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Characteristics of Phthalate Esters-exposed Boar Sperm during Boar Semen Storage

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Phthalate is a chemical endocrine disrupter and interfere with the action of hormones, estrogens, androgens and thyroid hormones. It also affect cardiovascular, metabolic, immune and reproductive system in the human and animals. Curcumin is antioxidant, anti-inflammatory activity and -cancer properties in the human. We studied whether phthalates damage viability, mitochondrial activity and membrane integrity of sperm in boar semen. We also treated curcumin with/without phthalates in the boar semen. Fresh boar semen was treated with phthalates and/or curcumin for examining sperm characteristics. Sperm characteristics, sperm motility, viability, mitochondrial activity, and membrane integrity were determined during storage of boar semen. Sperm motility and viability in dose-dependent manner decreased by di-n-butyl phthalate (DBP), mono-n-butyl phthalate (MBP) and di-2-ethylhexyl phthalate (DEHP, $p<0.05$). Phthalates also decreased mitochondrial activity and membrane integrity of sperm ($p<0.05$). However, sperm motility and viability were higher than untreated-curcumin when DBP, MBP and DEHP treated with a curcumin in boar semen ($p<0.05$). Mitochondrial activity and membrane integrity of sperm were higher in DBP- and MBP-treated semen with curcumin ($p<0.05$). In conclusion, phthalates can damage sperm viability and quality during the boar semen storage, and curcumin may protect the boar sperms from phthalates during storage term.

Key words : Boar semen, phthalate, spermatozoa

Introduction

Phthalate esters used for imparting the flexibility and durability, when producing plastic products [12, 36]. Di-2-ethylhexyl phthalate (DEHP), di-n-butyl phthalate (DBP), dimethyl phthalate (DMP) and mono-n-butyl phthalate (MBP) are phthalate esters. They commonly used in modern life [34, 41]. Briefly, phthalates are used as plasticizers of polyvinyl chloride materials, dietary supplements, flooring, roofing, carpeting, packaging equipment, automotive parts, medical equipment, toys [13].

Moreover, phthalate is an endocrine disrupter, endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, or population [2, 4, 6, 15, 23, 36]. Endocrine disruptors are chemicals that can interfere with action of hormones, estrogens, androgens and thyroid hormones, and affect cardiovascular, metabolic, immune and reproductive system [33]. Especially, it is possible to affect the reproductive system and sexual development [22]. Reduction of testicular weight and seminiferous tubule atrophy were induced by phthalates in males, and increased DNA damage on sperm [32]. Phthalate also induces premature, breast development, shortened pregnancy in females [32]. Therefore, phthalate exposed-sperm has a harmful influence on characteristics and viability of sperm.

Additionally, a curcumin (1,7-bis(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione) is the yellow substance contained in the curry or turmeric (curcuma longa). In recent studies, it has been shown that curcumin has various biological activities [38]. Curcumin is an antioxidant, which has anti-inflammatory activity and -cancer properties in the human [1, 8, 16, 40]. Oguzturk *et al*. and Lonare *et al*. reported that curcumin protects from cadmium and imidacloprid in mice [21, 26]. Also, curcumin eliminated 2,3,7,8-tetracholorodibenzo-p-dioxin-induced oxidative stress in rat liver [5]. The above results indicate that curcumin is a potential factor in reproductive function and infertility in pigs. Thus, we studied whether the phthalate damages sperm viability and

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quality in bore semen, also we have tested curcumin can protect sperm from phthalate.

Material and Methods

Reagents

DBP, DEHP and curcumin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). MBP was purchased from Tokyo Chemical Company (Chuo-ku, Tokyo, Japan).

Preparation of semen and treatments

Semen was collected from five pigs by gloved-hand technique at the local AI center (Wonju, Kangwon, South Korea). After collection, semen was diluted to concentration of 1.0×10^7 sperm in 1 ml Modena extender (Table 1). Then semen was transported to the laboratory at 17℃ within 2 hr [9, 20]. The semen was incubated with phthalates (50, 100, and 200 μM) and/or curcumin (5 and 10 μM) for 3, 6, and 9 hr at 37℃. Sperm samples were also treated phthalate (50 μM) with/without curcumin (5 μM) for 3 hr. Then, sperm motility, viability, mitochondria activity and membrane integrity were evaluated (n=5).

Motility and viability of sperm

The motility and viability of spermatozoa were evaluated after treatment with DBP, MBP, DEHP, and/or curcumin for 3, 6, and 9 hr. 20 μl of sperm samples (1.0×10^6 /ml) were placed on pre-warmed slide glass, then sperm progressive motility was analyzed using a phase contrast microscope (Nikon Eclipse TE300, Tokyo, Japan). Hoechst 33342 (HO) / propidium iodide (PI) staining method used for measuring sperm viability. 100 μl sample was stained with 10 μl PI (0.5 mg/ml in PBS), and incubated for 5 min at 37℃, and treated 10 μL HO (0.5 mg/ml) for 10 min at 37℃ in the dark. After incubation, 20 μl of sperm sample was placed on slide glass, and observed under an epifluorescence microscope at 400× magnification (Ziess Axioskop, Jena, Germany). The fluorescence spectra were 460/500 nm for HO, and PI emits at more than 600 nm.

Table 1. Composition of Modena extender

Compound	Original solution (mM)	Modified solution (mM)
Glucose (monohydrate)	138.75	-
Glucose (anhydrate)	-	152.61
Sodium citrate	23.46	23.46
Sodium bicarbonate	11.90	11.90
EDTA, disodium salt	6.99	6.99
Tris	446.66	46.66
Citric acid	15.10	15.10
Gentamicin	-	10 mg/l

Sperm membrane integrity

The plasma membrane integrity was evaluated using hypoosmotic swelling test (HOST). 100 μl sperm sample was mixed with 1 ml hypo-osmotic solution (150 mOsm, 7.35 g Na-citrate and 13.51 g fructose in 1 l water), and incubated for 30 min at 37℃. Finally, 20 μl sample was placed on a glass slide, and covered with a cover glass. The swell of sperm tails was observed under a phase contrast microscope at 400× magnification (Nikon Eclipse TE300, Tokyo, Japan).

Mitochondrial activity in sperm

Mitochondrial activity was assessed by Rhodamin123 (R123) staining. Briefly, 3 μl of R123 (0.1 mg/ml) was added in 1 ml sperm sample, and stained for 15 min at 37℃ in dark room. After incubation, the sample was centrifuged at 1,500× g for 5 min, then diluted with 1 ml PBS, and then mixed with 10 μl PI for 10 min. After incubation, 20 μl of sperm sample was placed a slide glass, and covered with a glass coverslip. Samples were evaluated by epifluorescence microscope at 400× magnification (Ziess Axioskop, Jena, Germany). R123 and PI were measured in 490/515 and 545/590 nm, respectively. Mitochondrial activity is determined on the mid-piece region of sperm.

Statistical analysis

The data was analyzed using a Statistical Analysis System software version 9.2 (SAS Institute Inc. USA). Treatment groups were compared for differences using of Duncan's modified multiple range tests. All values are presented as mean ± the standard effort of the mean (SEM). *P*-value of 5% was considered significant.

Results

In this study, we treated high concentration of phthalate in the boar semen, since sperm has different cell type of cells in the human. Also low concentration of phthalate did not effect on sperm characteristics.

Dose-dependent effect of phthalate on sperm motility and viability in boar semen

Table 2. Effect of phthalates on sperm motility

Treatments (μM)		Motility (%)		
		3 hr	6 hr	9 hr
Control		66.6±3.3^{a}	51.1±4.2^{a}	35.5±3.3^{a}
DBP	50	54.8±3.2^{b}	44.4±3.7^{a}	30.0±3.7^{a}
	100	52.2±2.7^{b}	32.2±2.2^{b}	20.0±2.3^{b}
	200	42.2±3.2^{c}	28.8±2.3^{b}	10.5±2.1^{c}
MBP	50	56.6±2.8^{b}	44.4±4.1^{a}	30.5±3.1^{ab}
	100	50.1±2.3^{bc}	41.1±4.2^{ab}	23.8±3.0^{b}
	200	43.3±3.3^{c}	32.2±3.2^{b}	12.7±1.4^{c}
DEHP	50	51.1±2.6^{b}	38.3±2.0^{b}	19.4±1.7^{b}
	100	40.0±2.3^{c}	28.8±2.3^{c}	11.1±1.8^{c}
	200	33.3±4.4^{c}	21.1±3.5^{c}	7.3±1.4^{c}

DBP, Di-n-butyl phthalate; MBP, monobutyl phthalate; DEHP, Di (2-ethylhexyl) phthalate. $^{a\sim c}$Values with different superscripts within same column are significantly different, $p<0.05$.

Table 3. Effect of phthalates on viability of sperm

Treatments (μM)		Viability (%)		
		3 hr	6 hr	9 hr
Control		64.0±3.0^{a}	48.3±3.3^{a}	39.8±1.8^{a}
DBP	50	52.6±4.1^{b}	39.2±2.6^{b}	31.3±2.2^{b}
	100	45.4±2.5^{bc}	35.2±1.6^{b}	28.6±1.6^{b}
	200	42.3±1.9^{c}	33.0±1.6^{b}	28.2±1.7^{b}
MBP	50	48.8±6.1^{b}	42.7±3.5^{ab}	36.0±2.9^{a}
	100	44.7±1.9^{c}	34.3±2.4^{bc}	27.4±1.5^{b}
	200	38.0±0.9^{c}	31.7±2.4^{c}	24.5±1.2^{b}
DEHP	50	54.3±3.6^{b}	43.2±4.0^{a}	37.5±3.0^{a}
	100	40.7±0.7^{bc}	35.0±2.2^{b}	31.7±2.2^{b}
	200	37.1±0.9^{c}	28.8±1.8^{b}	23.7±1.4^{b}

DBP, Di-n-butyl phthalate; MBP, monobutyl phthalate; DEHP, Di (2-ethylhexyl) phthalate. $^{a\sim c}$Values with different superscripts within same column are significantly different, $p<0.05$.

Phthalate reduced the motility and viability of sperm in a dose-dependent manner (Table 2 and Table 3). 50 μM of DBP, MBP, and DEHP were found to significantly decrease the motility and viability of sperm for 3, 6, and 9 hr ($p<0.05$). Thus, we used 50 μM phthalate concentration in the study.

Dose-dependent effect of phthalate on sperm mitochondrial activity and membrane integrity in boar semen

DBP, MBP, and DEHP decreased sperm mitochondrial activity and membrane integrity in boar semen (Table 4 and Table 5). 50 μM of phthalate significantly inhibited mitochondrial activity of sperm at 3, 6, and 9 hr (Table 4, $p<0.05$). Membrane integrity was decreased by phthalate at all concentrations (50, 100, and 200 μM), and all incubation times (Table 5, $p<0.05$).

Table 4. Effect of phthalates on sperm mitochondrial activity

Treatments (μM)		Mitochondrial activity (%)		
		3 hr	6 hr	9 hr
Control		64.6±2.3^{a}	51.5±3.7^{a}	43.1±2.8^{a}
DBP	50	56.4±1.9^{b}	50.6±4.2^{a}	41.2±2.5^{a}
	100	51.1±2.0^{bc}	43.7±3.3^{ab}	31.4±3.1^{b}
	200	45.5±1.6^{c}	35.0±2.6^{b}	25.2±3.7^{b}
MBP	50	55.8±1.4^{b}	50.0±2.1^{a}	43.8±3.8^{a}
	100	52.4±2.7^{bc}	42.4±2.6^{ab}	33.3±3.0^{b}
	200	44.8±3.7^{c}	35.3±3.9^{b}	26.6±3.3^{b}
DEHP	50	53.6±3.2^{b}	41.1±2.6^{b}	33.2±2.7^{b}
	100	44.7±3.3^{bc}	34.5±2.8^{b}	27.1±2.0^{b}
	200	35.6±4.1^{c}	25.2±2.6^{c}	15.2±1.7^{c}

DBP, Di-n-butyl phthalate; MBP, monobutyl phthalate; DEHP, Di (2-ethylhexyl) phthalate. $^{a\sim c}$Values with different superscripts within same column are significantly different, $p<0.05$.

Table 5. Effect of phthalates on membrane integrity of sperm

Treatments (μM)		Membrane integrity (%)		
		3 hr	6 hr	9 hr
Control		30.4±2.7^{a}	18.7±1.8^{a}	12.8±1.0^{a}
DBP	50	18.8±1.4^{b}	9.4±1.5^{b}	7.3±0.7^{b}
	100	14.8±1.1^{b}	9.7±1.5^{b}	5.4±0.8^{b}
	200	18.7±2.5^{b}	10.6±1.3^{b}	6.5±0.9^{b}
MBP	50	15.0±1.7^{b}	10.4±1.5^{b}	8.7±1.0^{b}
	100	12.8±1.7^{b}	7.5±0.7^{b}	5.1±0.8^{b}
	200	15.0±1.7^{b}	9.3±1.0^{b}	5.5±0.6^{b}
DEHP	50	13.5±1.5^{b}	8.1±1.2^{b}	6.4±1.0^{b}
	100	11.2±0.9^{b}	6.2±1.1^{b}	3.4±0.8^{b}
	200	15.8±2.3^{b}	9.3±1.3^{b}	6.1±0.9^{b}

DBP, Di-n-butyl phthalate; MBP, monobutyl phthalate; DEHP, Di (2-ethylhexyl) phthalate. $^{a\sim b}$Values with different superscripts within same column are significantly different, $p<0.05$.

Effect of curcumin, DBP, MBP and DEHP on sperm motility and viability in phthalate-treated semen

Sperm motility was increased at 5 μM curcumin for 3 and 9 hr (Fig. 1A, $p<0.05$), but not at higher concentration (10 μM). Viability of sperm was also increased at 5 μM curcumin for 3 hr (Fig. 2A, $p<0.05$). Therefore, a concentration of 5 μM of curcumin was used in the next experiments. Sperm motility increased in curcumin-treated samples with DBP, MBP and DEHP (Fig. 1B, $p<0.05$). Sperm viability also was higher in curcumin-treated sperm (Fig. 2B, $p<0.05$).

Effect of curcumin, DBP, MBP and DEHP on mitochondrial activity and membrane integrity in phthalate-treated semen

Mitochondrial activity and membrane integrity increased by curcumin (Fig. 3A and Fig. 4A, $p<0.05$). The mitochon-

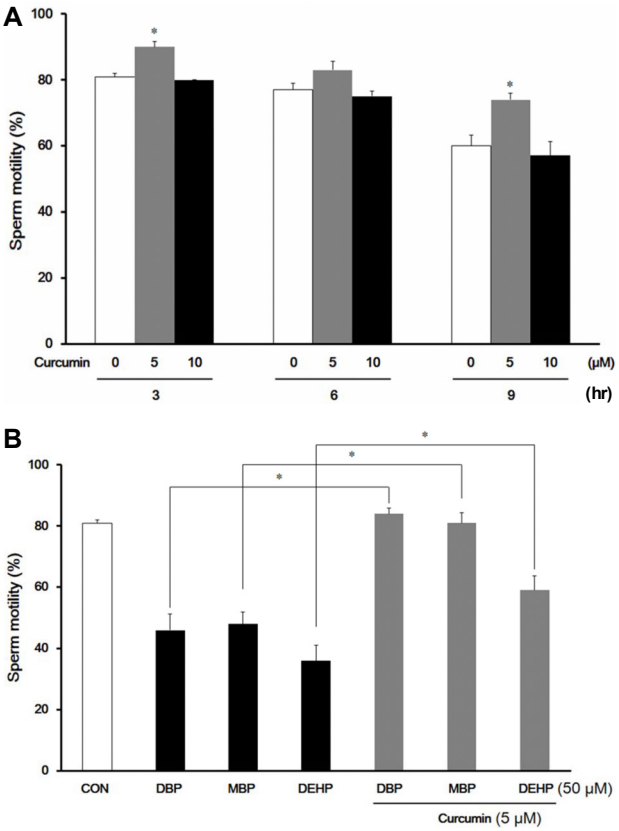

Fig. 1. Effect of DBP, MBP, DEHP (50 μM) and/or curcumin (5 μM) on sperm motility in boar semen. Asterisks indicate significant differences compared with phthalate-treated sperm. Values represented as means ± SEM (p<0.05).

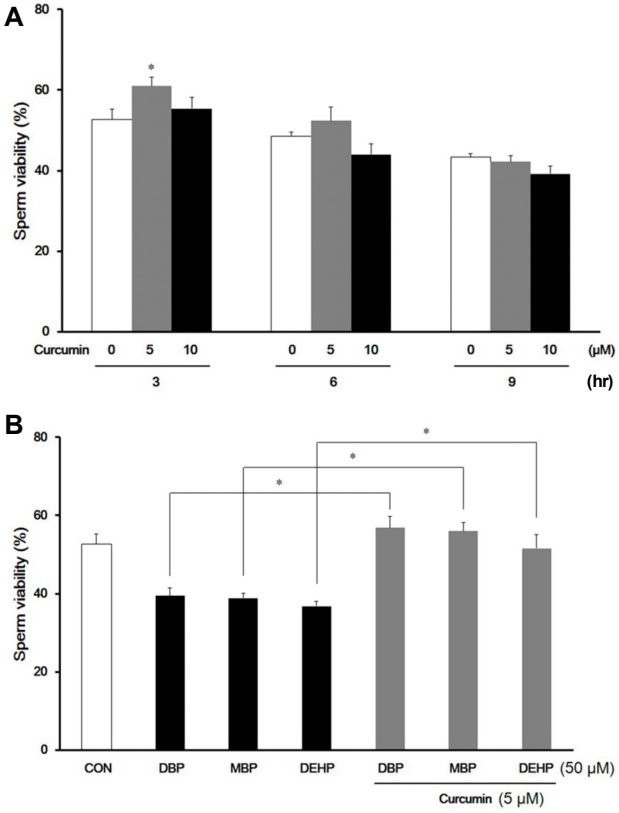

Fig. 2. Effect of DBP, MBP, DEHP (50 μM) and/or curcumin (5 μM) on viability of sperm in boar semen. Asterisks indicate significant differences compared with phthalate-treated sample. Values represented as means ± SEM (p<0.05).

drial activity and membrane integrity of sperm were higher in phthalate-treated sperm with curcumin (Fig. 3B and Fig. 4B, p<0.05), but DEHP-treated sperm was not affected by curcumin.

Discussion

Phthalate known to induce metabolic dysfunction and oxidative stress, and reactive oxygen species production by phthalate in the cells is rapidly increased [7, 28]. Also reactive oxygen species formation causes DNA damage, lipid peroxidation, protein denaturation, and apoptosis. Moreover, oxidative stress by reactive oxygen species induced the infertility of male [18]. Endocrine disruptors, bisphenol A, methoxychlor, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and genistein, are very important play in the infertility of male [27], and chemical or synthetic function has harmful effects in normal hormone mechanisms [36]. Therefore, antioxidant is an important factor for protecting cells from oxidative damage [11, 25]. Thus, we investigated whether phthalate can damage sperm characteristics as endocrine disruptor. Sperm motility, viability, membrane integrity and mitochondrial activity were inhibited by phthalates, suggesting phthalate is can trigger the infertility of male in mammals, and may play an endocrine disruptor in boar sperm.

In this study, curcumin enhanced sperm characteristics, motility, viability, mitochondrial activity, and membrane integrity of boar sperm. We showed time-dependent effects of curcumin, and found that a low concentration of curcumin elevated the quality of sperm, but not a high concentration. Some other studies reported that sperm motility decreased by a high concentration of curcumin, indicating curcumin plays a selective immobilizing effect in sperm [25, 29]. Also Tvrada *et al*. (2015) reported that curcumin could be beneficial for a complex enhancement of bovine spermatozoa activity and protection against complications resulting from in vitro culture experiments [39]. Moreover, curcumin can use a supplementation to medium for boar semen cry-

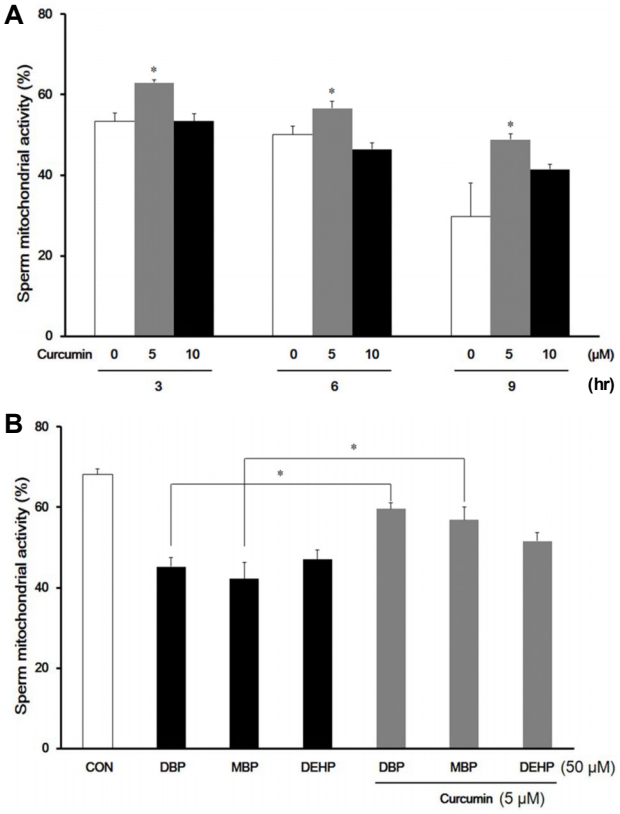

Fig. 3. Effect of DBP, MBP, DEHP (50 μM) and/or curcumin (5 μM) on mitochondrial activity in boar semen. Asterisks indicate significant differences compared with phthalate-treated sample (p<0.05).

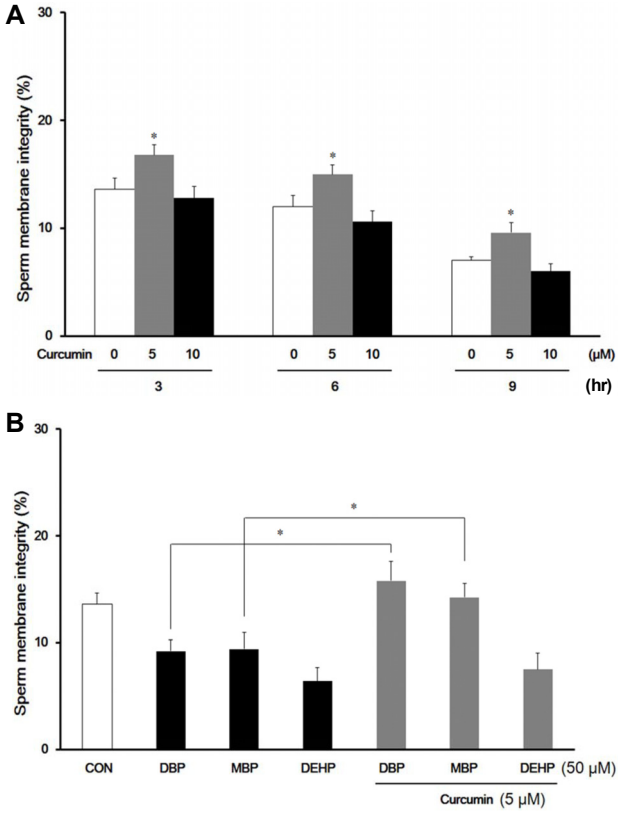

Fig. 4. Effect of DBP, MBP, DEHP (50 μM) and/or curcumin (5 μM) on membrane integrity of sperm in boar semen. Asterisks indicate significant differences compared with phthalate-treated sperm. Values represented as means ± SEM (p<0.05).

opreservation [2]. Thus, we used a low concentration of curcumin for protecting sperm damage from phthalate.

In fact, studies about the effect of curcumin on cell damage has been reported in various fields, diabetes, inflammation, neurodegenerative diseases, and testicular tissue and tubule tissues in human and animal [2, 14]. However, the sperm cell damaged by phthalate has not studied. Thus, we thought that curcumin can inhibit the function of phthalate, and protect sperm cell. Since phthalate induced sperm damage in this study, we need to investigate the function of curcumin in phthalate-damaged boar sperm.

For improving sperm quality, the motility and viability of spermatozoa are potential in pigs. Our results showed that curcumin protects sperm cells from exposure of phthalate, suggesting curcumin can interrupt a play of phthalate in boar sperm. However, some studies indicated that high concentration of curcumin causes a damage of sperm [19]. Therefore, a low concentration of curcumin is optimal for enhancing sperm characteristics in pigs. Moreover, mitochondria activity is an important factor for moving sperm. Generally, the energy of movement of the sperm is adenosine triphosphate, it is generated in the mitochondria. Therefore, the mitochondria activity in the sperm motility is regulated [31]. Our result showed that mitochondria activity increased in curcumin-treated sperm with phthalate than un-treated curcumin. Muthumani *et al* reported that curcumin reduces mitochondrial toxicity by arsenic [24]. Also curcumin improves a mitochondrial function [42]. These results suggest that curcumin stimulates the mitochondrial activity, protects viability and motility of sperm from phthalates damage.

Moreover, we determined the membrane integrity of sperm in pigs. The membrane is a major organelle which border distinguishing the internal and external of the cell, and plays a role of substance transporter and signal transfer. Lipid component of mammalian sperm membrane contains a high unsaturated-fatty acid including phospholipids than somatic cell, and reactive oxygen species and free radical are particularly stimulated and induced [17, 30]. The mem-

brane integrity is an important play for improving fertility via acrosome reaction [35]. Thus, we examined that sperm membrane integrity in phthalate- and curcumin-treated boar sperm samples. The membrane integrity of sperm was significantly increased in curcumin-treated sperm with phthalate, our result suggests that curcumin inhibits the damage of cell membrane via phthalate, as well as antioxidant.

In conclusion, phthalates can damage the viability and quality of sperm during semen storage, and curcumin will protect the spermatozoa from phthalates. Therefore, curcumin may play an anti-phthalate activator in boar semen.

Ethics approval

All procedures involving the use of animal experiments were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139).

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초록 : 돼지 정액을 보관하는 동안 phthalate esters에 노출된 정자의 특성

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Phthalate는 내분비 교란물질로 호르몬의 변화, 에스토젠, 앤드로젠, 갑상선 호르몬의 분비를 방해한다. 또한, 인간과 동물에서 심혈관질환, 대사작용, 면역 및 번식체계에 영향을 끼친다. Curcumin은 항산화물질로 항염증 활성 및 항암작용에 영향을 미치는 것으로 알려져 있다. 본 연구는 phthalate가 정자의 운동성, 생존율, 미토콘드리아 활성 및 세포막 기능에 미치는 영향을 알아보고자 실시하였다. 또한, curcumin을 처리하여 phthalate에 노출된 정자에 미치는 영향을 분석하였다. 정자의 운동성과 생존율은 di-n-butyl phthalate (DBP), mono-n-butyl phthalate (MBP) 및 di-2-ethylhexyl phthalate (DEHP)을 처리하였을 때 감소하였다(p<0.05). Phthalate는 정자의 미토콘드리아 활성 및 세포막의 기능을 감소시켰다(p<0.05). 그러나, 정자의 운동성과 생존율은 curcumin을 처리하지 않은 것보다 처리한 정자에서 높게 나타났으며(p<0.05), 정자의 미토콘드리아 활성 및 세포막 기능에서도 높게 나타났다(p<0.05). 결론적으로, phthalate는 정자의 생존율과 세포의 기능에 영향을 미칠 수 있고, 이로부터 세포의 기능을 보호하기 위해서는 curcumin의 처리가 필요 할 것으로 생각된다.