether or not curcumin exerts its effects on sperm characteristics during *in vitro* storage of boar semen has not been attempted.

Therefore, this study was to examine the protective effects of curcumin on sperm characteristics such as motility, membrane integrity, lipid peroxidation, ROS production and DNA fragmentation during *in vitro* storage of boar semen.

MATERIALS AND METHODS

Semen Source and Treatments

Sperm-rich fraction was collected from 1～3 crossbred boar of known reproductive history in Won Ju A.I center and transported to the laboratory at 17℃ within 1 hr. Semen was randomly treated with curcumin(1～10 μM) in the presence or absence of H_2O_2 (250 μM). The semen of each treated group was examined the sperm characteristics every 3 hr during 9 hr incubation periods at 37℃ in high humidified air. All of the treatments were repeated at least three times with semen sample from different boars. All chemicals used in this study were purchased from Sigma Aldrich unless otherwise stated and were analytical grades.

Analysis of Semen Characterics

The analysis of semen characterics was evaluated based on the motility, MI, LPO, ROS production and DNA fregmentation.

Sperm Motility

Sperm motility was subjectively assessed by visual estimations. The semen sample was examined at a magnification of × 400 by using inverted phase contrast microscope (Nicon, Japan). Sperm motility was measured by determining the percentage of spermatozoa showing any movement of the flagellum. A 200 spermatozoa in 3 different microscopic fields were evaluated according to the movement criteria.

Membrane Integrity(MI)

The spermatozoal plasma membrane integrity was evaluated by hypoosmotic swelling test (HOST) described by Jang *et al*. (2007), modified as indicated below. The assay was performed by mixing a 50 μl semen sample with 1 ml of hypoosmotic solution (150 mOsm, 7.35 g Na-citrate and 13.51 g fructose in 1 l of distilled water) and then incubated for 30 min at 37℃. The spermatozoa showing coiled or swollen were determined as viable spermatozoa.

Quantification of Lipid Peroxidation (LPO)

LPO was measured by using the thiobarbituric acid (TBA) reaction for malondialdehyde (MDA). Semen treated with 0.01 M sodium phosphate buffer (pH 7.4) containing 1.15 % (w:v) KCl was adjusted in Ca^+ and Mg^+ free D-PBS (Gibco, USA) to give a concentration to $20×10^6$ spermatozoa/ml. For the increasing of lipid peroxide to MDA, LPO was promoted using a combination of ferrous sulfate and sodium ascobate. Sperm suspension (1 ml) was mixed with 10 μl of 1 mM ferrous sulfate and 10 μl of 5 mM sodium ascorbate, and then incubated for 1 hr at 37℃. The reaction mixture was added 250 μl of 40 % trichloacetic acid, held for 10 min at 0℃, and centrifuged (2,500×g) for 10 min. Supernatants (1 ml) mixed with TBA were boiled with hot water for 10 min. The amount of MDA produced was quantified against a standard curve at 532 nm wavelength in spectrophotometer.

ROS Measurement

ROS in sperm was measured by the method described in LeBel *et al*.(1992), modified as indicated below. Levels of ROS in sperm were determined by a fluorescence spectrophotometer using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as probe. The sperm treated with curcumin (1～10 μM) in the presence or absence of H_2O_2 (250 μM) were incubated with 5 μM DCFH-DA for 1 hr at 37℃ followed by washing and adjusting of sperm concentration ($2×10^7$ spermatozoa/ml) with PBS. The conversion of DCFH to the fluorescent product DCF was measured using a fluorescence spectrophotometer (BIO-RAD, VersaFlora^{TM}) with excitation at 480 nm and emission at 520 μm. ROS production was quantified from a DCF standard curve and results expressed as intensity of DCF fluorescence.

DNA Fragmentation

SCD test was measured by the method modified by Fernandez *et al*.(2003). Semen sample was diluted in PBS medium to obtain the concentration of $2×10^7$ spermatozoa/ml. The sperm suspensions were mixed with 1 % low-melting point aqueous agarose (to obtain a 0.7 % final agarose concentration) at 37℃. Aliquots of 50 μl of the mixture were pipetted onto a glass slide precoated with 0.65 % standard agarose dried at 80℃, covered with a coverslip, and left to solidify at 4℃ for 5 min. Coverslips were carefully removed, and the slides was horizontally placed in neutralizing and lysing

solution Ⅰ(0.4 M Tris, 0.4 M DTT, 1 % SDS and 50 mM EDTA, pH 7.5) for 30 min at room temperature, which was followed by incubation in neutralizing and lysing solution II (0.4 M Tris, 2 M NaCl, 1 % SDS and 1 % Triton X-100, pH 7.5) for 30 min at 50 ℃. Slides were thoroughly washed in Trisborate- EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 min, dehydrated 2 minutes each in sequential 70 %, 90 %, and 100 % ethanol baths, and air dried. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 2 μg/ml) for fluorescence microscopy. DNA fragmentation index (DFI) was determined the methods as indicated below. Briefly, a total of 200 sperm were evaluated manually on each slide for halo size and dispersion pattern; 1) nuclei with large DNA dispersion halos, 2) nuclei with medium size halos, 3) nuclei with small size halos, and 4) nuclei with no halo. The nuclei with large to small size halo were considered sperm with fragmented DNA, whereas nuclei with no halo were considered sperm with non-fragmented DNA.

Statistical Analysis

Statistical analysis of replicated experiment results was used for treatment comparisons and was carried out one-way analysis of variance (ANOVA) using the SAS GLM procedure (SAS). Duncan's multiple range tests was used to compare the mean value of each treatment groups. A *p*-value below 0.05 was considered significant. Repeated measures ANOVA were used to test the main effects of curcumin and hydrogen peroxide, incubation periods on sperm characteristics. The treatments (control, curcumin, curcumin plus hydrogen peroxide and hydrogen peroxide alone) and incubation periods nested whthin the treatments and their interaction effects was included in the analytical model. The correlation coefficients among the evaluation methods were analyzed using the nested model; motility, MI, LP, ROS and DF for sperm quality were considered.

RESULTS

This study was designate to investigate the effects of curcumin on sperm characteristics during *in vitro* storage of boar semen. Effects of different concentration of curcumin (1, 5 and 10 μM) against H_2O_2 were evaluated to based on the sperm characteristics such as motility, MI, LPO, ROS level and DF at 3, 6 and 9 hr incubation periods at 37℃ in boar semen. Those results were shown in Fig. 1～5.

The overall mean of sperm motility in H_2O_2 group (negative control, 47.8 %±6.8) significantly dropped when compare to any other groups (73.3 %±5.5 in control, 79.8 %±2.7 in curcumin and 61.8 %±3.6 in curcumin plus H_2O_2 group irrespective of incubation periods (Fig. 1). The sperm motility gradually decreased in a incubation period dependent manner through all experimental groups, but in H_2O_2 group was dramatically dropped post 6 hr incubation period. The MI in control and curcumin groups at every incubation period (3, 6 and 9 hr) greatly increased when compare to H_2O_2 group and curcumin plus H_2O_2 group (Fig. 2). The MI in 1 μM curcumin group obtained the highest result among the experimental groups. The overall mean of MI in control and curcumin groups (35.3 %±1.8 and 34.6 %±1.0) was significantly increased in H_2O_2 group and curcumin plus H_2O_2 group (24.8 %±2.2 and 27.0 %±1.2). The LPO of spermatozoal plasma membrane was measured by TBA reaction for malondialdehyde (MDA), MDA level was 9.49 nmol/L×10^6 in control and 9.15～9.84 nmol/l×10^6 in curcumin group and these levels were lower than those of curcumin plus H_2O_2 or H_2O_2 group (11.51～15.72 nmol/l×10^6) at 3 hr incubation period and at 6 to 9 hr incubation periods, there were a similar trend to 3 hr incubation period in all experimental groups (Fig. 3). The MDA level in control and curcumin groups significantly lower than those of H_2O_2 group and curcumin plus H_2O_2 group irrespective of incubation periods (p<0.05). The amount of ROS was measured by DCF intensity. ROS level in control at 3, 6 and 9 hr was slightly lower then those of curcumin or curcumin plus H_2O_2 groups, however, there were no significantly different (Fig. 4). ROS levels were slightly increased according to the lapse of incubation periods through all experimental group. DNA fragmentation (DF) in boar sperm treated with curcumin (1～10 μM) and curcumin plus H_2O_2 (1～10 μM curcumin + 250 μM H_2O_2) was not significantly different among the experimental gr-

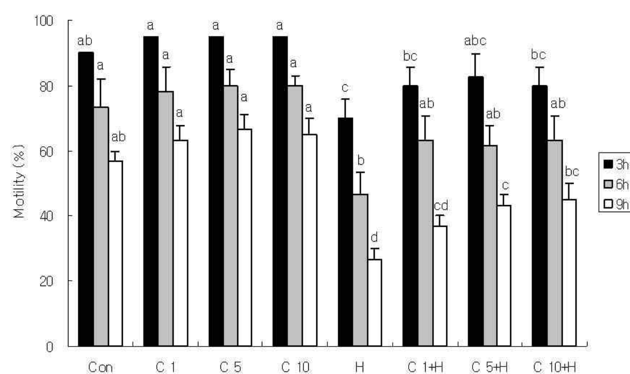

Fig. 1. Protective effects of curcumin against hydrogen peroxide on sperm motility in pig. Con; Control, C1; 1 μM curcumin, C5; 5 μM curcumin, C10; 10 μM curcumin, H; 250 μM hydrogen peroxide, C1+H; 1 μM curcumin + 250 μM hydrogen peroxide, C5+H; 5 μM curcumin +250 μM hydrogen peroxide, C10+H; 10 μM curcumin +250 μM hydrogen peroxide. [a~d] Different superscripts are significantly differ, p<0.05. Values are expressed as mean± S.E.M of three experiments.

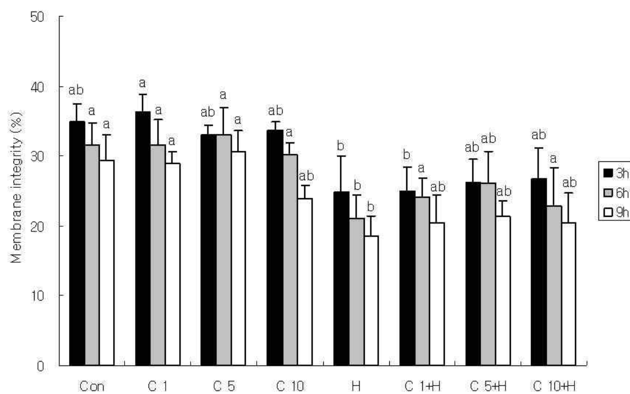

Fig. 2. Protective effects of curcumin against hydrogen peroxide on sperm membrane integrity in pig. [a,b] Different superscripts are significantly differ, $p<0.05$. Values are expressed as mean±S.E.M of three experiments.

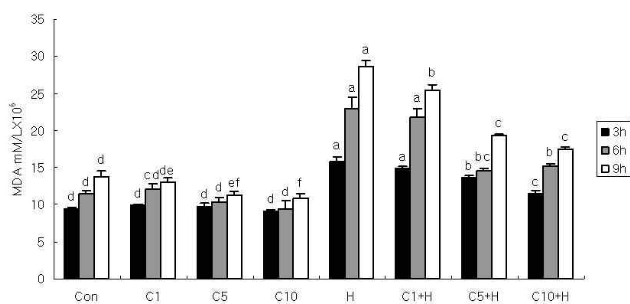

Fig. 3. Effect of curumin against hydrogen peroxide on the lipid peroxidation in pig. [a~f] Different superscripts are significantly differ, $p<0.05$. Values are expressed as mean±S.E.M of three experiments.

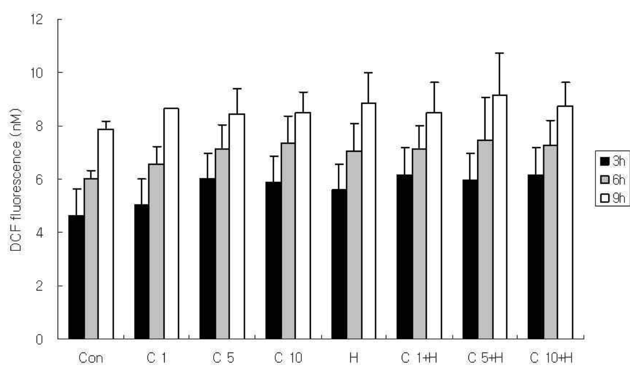

Fig. 4. Effects of curcumin against hydrogen peroxide on sperm ROS production during *in vitro* storage in pig. Values are expressed as mean±S.E.M of three experiments.

oups. But DF in curcumin and curcumin plus H_2O_2 groups at 3, 6 and 9 hr shown a little lower than that of H_2O_2 single treatment group (Fig. 5).

Correlation among evaluation methods for sperm quality were evaluated by motility, MI, DF, ROS and

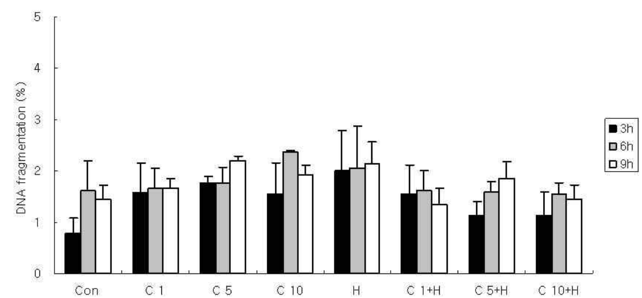

Fig. 5. Protective effects of curcumin against hydrogen peroxide on sperm DNA fragmentation in pig. Values are expressed as mean±S.E.M of three experiments.

LPO in boar sperm treated with curcumin in the presence or absence of H_2O_2 (Table 1). There were positively correlated in motility vs MI and DF vs ROS (r= 0.36～0.63 and r=0.22～0.44) irrespective of treatments. The other hand, motility vs DF, motility vs ROS, motility vs LPO, MI vs ROS and MI vs LPO were negatively correlated. Specially, in curcumin or curcumin plus H_2O_2 groups, motility vs MI were significantly correlated ($p<0.05$ and $p<0.01$), but control and H_2O_2 groups were not significantly different.

DISCUSSION

The generation of ROS is an essential prerequisite for the normal function of many cells, however, excessive formation can lead to cellular damage and pathology (Halliwell and Gutteridge, 1986). ROS are highly reactive with complex cellular molecules such as protein, lipid and DNA, and cause serious dysfunction such as enzyme inactivation, mitochondrial abnormality or DNA fragmentation (Guerin *et al.*, 2001). Recent attention has now focused on reactive oxygen species (ROS) as major causal factors decreasing of sperm motility and viability during *in vitro* storage (Ashok *et al.*, 2003). Boar sperm seems to be especially sensitive to ROS damage due to the relative high content of unsaturated fatty acids in the phospholipids of the boar sperm membrane and their cytoplasm contains low concentrations of scavenging enzymes (Cerolini *et al.*, 2000; Aitken *et al.*, 2004). Exposure of sperm to ROS is associated with decreased fertility, and the formation of lipid peroxidation and DNA damage (Aitken *et al.*, 1989; Potts *et al.*, 2000). In addition, the production of lipid peroxidation in sperm due to oxidative stress has been associated with a loss of cell motility and fertility (Aitken *et al.*, 1989; Alvarez and storey, 1989) and increasing of lipid peroxidation also related to the loss of spermatozoal membrane integrity (Kasimanickam *et al.*, 2007). The lipid peroxidation cascade is initiated

Table 1. Correlation coefficients among different evaluation methods to verify the effects of curcumin against hydrogen peroxide in boar semen

Treatments		Fertility index				
		Motility	MI	DF	ROS	LPO
Con	Motility	-	0.36	−0.26	−0.91**	−0.89**
	MI		-	0.24	−0.46	−0.61
	DF			-	0.42	0.24
	ROS				-	0.97**
	LPO					-
C	Motility	-	0.44*	−0.18	−0.82**	−0.75**
	MI		-	−0.23	−0.30	−0.26
	DF			-	0.29	−0.02
	ROS				-	0.54**
	LPO					-
H	Motility	-	0.55	−0.29	−0.86**	−0.89**
	MI		-	−0.86**	−0.56	−0.30
	DF			-	0.22	−0.08
	ROS				-	0.84**
	LPO					-
C+H	Motility	-	0.63**	−0.38*	−0.61**	−0.66**
	MI		-	−0.62**	−0.30	−0.22
	DF			-	0.44*	−0.10
	ROS				-	0.49**
	LPO					-

Con; Control, C; Curcumin, H; H_2O_2, C+H; Curcumin + H_2O_2.
MI; membrane integrity, DF; DNA fragmentation, ROS; reactive oxygen species, LPO; lipid peroxidation.
Significant level; * $p<0.05$, ** $p<0.01$.

when spermatozoa are attacked by ROS, which results in a loss of unsaturated fatty acids from plasma membrane and a decline in the motility and fertilizing ability of spermatozoa during storage (Aitken *et al*, 2004). Excessive production of ROS can lead to DNA fragmentation and its related to defective apoptosis. Exposure to environmental or industrial toxin, genetic and oxidative stress are known to cause sperm DNA fragmentation (Saleh *et al*., 2002; Wang *et al*., 2003), and also sperm storage for long times and handling such as centrifugation, commonly used prior to IVF or scientific studies are associated with the generation of ROS and an increase in the level of DNA damage. Sperm DNA fragmentation could also be involved in early loss of embryo because of abnormal development (Virro *et al*., 2004). ROS occurring regents such as H_2O_2 can reduce the antioxidant defense of spermatozoa and increase the peroxidation of plasma membrane (Griveau *et al*., 1995). Hydrogen peroxide was major ROS responsible for the loss of motility that occurred in response to an ROS challenge in boar spermatozoa. ROS are regulated by antioxidant enzymes as an enzymatic or non-enzymatic properties and this antioxidant enzymes play a role in protective action from oxidative stress-induced cell death, but the exact antioxidant mechanism is still unclear. Supplementation with ROS scavengers, which are normally present in the genital tract, has been reported to improve the sperm characteristics (Bilodeau *et al*., 2001; Pena *et al*., 2003).

Curcumin has a ROS scavenging property in addition to anti-inflammatory and immnosuppressive activity resulting from the inhibition of NF-KB and/or AP-1 and inhibit ROS production, nitric oxide synthase activity, lipoxygenase involved in inflammatory pathway (Ruby *et al*., 1995; Bobrovnikova-Marjon *et al*., 2004). Curcumin exhibits protective effects against oxidative damage and it is considered to be a potent cancer chemopreventive agent (Aggarwal *et al*., 2005). The antioxidative effects of curcumin may be due to its oxygen free radical scavenging property and activation of cellular antioxidant defense mechanisms (Rukkumani *et al*., 2003).

However, whether or not curcumin exerts its effects on sperm characteristics during *in vitro* storage of boar semen has not been attempted. Therefore, the present study was designed to examine whether or not curcu-

min has an antioxidative feature on sperm characteristics during *in vitro* storage of boar semen.

In this present studies, the sperm motility and MI in curcumin groups were significantly higher than in H_2O_2 group irrespective of incubation periods or curcumin concentration, and also curcumin plus H_2O_2 groups were obtained a little better results than H_2O_2 group. The LPO of spermatozoal plasma membrane between curcumin and control group were not greatly differ, but H_2O_2 addition into semen extender was significantly increased in incubation periods dependent manner than those of both curcumin and control group. The ROS production was slightly increased according to the incubation periods irrespective of treatments, but there were no significantly different. The DF in all experimental groups was not over 3% regardless of incubation periods and treatments. Taraglione and Ritta (2004) concluded that motility and MI with exposure of sperm to ROS was positively correlated the fertilizing ability in bulls. LPO and DF have been correlated with exposure of sperm to ROS (Lopes *et al*., 1998). Addition of antioxidants to the media produced a significant decrease in DNA strand breaks and thiobarbituric acid reactive species (Potts *et al*., 2000). Our result consistent with those with Taraglione and Ritta(2004), Lopes *et al*.(1998) and Potts *et al*.(2000). This present study indicated that curcumin supplementation into semen extender were improved the sperm characteristics such as motility, MI, LPO, ROS and DF on boar semen during *in vitro* incubation, indicating that curcumin has a antioxidative effects during *in vitro* storage of boar sperm.

The other hands, the results of this study in relationship of semen evaluation methods indicate that motility vs MI and DNA fragmentation vs ROS were positively correlated, but motility vs DNA fragmentation and ROS vs LPO were negatively correlated irrespective of treatments. This results agree with the finding of numerous investigators that the sperm motility and MI were generally used as a sperm viability index and LPO, ROS and DNA fragmentation was deeply correlated the fertility(Perez-Llano *et al*., 2003; Chohan *et al*., 2006; Kasimanickam *et al*., 2007; de Lamirande and O'Flaherty, 2008; Saleh and Agarwal, 2002).

In conclusion, to our knowledge, this is the first report in which the effect of curcumin exposure has been shown to be beneficial on sperm characteristics during *in vitro* storage of boar semen. The beneficial effects on improvement of boar semen quality are thought to be due to antioxidative function provided by curcumin supplementation. It is also possible that antioxidant genes may be expressed in sperm, and antioxidants gene to be expressed may mediate its effects on improvement of semen quality in pig.

The present study demonstrates that addition of curcumin can enhances the semen quality in *in vitro* storage of boar semen through its ROS scavenging action as an antioxidant. Although our data from enhancements of sperm characteristics during *in vitro* storage in boar semen can not be directly applied to mammalian species, curcumin might improve the sperm characteristics during preservation.

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(Received: 15 June 2009/ Accepted: 20 June 2009)