Curcumin and Vit. E Alleviate Alone or Synergetically Hydrogen Peroxide Induced-Oxidative Stress on Boar Sperm Characteristics during *In Vitro* Storage

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ABSTRACT

Antioxidants partially ameliorated the detrimental effects of reactive oxygen species (ROS) on sperm characteristics during *in vitro* storage. The objective of the present study was to investigate the single or synergetic antioxidative effect of curcumin and Vit. E on the characteristics of fresh boar sperm during *in vitro* storage. The sperm viability in curcumin, Vit. E supplementation and curcumin+Vit. E+H_2O_2 groups remained over 85.0% in 3 hr incubation period, but in 6 hr incubation period, curcumin+Vit. E+H_2O_2 groups was sharply dropped than those of curcumin and Vit. E group. The membrane intergrity in all evaluated groups except for H_2O_2 group did not significantly difference in 3 hr incubation period. The viability in curcumin or Vit. E supplementation were significantly increased than in curcumin+H_2O_2 and Vit. E+H_2O_2 group in 6 hr incubation period. The percentage of mitochondrial activity and acrosome intergrity obtained similar trends within same incubation periods irrespective of treatment. The lipid peroxidation of spermatozoal plasma membrane ranged from 11.6～17.5 nM/*l*×10^6 and 14.0～19.0 nM/*l*×10^6 in 3 hr and 6 hr incubation periods. In conclusion, curcumin or Vit. E surpplementation alone or cooperatively improved sperm viability index (motility, membrane intergrity, viability and survival rates) and fertility index (mitochondria activity, acrosome intergrity and lipid peroxidation) of fresh boar sperm, indicating that curcumin and Vit. E have a antioxidative properties through its scavenging activity against hydrogen peroxide.

(Key words : Curcumin, Vitamin E, Antioxidant, Hydrogen peroxide, Sperm characteristics)

INTRODUCTION

Cells living under aerobic conditions constantly face the oxygen(O_2) environment. O_2 is inevitablely required to support life, but its metabolites such as reactive oxygen species (ROS) can modify cell functions, endanger cell survival or both. Althrough ROS are essential for various sperm specific physiological processes such as acrosome reaction and capacitation, high concentration of ROS has a detrimental effects on sperm characteristics, leading to ATP depletion, DNA fregmentation, motility loss, and lipid peroxidation in spermatozoa (Agarwal *et al.,* 2003).

Spermatozoa, unlike other cells, are unique in structure, function and very susceptible to damage by ROS. ROS induce membrane lipid peroxidation in sperm and that the toxicity of generated fatty acid peroxides are important causes of decreased sperm function. Oxidative damaging process in sperm function, which is one of the major obstacles to sperm functionality, both *in vivo* and *in vitro*, is based on the fact that mammalian spermatozoa membranes are rich in polyunsaturated fatty acids that make them very fluid but at the same time very susceptible to free radical-induced peroxidative damage (de Lamirande and O' Flaherty, 2008; Sikka, 2004).

Boar spermatozoa are particularly susceptible to ROS-induced damage because their plasma membrane contain relatively large percentages of polyunsaturated fatty acids and their cytoplasm contains relatively low concentration of scavenging enzymes (Cerolini *et al.,* 2000).

Antioxidants, in general, are free radical scavengers that suppress the formation of ROS and/or oppose their actions.

Many flavonoids are a group naturally occurring antioxidants, usually found in plants, fruits and vegetables(curcumin, quercertin, luteolin, genistein and epigallocatechin, etc.) and may prevent oxidative stress induced-sperm damage (Cai *et al.,* 1997; Johnson and Loo, 2000; Mathuria and Verma, 2008).

Curcumin(1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) is a naturally occuring phenolic compound isolated as a yellow pigment from turmeric (*Curcuma longa*), which is commonly used as a spice and

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food colorant. The compound has been reported to possess a variety of biological and pharmacologial activities including antioxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic and anti-angiogenic (Duvoix *et al.*, 2005; Jackson *et al.*, 2006).

Vit. E is a group of naturally occuring lipid - soluble antioxidants and the major chain-breaking antioxidant in membranes, the tocopherols and the tocotrienols, that are found in certain plant oils. Vit. E is a potent lipid soluble antioxidant in biological systems with the ability to directly quench free radicals and function as membrane stabilizer (Sikka, 2004; Subudhi *et al.*, 2008). The application of ROS scavengers such as curcumin and Vit. E is likely to improve sperm characteristics and fertility (Sahoo *et al.*, 2008; Saleh, 2002).

In the present study, two potent antioxidant, i.e. curcumin and Vit. E, have been selected to evaluate its protective effect against oxidative stress on boar sperm characteristics during *in vitro* storage. Therefore, the object of this study is to examine the antioxidative effect on sperm characteristics when alone or in combination with the curcumin or Vitamin E was added into semen extender during *in vitro* storage.

METERIALS AND METHODS

Sperm Collection and Preparation

Sperm-rich fractions(30 to 50 ml) were collected from 1～3 pure breed (Duruc, Yorkshire and Landrace) with more than 85% motile sperm by the gloved hand method at the local A. I. center (Wonju). After collection, fresh semen was immediately diluted with semen extender(Gene sperm®) and transported to the laboratory at 17℃ within 2 hr. Semen was treated with 5 uM curcumin or 200 uM Vit. E in the presence or absence of H_2O_2 (50 uM). All of the experiments were repreated at least 3 times with semen samples from the different boars. The semen of each treated group was incubated for 3 and 6 hr at 37℃ under 5% CO_2 in high humidifird air.

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich(U.S.A.) and were analytical grade.

Sperm Evaluations

1) Sperm Viability Index

Sperm Motility

Sperm motility was subjectively assesed by visual estimations. A drop of semen (10～20 ul) was placed on clean slide glass that was pre-heated to 37℃ and then covered with coverslip. The semen samples was examined at a magnification of ×400 using inverted phase contrast microscope(Nicon, Japan). Sperm motility was measured by determining the percentage of spermatozoa showing any movement of the flagellum, considering motility of individual spermatozoa from wave to progressive motion.

Membrane Integrity

Spermatozoal plasma membrane integrity was evaluated by hypo-osmotic swelling test(HOST). The HOST solution as a hypo-osmotic solution (150 mOsm) was consist of 7.35 g Na-citrate and 13.51 g fructose in 1 *l* of distilled water. A 50 ul of sperm suspension was mixed with 1 ml of hypo-osmotic solution and then incubated for 30 min at 37℃ under 5% CO_2 in high humidified air. Viable spermatozoa (HOS positive) had coiled or swollen tails whereas non-viable spermatozoa (negative) had not changed tails when observed under inveted phase contract microscope.

Viability

MTT(3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction rates assay that depend on the ability of metabolically active cells to reduce the tetrazolium salt to formazan was used to evaluate sperm viability. Fresh semen samples of three boar semen with good quality (more than 80% motile sperm) were used to obtain the standard curve and the relationship between the MTT reduction rates and sperm viability. The semen samples were washed twice with Hepes-BSA sol. and adjusted to 30×10^6 spermatozoa/ml. The 100 ul of semen samples plus 10 ul of MTT stock sol. (5 mg MTT/ml of PBS) was introduced in each well of 96-well microplate and incubated at 37℃ for 1 hr. After incubation, MTT reduction rates assay was measured against a standard curve at 550 nm wavelength in a ELISA Reader (Packard, USA).

Survival Rates

The sperm survival rates was examined using Hochest 33342(HO)/ propidium iodide(PI) staining method. Briefly, a 100 ul aliquot of fresh semen added 10 ul PI (0.5 mg/ml in PBS) incubated for 5 min, and then 10 ul HO (0.5 mg/ml) was mixed and stained for 10 min at 37℃ in the dark condition. After incubation, 10～20 ul of stained spermatozoa suspension was placed on clean slide glass, coverslipped and evaluated immediately at 400× magnification by epifluorescence microscope (Ziess, Germany) equipped with excitation/barrier filter of 460/500 nm. Two hundred sperm cell per slide were examined and classified based on the fluorescene emitted. Viable spermatozoa(live) emitted blue whereas non-viable spermatozoa(dead) emitted red.

2) Sperm Fertility Index

Mitochondria Activity

The percentage of live spermatozoa with functional mitochondria was evaluated using a combination of fluorescent stains, rhodamine 123(R 123) and propidium iodide(PI). For this assay, 3 ul of R123 solution (2 mg/ml in DW) were added to 1 ml of semen sample (20×10^6 spermatozoa/ml) and incubated for 15 min at 37℃ in the dark. Subsequently, semen sample were stained for 10 min at 37℃ with 10 ul of PI. Following incubation, the supernatant was removed after centrifugation and the sperm pellets was resuspended in 1 ml PBS. A 10 ul drop of suspension were placed on slide glass, coveslipped and examined at 400× magnification under epifluorescence microscope equipped with excitation/barrier filter of 490/515 nm for R123, excitation/barrier filter of 545/590 nm for PI. Sperm cells displaying only green fluorescense at the middle piece region of tail were considered viable spermatozoa having strong mitochondria activity. Sperm celll displaying red or faint green fluorescence at middle piece were considered non-viable spermatozoa.

Acrosome Integrity

Staining with FITC-PNA(fluorescein-labeled peanut agglutinin) was used for evaluation of acrosome intergrity. Briefly, an aliquot (10 ul) of the sperm sample was smeared on the glass slide, air-dired, and membrane-permeabilized with 95% ethanol for 30 sec. A 90 ul aliquot of FITC-PNA(100 ul/ml in PBS) was mixed with 5 ul of propidium iodide (PI, 340 mM in PBS, final concentration of 18 mM), and 20 ul of this solution was spread over the smeared slide. The slide was incubated in a dark humidified chamber at 4℃ for 30 min. After incubation, the slide was rinsed with cold distiled water and air-dried in the dark at 4℃. Two hundred spermatozoa were evaluted under the epiflourescent microscope(excitation 460～500 nm, emission 550 nm) and classified into three categories: intact acrosome (stained bright green from FITC-PNA at the acrosomal cap), damaged acrosome(stained green and red). and missing acrosome(stained red from PI).

Quantification of Lipid Peroxidation(LPO)

Lipid peroxidation was measured by using the thiobarbituric acid (TBA) reaction for malondialdehyde. Semen treated with 0.01 M sodium phosphate buffer (pH 7.4) containing 1.15% (w:v) KC1 was adjusted in Ca_2^+ and Mg_2^+ free D-PBS (Gibco, USA) to give a concentration to 20×10^6 spermatozoa/ml. For the increasing of lipid peroxide to malondialdehyde, lipid peroxidation was promoted using a combination of ferrous sulfate and sodium ascorbate. Sperm suspension(1 ml) was mixed with 10 ul of 1 mM ferrous sulfate and 10 ul of 5 mM sodium ascorbate, and then incubated for 1 hr at 37℃. The reaction mixture was added with 250 ul 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 quantified against a standard curve at 532 nm wave length in a spectrophotometer.

Statistical Analysis

Statistical analysis of replicated experiment results were used for treatment comparisions and were carried out one-way analysis of variance (ANOVA) using SAS program. Ducan's multiple range test used to compare mean value of individual treatments. All results were expressed as mean±S.E.M. A p-value below 0.05 was considered significant. The treatments (curcumin, Vit. E and curcumin plus Vit. E in the presence or absence of H_2O_2) and incubation times(3 and 6 hr) nested within the treatments and their interaction effects were included in the analytical model. The pearson correlation test was used to calculate the corelations among sperm evaluation methods within treatments.

RESULTS

The single or synergetic effects of curcumin (50 μM) or Vit. E (200 μM) against hydrogen peroxide (50 μM) on sperm characteristics were evaluated in boar semen incubated during 3 and 6 hr at 37℃ under 5% CO_2 in high humidified air.

These results were analyzed by ANOVA and summarized in Table 1. There were significantly different between incubation periods or sperm treatments in all of sperm characteristics (p<0.0001). But in considering simultaneously two paraments as the sperm treatments and incubation periods, sperm motility was only indicated significantly different (p<0.0001).

The results of the viability index such as motility, membrane integrity, viability and survival rates in this study were summarized in Fig. 1. The motility in curcumin or Vit. E alone irrespective of incubation periods was superior to any other groups. The motility in curcumin+H_2O_2 or Vit. E+H_2O_2 at 3 hr incubation period was not significantly different when compare to control group and at 6 hr incubation period, there were significantly different between control and curcumin+H_2O_2 or Vit. E+H_2O_2 groups (p<0.05), and also curcumin or Vit. E plus H_2O_2 groups were significantly increased compare to H_2O_2 group (p<0.05).

The membrane integrity in all treatment groups ex-

Table 1. ANOVA sources in variation in boar sperm treated with curcumin or Vit. E in the presence or absence of H_2O_2 during *in vitro* storage

Source	Motility				Viability				Membrane integrity			
	d.f.	MS	*F*-value	*P*-value	d.f.	MS	*F*-value	*P*-value	d.f.	MS	*F*-value	*P*-value
Incubation periods	1	6132.07	406.16	<0.0001	1	4,536.48	282.89	<0.0001	1	792.00	69.50	<0.0001
Sperm treatments	6	937.67	62.11	<0.0001	6	648.71	40.45	<0.0001	6	182.07	15.98	<0.0001
Sperm treatments × Incubation periods	6	339.79	22.51	<0.0001	6	70.69	4.41	0.0031	6	5.32	0.47	0.8283
Replication	1	157.50	10.43	0.0024	1	33.88	2.11	0.1576	1	6.30	0.55	0.4614
Error	41	15.09			27	16.03			41	11.39		
R-Square	0.957				0.953				0.804			

Source	Survival rates				Mitochondria function				Acrosome integrity			
	d.f.	MS	*F*-value	*P*-value	d.f.	MS	*F*-value	*P*-value	d.f.	MS	*F*-value	*P*-value
Incubation periods	1	6,007.14	390.76	<0.0001	1	1,639.44	57.43	<0.0001	1	1,097.52	100.49	<0.0001
Sperm treatments	6	251.11	16.34	<0.0001	6	702.78	24.62	<0.0001	6	159.92	14.64	<0.0001
Sperm treatments × Incubation periods	6	2.93	0.19	0.9777	6	30.30	1.06	0.4011	6	43.56	3.99	0.0055
Replication	1	270.08	17.57	0.0001	1	386.57	13.54	0.0007	1	79.90	7.32	0.0117
Error	41	15.37			41	28.54			27	10.92		
R-Square	0.925				0.845				0.890			

Source	Lipid peroxidation			
	d.f.	MS	*F*-value	*P*-value
Incubation periods	1	77.08	78.99	<0.0001
Sperm treatments	6	21.33	21.86	<0.0001
Sperm treatments × Incubation periods	6	0.81	0.84	0.5529
Replication	1	0.04	0.04	0.8349
Error	27	0.97		
R-Square	0.888			

cept for H_2O_2 group at 3 hr incubation period ranged from 36.1%~40.8%, there were no significantly different (p>0.05). For 6 hr incubation period, curcumin+H_2O_2 or Vit. E+H_2O_2 groups were slightly lower than those of other groups (34.1% in control, 34.3% in curcumin, 31.0 % in Vit. E and 31.6% in curcumin+Vit. E+H_2O_2).

Sperm viability in control (84.7%), curcumin (85.5%), Vit. E (84.3%) and curcumin+Vit. E+H_2O_2 (81.0%) groups in 3 hr incubation period remained over 80%, there were no significantly different, but for 6 hr incubation period, only curcumin and Vit. E groups were obtained more then 70.0%. The H_2O_2 group for 3 and 6 hr incubation periods in sperm viability was significantly lower than those of any other groups.

The survival rates for 3 and 6 hr incubation periods were no significantly different among all treatment groups except for H_2O_2 group.

The fertility index such as mitochondria activity, acrosome integrity and lipid peroxidation were indicated in Fig. 2. The mitochondrial activity measured by fluorescent assay in curcumin group (77.0%) for 3 hr incubation period was obtained the highest result among all treatment groups whereas in H_2O_2 group (54.0%) was significantly the lowest in all treatment groups. For 6

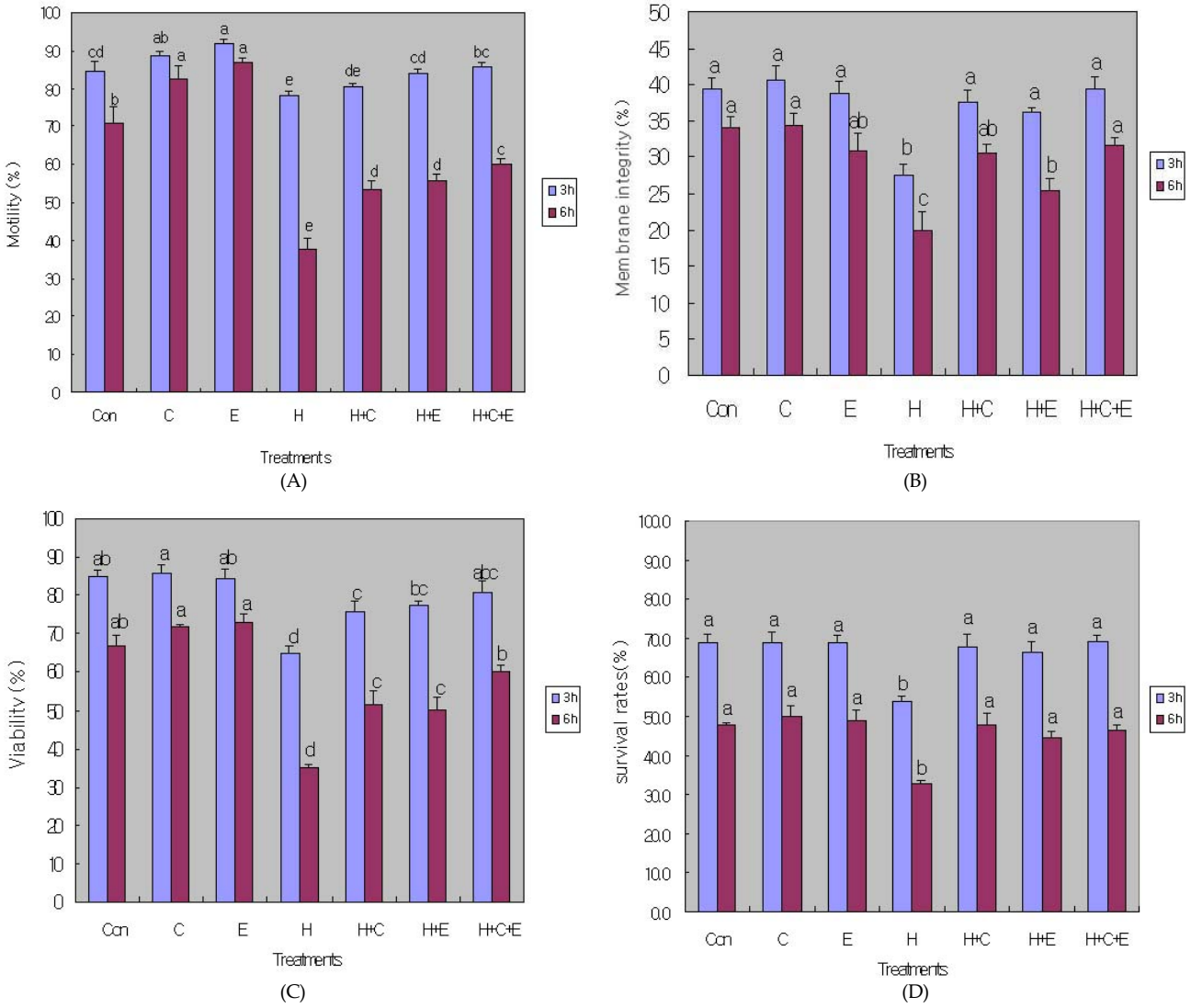

Fig. 1. Effect of curcumin and Vit. E on boar sperm viability index such as A) motility, B) membrane integrity, C) viability and D) suvival rates during *in vitro* storage. $^{a\sim d}$ Mean values with different superscripts within same incubation times are significantly differ, $p<0.05$. Data are expressed as mean±SEM of three experiments. Con: Control, H: 50 μM Hydrogen peroxide, C: 5 μM Curcumin, Vit. E: 200 μM Vitamin E, H+C: 50 μM Hydrogen peroxide+5 μM Curcumin, H+E: 50 μM Hydrogen peroxide+200 μM Vitamin E, H+C+E: 50 μM Hydrogen peroxide+5 μM Curcumin+200 μM Vitamin E.

hr incubation period, control (66.8%), curcumin (71.5%) and Vit. E (74.5%) groups were slightly higher when compare to curcumin+H_2O_2 (58.4%) or Vit. E+H_2O_2 (56.3%) or curcumin+Vit. E+H_2O_2 (58.0%) groups.

The acrosome integrity for 3 hr incubation period in all treatment groups remained over 80% (81.3~89.6%) and no difference were detected among those groups. For 6 hr incubation period, acrosome integrity in curcumin (82.5%), Vit. E (82.7%), curcumin+Vit. E+H_2O_2 (80.7%) and control (78.8%) groups were significantly higher than those of other groups (61.0% in H_2O_2, 72.7 in curcumin+H_2O_2 and 73.0% in Vit. E+H_2O_2 group; $p<0.05$).

In LPO measurement by TBA reaction of the spermatozoal plasma membrane, the MDA levels for 3 hr incubation period range from 11.63~17.53 nmol/l×10^6 and for 6 hr incubation period was 13.97~19.03 nmol/l×10^6 in all experimental groups. No significantly difference was detected in all experimental group except for H_2O_2 group during *in vitro* incubation.

Corelationships among sperm characteristics in curcumin, Vit. E, curcumin+ H_2O_2, Vit. E+H_2O_2, and curcumin+Vit. E+H_2O_2 groups were investigated by considering motility, membrane integrity, viability, survival rates, mitochondria activity, acrosome integrity and lipid peroxidation (Table 2). Among sperm characteristics wi-

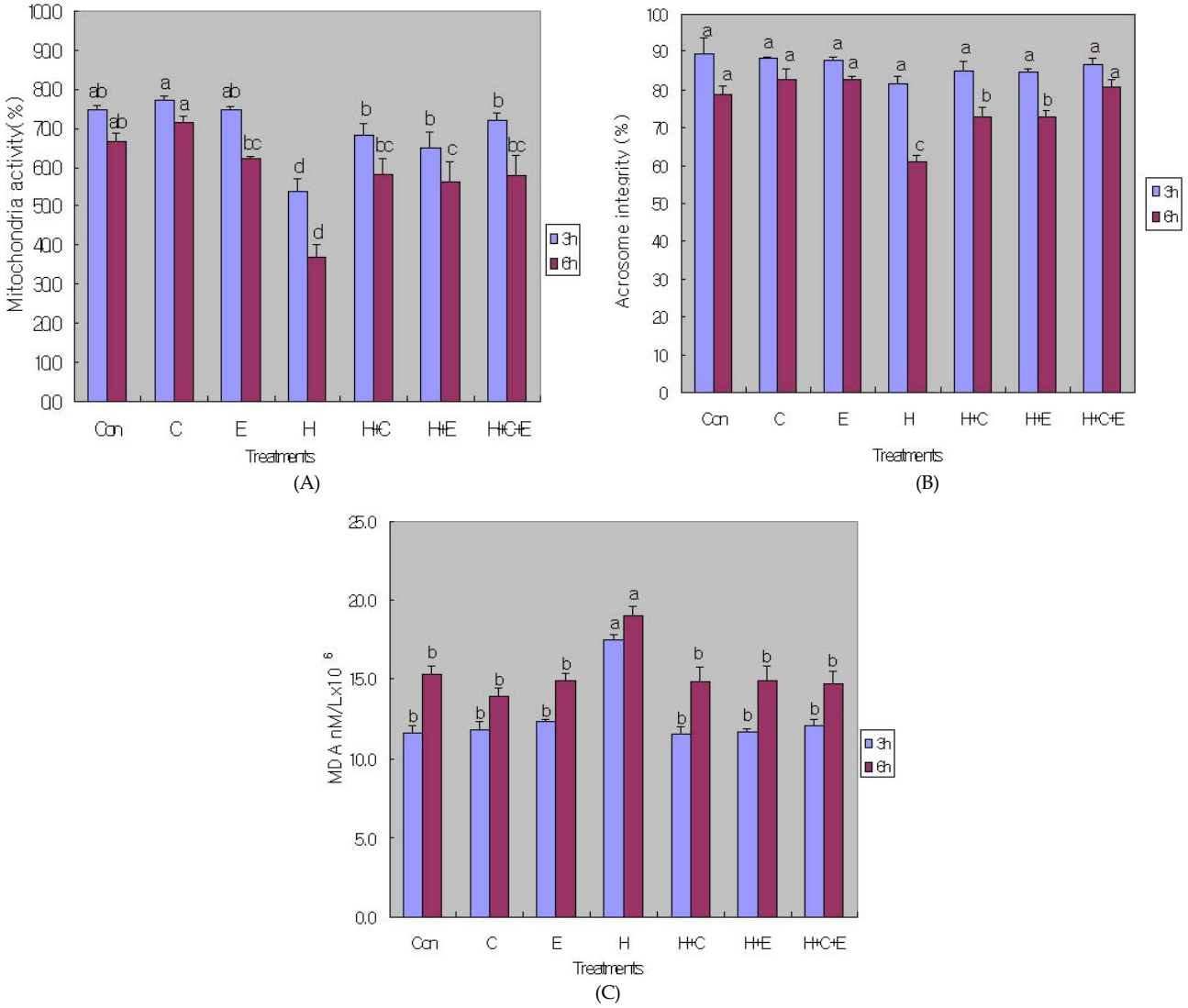

Fig. 2. Effect of curcumin and Vit. E on boar sperm fertility index such as A) mitochondria activity, B) acrosome integrity and C) lipid peroxidation of permatozoal membrane during *in vitro* storage. $^{a\sim c}$ Mean values with different superscripts within same incubation times are significantly differ, $p<0.05$. Data are expressed as mean±SEM of three experiments.

thin treatments, lipid peroxidation vs other parameters irrespective of treatments were negatively correlated and between motility, membrane integrity, viability, survival rates, mitochondria activity and acrosome integrity were positively correlated in all treatments.

DISCUSSION

This study was conducted to examine whether curcumin or Vit. E acted alone or synergetically on boar sperm characteristics during *in vitro* storage as an antioxidant through scavenging ROS.

The generation of ROS has a detrimental effect on sperm quality and function during *in vitro* storage or cryopreservation and also is implicated in the induction of apoptotic cell death (Kumareskan *et al.*, 2009). The boar sperm expose to high concentration of oxygen, which will increase ROS production (Guthrie *et al.*, 2008) and LPO (Cerolini *et al.*, 2000; Gro β feld *et al.*, 2008), resulting in DNA damage, decreased chromatin quality, decline of motility and fertility (Chen *et al.*, 1997). In order to scavenge ROS and reduce their damaging action under normal physiological conditions, a complex antioxidant defence system against free radicals is present in sperm and seminal plasma. But antioxidant system of the spermatozoa is not potent enough to prevent ROS and LPO completely, especially during *in vitro* storage or cryopreservation when the production of free

Table 2. Correlation coefficients among various evaluation methods on sperm characteristics of boar semen preserved in semen extender containing curcumin or Vit. E in the presence or absence of hydrogen peroxide

Treatments		Motility	MI	Viability	SR	MA	AI	LPO
	Motility	-	0.79*	0.67	0.63	0.79*	0.55	−0.67
	MI	0.79*	-	0.73*	0.67	0.59	0.45	−0.58
	Viability	0.67	0.73*	-	0.88**	0.74*	0.74*	−0.90**
Con	SR	0.63	0.67	0.88**	-	0.70	0.69	−0.86**
	MA	0.79*	0.59	0.74*	0.70	-	0.41	−0.64
	AI	0.55	0.45	0.74*	0.69	0.41	-	−0.91**
	LPO	−0.67	−0.58	−0.90**	−0.86**	−0.64	−0.91**	-
	Motility	-	0.64	0.56	0.18	0.03	0.34	−0.49
	MI	0.64	-	0.83*	0.52	0.22	0.24	−0.59
	Viability	0.56	0.83*	-	0.86**	0.68	0.66	−0.74*
C	SR	0.18	0.52	0.86**	-	0.88**	0.64	−0.71*
	MA	0.03	0.22	0.68	0.88**	-	0.68	−0.59
	AI	0.34	0.24	0.66	0.64	0.68	-	−0.59
	LPO	−0.49	−0.59	−0.74*	−0.71*	−0.59	−0.59	-
	Motility	-	0.83*	0.79*	0.68	0.79*	0.71*	−0.70
	MI	0.83*	-	0.90**	0.58	0.69	0.70	−0.85**
	Viability	0.79*	0.90**	-	0.79*	0.84**	0.75*	−0.91**
E	SR	0.68	0.58	0.79*	-	0.88**	0.87**	−0.83*
	MA	0.79*	0.69	0.84**	0.88**	-	0.88**	−0.87**
	AI	0.71*	0.70	0.75*	0.87**	0.88**	-	−0.92**
	LPO	−0.70	−0.85**	−0.91**	−0.83*	−0.87**	−0.92**	-
	Motility	-	0.78*	0.97**	0.98**	0.89**	0.97**	−0.81*
	MI	0.78*	-	0.69	0.68	0.95**	0.80*	−0.89**
	Viability	0.97**	0.69	-	0.97**	0.81*	0.97**	−0.72*
H	SR	0.98 **	0.68	0.97**	-	0.83*	0.93**	−0.73*
	MA	0.89	0.95**	0.81*	0.83*	-	0.89**	−0.89**
	AI	0.97**	0.80*	0.97**	0.93**	0.89**	-	−0.82*
	LPO	−0.81*	−0.89**	−0.72*	−0.73*	−0.89**	−0.82*	-
	Motility	-	0.82*	0.97**	0.84**	0.71*	0.91**	−0.86**
	MI	0.82*	-	0.75*	0.71*	0.42	0.80*	−0.53
	Viability	0.97**	0.75*	-	0.79*	0.67	0.94**	−0.86**
H+C	SR	0.84**	0.71*	0.79*	-	0.38	0.72*	−0.76*
	MA	0.71*	0.42	0.67	0.38	-	0.44	−0.76*
	AI	0.91**	0.80*	0.94**	0.72*	0.44	-	−0.68
	LPO	−0.86*	−0.53	−0.86**	−0.76*	−0.76*	−0.68	-
	Motility	-	0.95**	0.94**	0.91**	0.50	0.93**	−0.94**
	MI	0.95**	-	0.91**	0.83*	0.45	0.91**	−0.89**
	Viability	0.94**	0.91**	-	0.95**	0.47	0.97**	−0.80*
H+E	SR	0.91**	0.83*	0.95**	-	0.45	0.93**	−0.76*
	MA	0.50	0.45	0.47	0.45	-	0.34	−0.30
	AI	0.93**	0.91**	0.97**	0.93**	0.34	-	−0.84
	LPO	−0.94**	−0.89**	−0.80*	−0.76*	−0.30	−0.84	-
	Motility	-	0.85**	0.94**	0.96**	0.75*	0.72*	−0.86**
	MI	0.85**	-	0.71*	0.90**	0.52	0.66	−0.92**
	Viability	0.94**	0.71*	-	0.91**	0.72*	0.87**	−0.79*
H+C+E	SR	0.96**	0.90**	0.91**	-	0.66	0.76*	−0.90**
	MA	0.75*	0.52	0.72*	0.66	-	0.55	−0.47
	AI	0.72*	0.66	0.87**	0.76*	0.55	-	−0.77*
	LPO	−0.86**	−0.92**	−0.79*	−0.90**	−0.47	−0.77*	-

MI; Membrane integrity, SR; Survival rate, MA; Mitochondria activity, AI; Acrosome integrity, LPO; Lipid peroxidation.
Singnification level *p<0.05, **p<0.01.

radicals could be significantly enhanced as a result of metabolic change (Tavilani *et al.,* 2008). Therefore, addition of ROS scavenger as a antioxidants into semen extenders attenuate the oxidative stress and had positive effects on boar sperm motility and mitochondria membrane potential during sperm preservation in *in vitro* or cryopreservation (Pena *et al.,* 2003).

Curcumin is the active component of turmeric (diferuloyl-methane). Tumeric and its active ingredient curcumin have been shown to scavenge free radicals, thereby acting as good antioxidants. It was shown to be a potent scavenger of a variety of ROS including hydroxyl radicals and nitrogen oxides radicals. Therefore, curcumin may play a role in reducing detrimental effects on sperm function (Mathuria and Verma, 2008).

Vit. E can break the covalent links that ROS have formed between fatty acids side chains in membrane lipids. This result indicates that Vit. E plays an important role in reducing membrane damage caused by excessive ROS production during *in vitro* storage or cryopreservation. Vit. E supplementation into semen extender during *in vitro* storage significantly improved sperm motility, progressive motility, membrane integrity, and DNA integrity of cat spermatozoa (Thuwamut *et al.,* 2008). In boar semen, Vit. E supplementation provided protection to sperm viability and was able to inhibit lipid peroxidation (Corelini *et al.,* 2000).

In the present study, supplementation of curcumin (500 uM) or Vit. E (200 uM) into semen extender improved alone or synergetically the semen quality during *in vitro* storage. The sperm viability index such as motility, membrane integrity, viability and survival rates in control, antioxidants (curcumin, Vit. E), antioxidant plus H_2O_2, and curcumin +Vit. E+H_2O_2 group significantly increased than in H_2O_2 group, which is artificially induced hydrogen peroxide radical, irrespective of incubation periods (p<0.05). But the sperm viability index in curcumin and Vit. E groups did not significantly increased than those of curcumin +H_2O_2, Vit. E +H_2O_2 groups. We also evaluated mitochondrial function, acrosomal change and lipid peroxidation as a fertility index in spermatozoa. It is considered that those factors highly related to fertilizing ability. Mitochondrial activity and acrosome integrity did not significantly difference among all treatment groups excepts for H_2O_2 alone. But the mitochondrial activity and acrosome integrity in curcumin and Vit. E supplementation slightly improved, suggesting that both shown alone and collaboratively scavenging action against H_2O_2. Lipid peroxidation of spermatozoa in all experimantal groups excepts for H_2O_2 group increased according to incubation periods, but there were no significantly difference (p>0.05).

Results of the present study were consistent with the previous observations that curcumin have inhibited aflatoxin - induced toxicity in mice spermatozoa. Aflatoxin - treated mice had been shown a decreasing sperm viability and motility, which could be due to reduced mitochondrial function and/or decreased ATP and ATPase activity (Mathuria and Verma, 2008) and Vit. E supplementation to the freezing medium prevented oxidative damage and thus improved sperm characteristics (Breininger *et al.,* 2005).

The mechanism of action of curcumin and Vit. E in protection of boar sperm suggested to be related to inhibit ROS produced in semen extender during *in vitro* storage. Thus, curcumin and Vit. E has proved to have a highly ameliorative effect against H_2O_2 induced - toxicity in boar sperm (Eybl *et al.,* 2006; Mathuria and Verma, 2008; Pena *et al.,* 2003). The results of our study indicated that curcumin and Vit. E supplementation improved on sperm quality during *in vitro* storage through showing their efficiency in ameliorating oxidative stress. But Vit. E is more effective in protecting boar spermatozoa from oxidative stress in comparison to curcumin. This results is consistent with Sahoo *et al.* (2008) reported that both curcumin and Vit. E are cooperatively efficient in protecting testis from oxidative stress generated by T4.

To confirm the correlative effect of incubation periods and treatments on sperm characteristics, we analyzed the results in each evaluation methods by ANOVA. There were significantly difference between incubation periods or sperm treatment (p<0.0001), but sperm motility among evaluation methods was only significantly difference (p<0.0001). To validate the accuracy among sperm evaluation methods, the correlations of semen evaluation methods in each treatments were analyzed in this study. According to our results a positive correlation were observed between evaluation methods such as motility, membrane integrity, viability, survival rates, mitochondrial activity and acrosome integrity, but LPO vs the other parameters was shown to have a negative correlation. Our results were consistent with the results of Cerolini *et al.* (2000), who reported that the decrease in sperm motility and viability after *in vitro* storage was induced by an increase in the LPO in boar sperm.

In conlclusion, curcumin and Vit. E have a scavenging action against H_2O_2 toxicity when boar sperm are stored in *in vitro*.

Curcumin and Vit. E are not only having an antioxidant feature but also both are a synergistic effects on sperm characteristics during *in vitro* storage.

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