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Effects of Endocrine Disruptors (NP, DBP and BPA) on Sperm Characteristics and Development of *IVF* Embryos in Pig

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ABSTRACT

This study was to examine single or combined *in vitro* effects of environmental endocrine disruptors on boar sperm characteristics, oxidative stress damage in sperm and development of porcine *IVF* embryos. Addition of various concentration of NP (10, 20, 30μM), DBP (10, 50, 100 μM) and BPA (1, 5 or 10 μg/ml) on boar sperm characteristics such as percentages of sperm motility, viability, membrane integrity and mitochondrial activity were dose-dependently decreased within 3, 6 or 9 hr incubation period ($p<0.05$). The overall detrimental effects increased with incubation time increasement. NP, DBP and BPA showed the detrimental effects on sperm membrane and mitochondria of energy production organelles affecting cell viability with the dependancy of dose and incubation time.

In combination effects, NP (10 μM) + DBP (10 μM) significantly decreased boar general sperm characteristics for 3 or 6 hr incubation period compared with control ($p<0.05$). When both of NP and DBP concentrations (NP; 30 μM, DBP; 100 μM) increase, the detrimental effects on sperm characteristics were larger than those of low concentration combination ($p<0.05$). The inhibitory effects of NP (30 μM) + BPA (10 μg/ml) on sperm characteristics were larger than those of NP (10 μM) + BPA (1 μg/ml) ($p<0.05$). DBP (100 μM) + BPA (10 μg/ml) decreased sperm characteristics compared with the low concentration combination (DBP 10 μM + BPA 1 μg/ml, $p<0.05$). This result indicates the detrimental effects of both chemicals on sperm characteristics were dose dependent. Addition of NP (30 μM) + DBP (100 μM), NP (30 μM) + BPA (10 μg/ml), DBP (10 μM) + BPA (1 μg/ml) or DBP (100 μM) + BPA (10 μg/ml) significantly increased lipid peroxidation for 3 or 6 hr incubation period ($p<0.05$) compared with no addition control.

NP ($\geq$ 20 μM) decreased the percentages of *IVF* embryo development from morulae and blastocyst stages ($p<0.05$) and its detrimental effects were dose-dependant. BPA 0, 1, 5 or 10 μg/ml decreased significantly and dose-dependently the percentage of morulae plus and blastocysts ($p<0.05$). Combinations of DBP (100 μM) plus NP (30 μM) and DBP (100 μM) plus BPA (10 μg/ml) did not affect on morulae and blastocyst development, but NP (30 μM) plus BPA (10 μg/ml) has significant detrimental effect on embryo development at these stages ($p<0.05$). These overall results indicate that the partial detrimental effects on boar sperm characteristics and embryo development by NP, DBP, BPA or the combination of these chemicals might be due to the increasement of lipid peroxidation and free radical formation in the cell and there were no specific interaction effects on boar sperm and embryo degeneration among the combined treatments.

(**Key words :** NP. DBP, BPA, Endocrine disruptors, Sperm characteristics, *IVF* embryo development)

INTRODUCTION

Recently, the public and the scientific community have had the increasing concerns about endocrine disrupting effects by detrimental chemicals which are widely present in environment as results of industrial pollution. Endocrine disrupting chemicals known as endocrine disruptors interfere with synthesis, function, storage and metabolism of various hormones, resulting in disruption of the endocrine system of human and animal. The endocrine disruptors are easily and broadly exposed not only via contaminated foods and drinking water but also dermal absorption or inhalation of the pollutants (Bolt et al., 2001).

Endocrine disruptors alter steroidogenesis and mimic or antagonize the effects of natural hormones by binding to natural hormone's receptors (Uguz et al., 2009; Petro et al., 2012). Xeno-estrogens having estrogenic activity in environment have been shown to affect reproduction in wild animals and have adverse effects on human. These findings have sharply raised public concerns on human and animal health and reproduction.

NP (nonyl phenol), DBP (di-n-butyl phthalate) and BPA (bisphenol A) are multifunctional compounds used in a variety of commercial and industrial products and are known

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as the endocrine disruptors (Herath et al., 2005; Li et al., 2010; Xu et al., 2013). NP and BPA have estrogenic activity while DBP has anti-androgenic activity (Tinwell and Ashby, 2004; Zhang et al., 2004). Among endocrine disrupters, NP and DBP are commonly found in water environment and seriously threaten human and animal health and reproduction (Ying et al., 2002; Zafra-Gomez et al., 2008; Li et al., 2010). NP is widely used as lubricating oil additives, plasticizers and surface-active agents. NP is one of APE (alkyophenolpolyethoxylates) derivatives and has been shown to stay biologically active form for longer period than endogenous estrogens in the body. APE derivatives are nonylphenol, octylphenol and butylphenol, and frequently found in fresh fruits and vegetables, human breast milk, animal products, rice and contaminated drinking water (Guenther et al., 2002; Yang and Ding 2005; Lu et al., 2007).

BPA is a diphenyl compound containing two hydroxyl groups, having a estrogen property and structural similarity to synthetic estrogen, diethylstilbestrol. BPA can mimic estradiol action in inducing vaginal coalification, uterine vascular permeability, growth and differentiation of the mammary gland and c-fos gene expression in the female reproductive tracts. NP and BPA can reduce function of male gametogenic and accessory reproductive organs, alter the release of pituitary and gonadal hormones, and disrupt sperm production in adult male rates (Boockfor and Blake, 1997; Howdeshell et al., 1999; Kwack et al., 2002).

Phthalates including DEP, DEHP, DBP, DMP and DOP induce the decrease of testicular and epididymal weight, cryptorchidism, lesin intestes, leydig cell hyperplasia, deterioration of semen quality and the decrease in fertility index (Ying et al., 2002; Ichimura et al., 2003; Akingbemi et al., 2004; Fabjan et al., 2006; Rastogi, 2006). In addition, phthalate esters induce embryo toxic and teratogenic effects, reduction of anogenital distance, litter size and pup weight gain and the malformed epididymis. Unlike other anti-androgens, phthalates did not directly interact with androgen receptors but they exerted anti-androgenic effects by disrupting testosterone biosynthesis (Mylchreest et al., 1999; Gray et al., 2006). DBP is a widely distributed chemical used in a variety of industrial applications from creating plastic coating to producing cosmetics and is a well known member of the environmental endocrine disruptors (Pan et al., 2006).

In general, the exposure level of single environmental endocrine disruptor to human is low and less potent than endogenous natural hormones. Humans are often exposed to a mixture of these chemicals and the combined effects among these should be evaluated for the human exposure risks. The purpose of this study is to examine single or combined *in vitro* effects of environmental endocrine disruptors on boar sperm characteristics such as sperm motility, viability, membrane integrity and mitochondrial activity, oxidative stress damage in sperm, and development of porcine IVF embryos.

MATERIALS AND METHODS

1. Semen source and treatments

Sperm-rich fraction was collected from 1~3 purebred boar (Duruc, Yorkshire and Landrace) with more than 85% motile sperm by the grove hand method in Won Ju A.I center and transported to the laboratory within 2 hr of collection at 17 ℃. Semen washed and diluted with BTS extender, and treated with NP (10 ~ 30 μM), DBP (10 ~ 100 μM) and BPA (1 ~ 10 μg/ml) alone or mixture. Curcumin (5 μM) and H_2O_2 (100 μM) treatments was used as positive and negative control, respectively. For evaluation of semen characteristics, each treated group was examined every 3 hr interval for 9 hr incubation periods at 37℃ in high humidified condition at CO_2 incubator. All chemicals used in this study were purchased from Sigma Aldrich unless otherwise stated and were analytical grades.

2. Analysis of semen characteristics

The analysis of semen characteristics was evaluated based on the motility, viability, membrane integrity, mitochondrial activity and lipid peroxidation. All experiments were repeated at least three times with semen samples from different boars.

(1) Sperm motility

Sperm motility was subjectively assessed by visual estimations using inverted phase contrast microscope (Nikon, Japan). It was measured by determining the percentage of spermatozoa showing from wave to progressive motion.

(2) Survival rates

The sperm survival rates was examined using Hochest 33342 (HO) / propidium iodide (PI) staining method. Briefly, a

100 μl fresh semen sample was mixed with 10 μl PI (0.5 mg/ml in PBS) sol. and incubated for 5 min, and then 10 μl HO (0.5 mg/ml) was mixed and stained for another 10 min at 37℃ in the dark condition. After incubation, 10~20 μl of stained spermatozoa suspension was placed on clean slide glass, covered with a glass coverslip and evaluated at 400 × magnification by epifluorescence microscope (Ziess, Germany) equipped with excitation/barrier filter of 460/500 nm. Viable spermatozoa (live) emitted blue whereas non-viable spermatozoa (dead) emitted red.

(3) Membrane integrity

The spermatozoal plasma membrane integrity was evaluated by hypo-osmotic swelling test (HOST). The assay was performed by mixing a 50 μl semen sample with 1 ml of hypo-osmotic solution (150 mOsm, 7. 35 g Na-citrate and 13.51 g fructose in 1 liter of distilled water) and then incubated for 30 min at 37℃. Viable spermatozoa (positive) had coiled or swollen tails whereas non-viable spermatozoa (negative) had not damaged tails.

(4) Fluorescent assay of mitochondrial activity

The percentage of live spermatozoa with functional mitochondria was assessed by a dual fluorescence stain as a combination of Rhodamine 123 (R 123) and propidium iodide (PI). For this assay, 3 μl of R123 solution 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 with 10 μl of PI and incubated for 10 min at 37℃. Mitochondrial activity was examined at 400 × magnification under epifluorescence microscopy (Zeiss, Germany) equipped with excitation of 490/515 nm for R123, and 545/590 nm for PI. Sperm cells displaying only green fluorescence at the mid-piece region were considered viable spermatozoa with functional mitochondria.

(5) 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) KCl 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 lipidperoxide to malondialdehyde, lipid peroxidation was promoted using a combination of ferrous sulfate and sodium ascorbate. 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 with 250 μl of 40% trichloroacetic acid, held for 10 min at 0℃, and centrifuged (2,500 × g) for 10 min. Supernatant (1 ml) mixed with TBA were boiled with hot water for 10 min. The amount of malondialdehyde produced was quantified against a standard curve at 532 nm wave length in a spectrophotometer.

3. *In vitro* maturation (IVM), *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 hr 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. For IVF, 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 hr 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 single or mixture of NP, DBP and BPA and cultured for 6~8 days in 5% CO_2 in air at 38.5℃

4. Statistical Analysis

Statistical analysis of replicated experiment results was used for treatment comparisons and one-way analysis of variance was carried out using SAS program (SAS Institute Inc. USA). Duncan's multiple range test was used to compare the mean value of treatments. A p-value less than 0.05 were considered to be significant.

RESULTS

Fig. 1 shows NP effects on boar sperm characteristics during *in vitro* storage. Curcumin and H_2O_2 were used as positive and negative controls in this experiment. Curcumin did not decrease percentages of boar sperm motility, viability, membrane integrity and mitochondrial activity for each 3, 6 or 9 hr incubation period ($p>0.05$). Addition of H_2O_2 significantly decreased all boar sperm characteristics

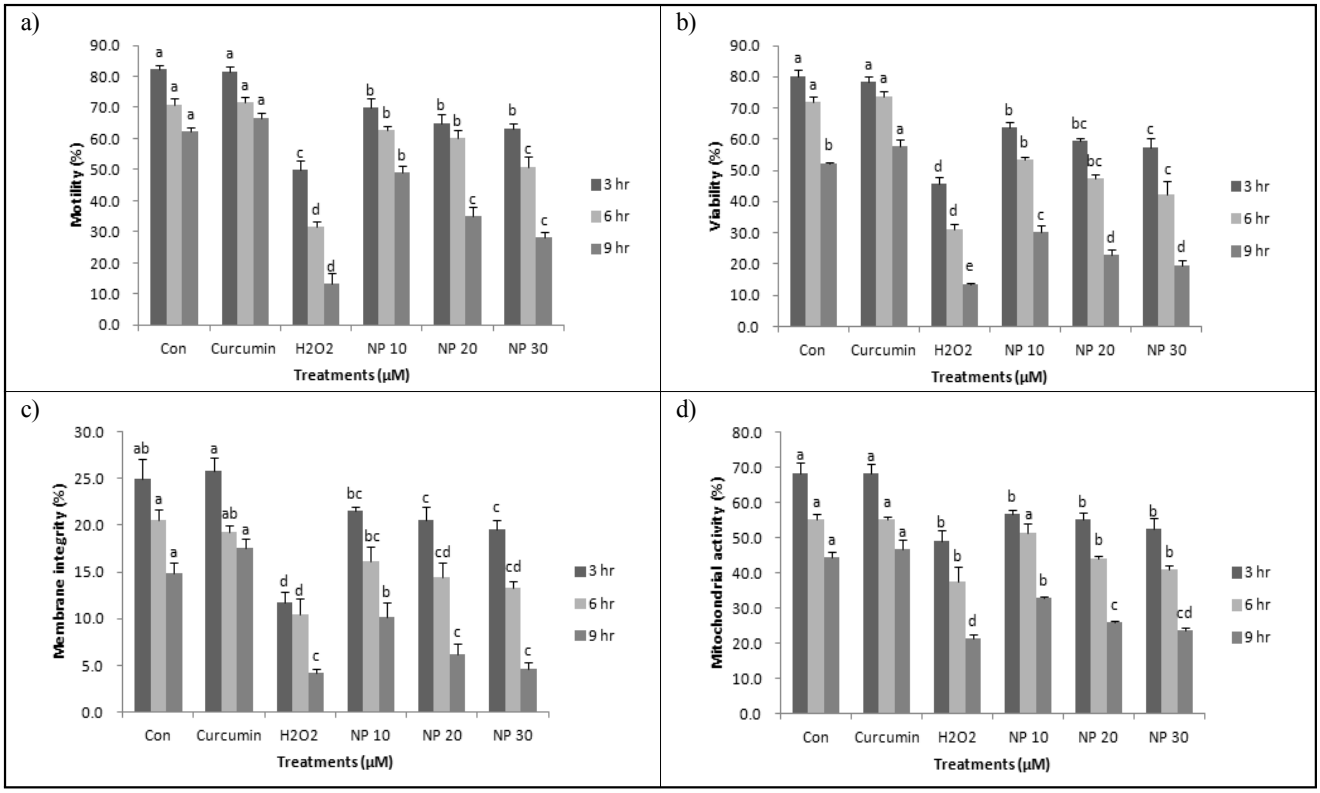

Fig. 1. Effects of NP on boar sperm characteristics during *in vitro* storage; Semen diluted with BTS was incubated for 9 hrs at 37℃ under 5% CO_2 in air. Each semen sample was evaluated at 3 hr interval in a) Motility, b) Viability, c) Membrane integrity and d) Mitochondrial activity.

Con; control, Curcumin; Curcumin 5 μM, H_2O_2; H_2O_2 100 μM.

[a~d] Different superscripts are significantly different, $p<0.05$. Different superscripts were compared among treatments within 3, 6, 9 hrs incubation times. Data are expressed as mean±SEM of three experiments.

such as percentages of sperm motility, viability, membrane integrity and mitochondrial activity compared with control for each 3, 6 or 9 hr incubation period ($p<0.05$). Addition of 10, 20 or 30 μM of NP also significantly decreased all boar sperm characteristics compared with control for each 3, 6 or 9 hr incubation period ($p<0.05$). When concentrations of NP increase from 0 to 30 μM concentration, percentages of most boar sperm characteristics were dose-dependently decreased within 6 or 9 hr incubation period. Addition of H_2O_2 for overall incubation periods showed the most detrimental effect on boar sperm characteristics among treatment groups.

Fig. 2 shows effects of DBP on boar sperm characteristics during *in vitro* storage. Curcumin did not affect general boar sperm characteristics for each 3, 6 hr incubation period ($p>0.05$). H_2O_2 treatment significantly decreased all boar sperm characteristics when these were compared with control for each 3, 6, or 9 hr incubation period ($p<0.05$). These results were consistent with those of Fig. 1. Addition of 10, 50 or 100 μM of DBP tended to decrease overall boar sperm characteristics with dose-dependency within incubation periods. Addition of 100 μM of DBP showed the most detrimental effect on boar characteristics among the DBP concentrations and these detrimental effects increased with incubation time increasement. Fig. 3 is to show effects of BPA on boar sperm characteristics during *in vitro* storage. The effects of curcumin and H_2O_2 treatments showed the similar trends compared with those of Fig. 1 and 2. Addition of 1, 5, 10 μg/ml of BPA decreased overall boar sperm characteristics with dose- dependency for each 3, 6 or 9 hr incubation period ($p<0.05$). Addition of 10 μg/ml of BPA showed the most detrimental effect on boar characteristics among the BPA concentrations and these detrimental effects increased with incubation time increasement.

The combination effects of NP, DBP and BPA on boar sperm characteristics during *in vitro* storage were examined in Fig. 4. There were no significant differences in percentages of sperm motility and membrane integrity among control, silymarin 50 μM and curcumin 5 μM treatments

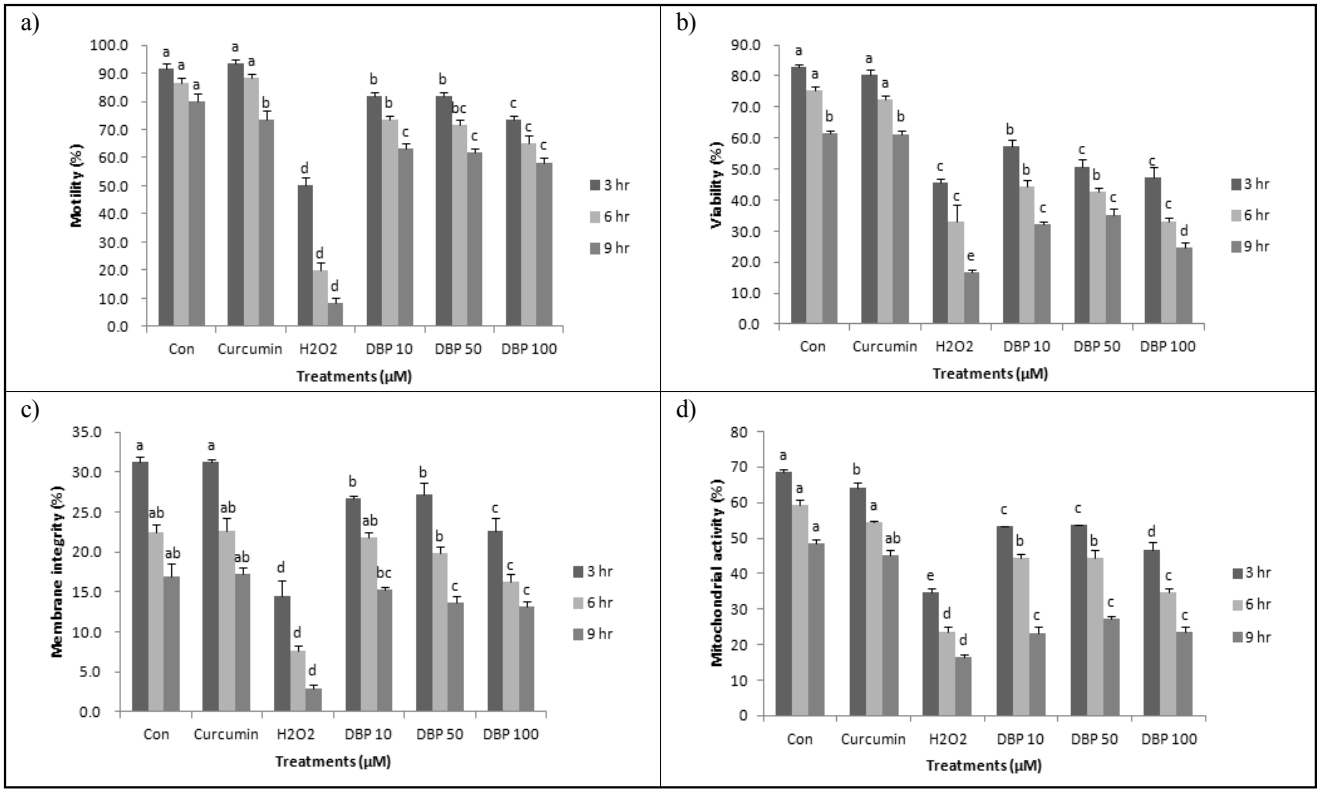

Fig. 2. Effects of DBP on boar sperm characteristics during *in vitro* storage; Semen diluted with BTS was incubated for 9 hrs at 37℃ under 5% CO_2 in air. Each semen sample was evaluated at 3 hr interval in a) Motility, b) Viability, c) Membrane integrity and d) Mitochondrial activity.

Con; control, Curcumin; Curcumin 5 μM, H_2O_2; H_2O_2 100 μM.

[a~e] Different superscripts are significantly different, $p<0.05$. Different superscripts were compared among treatments within 3, 6, 9 hrs incubation times. Data are expressed as mean±SEM of three experiments.

within 3 or 6 hr incubation period ($p>0.05$). Addition of silymarin 50 μM and curcumin 5 μM significantly decreased sperm viability for 3 hr incubation period ($p<0.05$) when compared to control but effects of silymarin and curcumin treatment on boar sperm characteristics were small. In Fig. 4 e), silymarin 50 μM and curcumin 5 μM as antioxidants, significantly decreased lipid peroxidation for 3 hr incubation period ($p<0.05$). H_2O_2 significantly increased lipid peroxidation of sperm for 3 or 6 hr incubation period ($p<0.05$). In combination effects, NP (10 μM) + DBP (10 μM) significantly decreased sperm motility within 3 or 6 hr incubation period compared with control. When both of NP and DBP concentrations (NP; 30 μM, DBP; 100 μM) increase, the detrimental effects on sperm characteristics were larger than those of low concentration combination (Fig. 4b)-d), $p<0.05$). NP (30 μM) + DBP (100 μM) significantly increased lipid peroxidation for 3 or 6 hr incubation period (Fig. 4 e), $p<0.05$). Addition of NP (10 μM) + BPA(1 μg/ml) or NP (30 μM) + BPA (10 μg/ml) significantly decreased all sperm characteristics for 3 or 6 hr incubation period compared with control or positive controls ($p<0.05$). In addition, the inhibitory effects of NP (30 μM) + BPA (10 μg/ml) on percentages of sperm viability, membrane integrity and mitochondrial activity were larger than those of NP (10 μM) + BPA (1 μg/ml) (Fig. 4 a)-d), $p<0.05$). Addition of NP (30 μM) + BPA (10 μg/ml) significantly increased lipid peroxidation for 3 or 6 hrs incubation period but NP (10 μM) + BPA (1 μg/ml) increased only for 6 hr incubation period (Fig. 4 e), $p<0.05$). Increase of both chemical concentrations did not much increase lipid peroxidation compared with low concentration combination.

Addition of DBP (10 μM) + BPA (1 μg/ml) or DBP (100 μM) + BPA (10 μg/ml) significantly decreased overall boar sperm characteristics for 3 or 6 hr incubation period ($p<0.05$). When DBP (100 μM) + BPA (10 μg/ml) was added, the percentages of overall boar sperm characteristics were lower than those of low concentration combination (DBP 10 μM + BPA1 μg/ml, Fig. 4 a)-d) $p<0.05$). This result indicates the detrimental effects of both chemicals on sperm

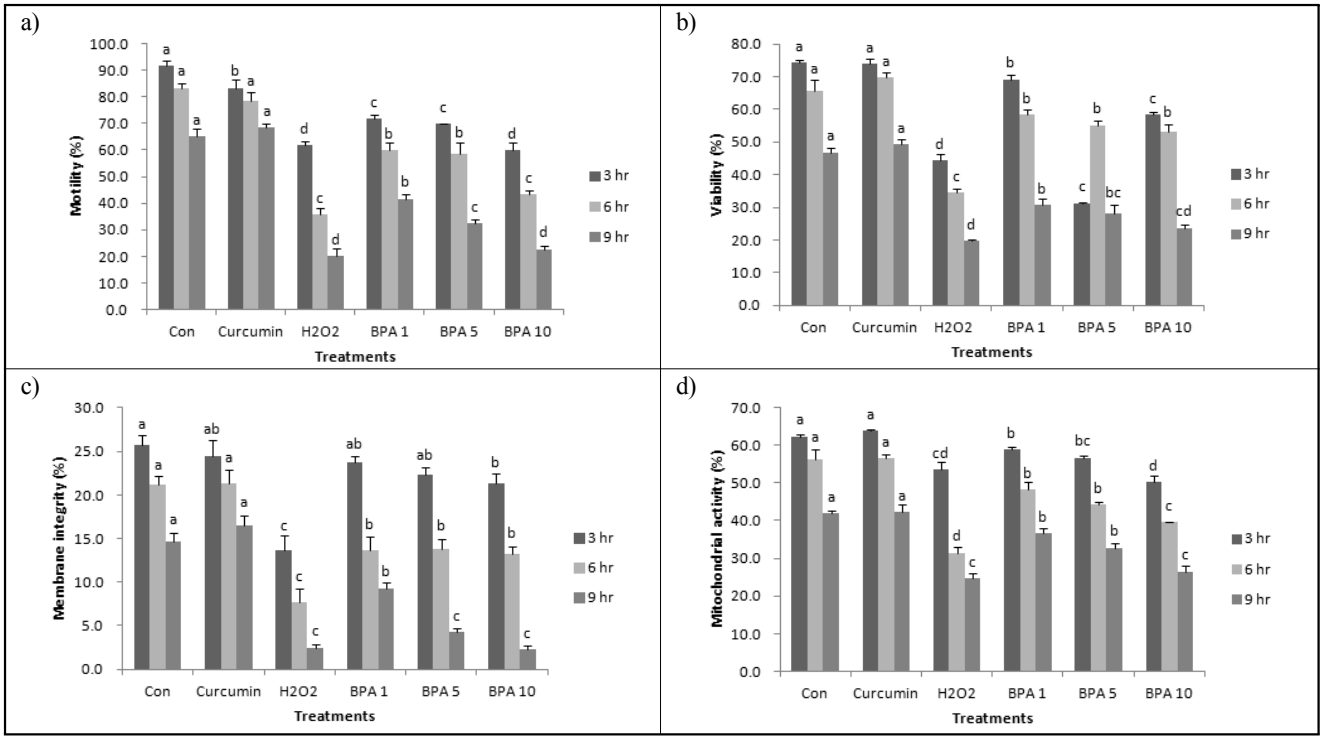

Fig. 3. Effects of BPA on boar sperm characteristics during *in vitro* storage; Semen diluted with BTS was incubated for 9 hrs at 37℃ under 5% CO_2 in air. Each semen sample was evaluated at 3 hr interval in a) Motility, b) Viability, c) Membrane integrity and d) Mitochondrial activity.

Con; control, Curcumin; Curcumin 5 μM, H_2O_2; H_2O_2 100 μM, BPA 1; BPA 1μg/ml, BPA 5; BPA 5μg/ml, BPA 10; BPA 10μg/ml.

[a~e] Different superscripts are significantly different, $p<0.05$. Different superscripts were compared among treatments within 3, 6, 9 hrs incubation times. Data are expressed as mean ± SEM of three experiments.

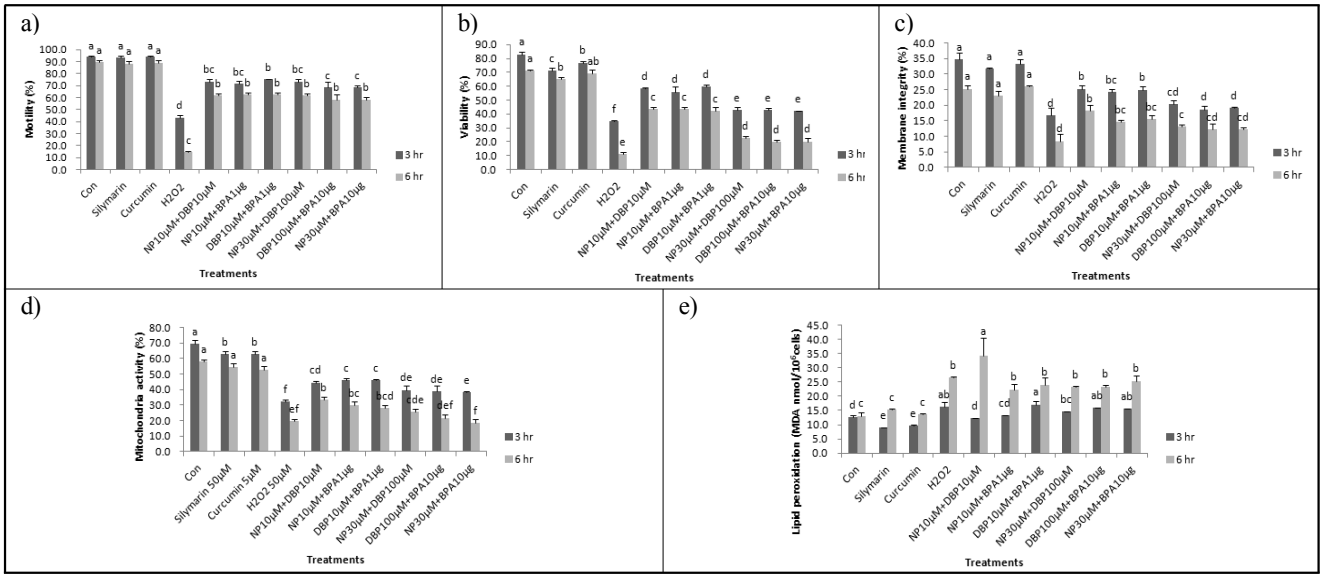

Fig. 4. Combination effects of NP, DBP and BPA on boar sperm characteristics during *in vitro* storage; Semen diluted with BTS was incubated for 6 hrs at 37℃ under 5% CO_2 in air. Each semen sample was evaluated at 3 hr interval in a) Motility, b) Viability, c) Membrane integrity, d) Mitochondrial activity and e) Lipid peroxidation.

Con; control, Silymarin; Silymarin 50 μM Curcumin; Curcumin 5 μM, H_2O_2; H_2O_2 100 μM.

[a~e] Different superscripts are significantly different, $p<0.05$. Different superscripts were compared among treatments within 3, 6 hrs incubation times. Data are expressed as mean ± SEM of three experiments

characteristics were dose-dependent but did not seem to show interaction effect among chemicals. Addition of DBP (10 μM) + BPA (1 μg/ml) or DBP (100 μM) + BPA (10 μg/ml) significantly increased lipid peroxidation for 3 or 6 hr incubation period compared with no addition control or positive controls (Fig. 4 e), $p<0.05$).

Table 1 shows the effects of NP on development of porcine *IVF* embryos. Addition of 20 or 30 μM of NP significantly increased percentage of premorulae embryos compared with no addition control or 10 μM of NP treatment ($p<0.05$). In otherwise, 20 or 30 μM of NP significantly decreased embryo development to morulae or blastocyst stages compared with control ($p<0.05$). In addition, 20 or 30 μM of NP significantly decreased percentage of morulae plus blastocysts ($p<0.05$). The decreases of percentages of embryo development at various stages by addition of NP were dose-dependent. These results indicate that NP has detrimental effect from molulae and blastocycst stage but did not affect at premorulae of early embryo development stage. Addition of 10 or 50 μM of DBP tends to increase premorulae development compared with control but its effect was very small. In addition, DBP 10, 50 and 100 μM did not affect porcine embryo development to morulae and blastocyst stages compared with control group ($p>0.05$, Table 2). Table 3 shows the effects of BPA on development of porcine *IVF* embryos. Addition of BPA 5 or 10 μg/ml increased percentage of premorulae embryos compared with control (control; 36.8%, BPA 5 μg/ml; 50.6%, BPA 10 μg/ml; 59.1%, $p<0.05$). These results were similar to those of NP treatments at premorulae embryo development stage showing slight increase of embryo development. In the contrast, addition of 5 and 10 μg/ml of BPA significantly decreased embryo development of morulae and blastocyst stages compared with control ($p<0.05$). BPA 0, 1, 5 or 10 μg/ml decreased significantly and dose-dependently percentage of morulae plus and blastocysts ($p<0.05$). The combination effects of NP, DBP and BPA on embryo development were shown in Table 4. When NP (10 μM), DBP (10 μM) and BPA (1 μg/ml) are combined each other, percentages of premorulae embroys slightly increased compared with control ($p<0.05$) but significantly decreased embryo development of morulae and blastocysts. There seem to be no specific interaction effects on embryo degeneration among the combined treatments. Table 5 shows the combination effect of NP (30 μM), DBP (100 μM) and BPA (10 μg/ml). The given combinations of these chemicals slightly increased embryo development of premorulae ($p<0.05$) and NP (30 μM) plus BPA (10 μg/ml) increase percentage of premorulae development by 2.7 fold to control ($p<0.05$). Combinations of DBP (100 μM) plus NP (30 μM) and DBP (100 μM) plus BPA (10 μg/ml) did not affect on morulae development, but NP (30 μM) plus BPA (10 μg/ml) has significant detrimental effect on embryo development of morulae and blastoccysts stages ($p<0.05$).

Table 1. Effects of NP on development of porcine *IVF* embryos

NP (μM)	No. of *IVM/IVF* embryos	No. of embryos developed (%);			Morulae plus Blastocysts
		Premorulae	Moulae	Blastocysts	
0	100	24(24.0)^{b}	54(54.0)^{a}	22(22.0)^{a}	76(76.0)^{a}
10	100	26(26.0)^{b}	51(51.0)^{ab}	23(23.0)^{a}	74(74.0)^{a}
20	100	37(37.0)^{a}	46(46.0)^{c}	17(17.0)^{b}	63(63.0)^{b}
30	100	36(36.0)^{a}	49(49.0)^{bc}	15(15.0)^{b}	64(64.0)^{b}

$^{a-c}$ Different superscripts within column are significantly differ, $p<0.05$.

Table 2. Effects of DBP on development of porcine *IVF* embryos

DBP (μM)	No. of *IVM/IVF* embryos	No. of embryos developed (%);			Morulae plus Blastocysts
		Premorulae	Moulae	Blastocysts	
0	75	16(21.3)^{b}	43(57.3)^{a}	16(21.3)^{a}	59(78.7)^{a}
10	78	21(26.9)^{a}	43(55.1)^{a}	14(17.9)^{a}	57(73.1)^{a}
50	79	22(27.8)^{a}	44(55.7)^{a}	13(16.5)^{a}	57(72.2)^{a}
100	75	20(26.7)^{b}	42(56.0)^{a}	13(17.3)^{a}	55(73.3)^{a}

^{a,b} Different superscripts within column are significantly differ, $p<0.05$.

Table 3. Effects of BPA on development of porcine *IVF* embryos

BPA (μg/ml)	No. of *IVM/IVF* embryos	No. of embryos developed (%);			Morulae plus Blastocysts
		Premorulae	Moulae	Blastocysts	
0	87	32(36.8)[c]	37(42.5)[a]	18(20.7)[a]	55(63.2)[a]
1	85	35(41.2)[c]	35(41.2)[a]	15(17.6)[b]	50(58.8)[b]
5	87	44(50.6)[b]	31(35.6)[b]	12(13.8)[c]	43(49.4)[c]
10	88	52(59.1)[a]	28(31.8)[b]	8(9.1)[d]	36(40.9)[d]

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

Table 4. Combination effects of NP, DBP and BPA on development of porcine *IVF* embryos

Treatments (μM)	No. of *IVM/IVF* embryos	No. of embryos developed (%);			Morulae plus Blastocysts
		Premorulae	Moulae	Blastocysts	
Con	126	36(28.6)[c]	67(53.2)[a]	23(18.3)[a]	90(71.4)[a]
NP10μM+DBP10μM	121	45(37.2)[a]	61(50.4)[b]	15(12.4)[b]	76(62.8)[b]
NP10μM+BPA1μg	106	38(35.8)[bc]	56(52.8)[c]	12(11.3)[b]	68(64.2)[c]
DBP10μM+BPA1μg	111	40(36.0)[b]	59(53.2)[bc]	12(10.8)[b]	71(64.0)[c]

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

Table 5. Combination effects of NP, DBP and BPA on development of porcine *IVF* embryos

Treatments (μM)	No. of *IVM/IVF* embryos	No. of embryos developed (%);			Morulae plus Blastocysts
		Premorulae	Moulae	Blastocysts	
Control	65	12(18.5)[b]	40(61.5)[a]	13(20.0)[a]	53(76.9)[a]
DBP100μM+NP30μM	64	16(25.0)[b]	42(65.6)[a]	6(9.4)[b]	48(75.0)[a]
DBP100μM+BPA10μg	61	16(26.2)[b]	38(62.3)[a]	7(11.5)[b]	45(73.8)[a]
NP30μM+BPA10μg	63	32(50.8)[a]	27(42.9)[b]	4(6.3)[b]	31(49.2)[b]

[a,b] Different superscripts within column are significantly differ, $p<0.05$.

DISCUSSION

NP, DBP and BPA are environmental toxicants and potential endocrine disrupting chemicals, giving estrogenic or anti-androgenic properties. They threaten intensely reproductive health of human and wildlifes, resulting in the reduction of testosterone level, sperm production and fertility, and change other reproductive parameters (Fisher et al., 2003; Han et al., 2004; Herath et al., 2004; Li et al., 2010). It has been addressed that there is necessity to be evaluated the exposure risks of single or mixture of two or more endocrine disrupters to human and animal health (Tinwell and Ashby, 2004; Brian et al., 2005; Li et al., 2010; Braun et al., 2012).

The present studies were to evaluate the deleterious effects of single or mixtures of NP, DBP and BPA on boar sperm characteristics and porcine IVF embryos development. The sperm motility, viability and mitochondrial activity by NP, DBP and BPA single treatments were significantly decreased to that of control (no addition) or curcumin treatment group (positive control) with dose and incubation-time dependancy. NP could induce oxidative stress, cytotoxicity and apoptosis through endoplasmic reticulum stress in testicular sertoli cell in rat. Exposure of NP to neonatal rat has adverse effects with various reproductive parameters including decrease in the size of testis, reduced sperm count and sperm motility (Lee et al., 1999). In our experiments, NP 30 μM significantly decreased percentages of most boar sperm characteristics such as motility, viability, membrane integrity and mitochondrial activity for each 3, 6 or 9 hr incubation period ($p<0.05$). These results indicate that NP as endocrine disruptor also has direct detrimental effect on sperm characteristics despite of indirect effect mediated through the testicular tissue degeneration (Lee et al., 1999; El-Dakdoky

and Helal, 2007). Addition of 10, 50 or 100 μM of DBP decreased overall boar sperm characteristics with dose-dependency for 3, 6 or 9 hr incubation period ($p<0.05$). These results are not compatible with other researcher's studies that DBP alone has not toxic effect in testicular leydig cells up to 100 μM concentration (Gang and Han, 2006; Hallmark et al., 2007; Gong et al., 2009) and did not induce apoptosis in rat sertoli cell at same concentration(Li et al., 2010). In our experiment, DBP was directly treated to boar sperm and caused the deleterious effect at 10, 50 or 100 μM DBP within short time periods. Thus, the detrimental effects of boar sperm characteristics at given concentrations of DBP in our experiment was direct and should be probable.

In our experiment, addition of 1, 5 or 10 μg/ml of BPA decreased overall boar sperm characteristics with dose dependency for 3, 6 or 9 hr incubation period ($p<0.05$). When mouse was treated with BPA at environmental exposure levels (20-100 μg/kg body weight/day), BPA appeared to be potent disruptor of meiosis and cell division process, resulting the damage of sperm or embryos (Hunt et al., 2003). Thus, BPA is also environmental toxicant and can directly cause detrimental effect on boar sperm characteristics. The results of our study indicate that NP, DBP and BPA alone basically have detrimental effects. These results are consistent with previous studies (Hunt et al., 2003; Gang and Han, 2006; El-Dakdoky and Helal, 2007; Hallmark et al., 2007; Gong et al., 2009; Bao et al., 2011). The present study attempted to evaluate the combination effects of low and high dose mixtures of NP, DBP and BPA on boar sperm characteristics. The sperm characteristics at both low (NP 10 μM + DBP 10 μM, NP 10 μM + BPA 1 μg/ml and DBP 10 μM + BPA 1 μg/ml) and high concentration (NP 30 μM + DBP 100 μM, NP 30 μM + BPA 10 μg/ml and DBP 100 μM + BPA 10 μg/ml) of mixture groups significantly deteriorated than those of control or positive control groups (silymarin and curcumin). There were no interaction effects between chemicals on boar sperm characteristcs. The lipid peroxidations in boar sperm significantly increased at low and high concentration mixtures for 3 and 6 hr incubation periods compared to silymarin or curcumin groups (antioxidants) as positive controls ($p<0.05$). The biological activity of sperm characterisitics by silymarin or curcumin treatment groups were much higher than those of low or high mixture groups. Overall results indicate that the detrimental effects on overall boar sperm characeristics should be partially resulted from endogenous lipid peroxidation and generation of free radicals in sperms by various endocrine disruptors.

In the experiment of porcine IVF embryo development, NP or BPA alone significantly decreased the percentages of embryo development from morulae and blastocyst stages ($p<0.05$) and its detrimental effects were dose-dependant. In addition, combinations of NP (30 μM) plus BPA (10 μg/ml) has significant detrimental effect on embryo development at these stages ($p<0.05$). NP and BPA have estrogenic activity, while DBP has anti-androgenic activity (Tinwell and Ashby, 2004; Zhang et al., 2004). BPA can mimic estradiol action in inducing vaginal coalification, uterine vascular permeability, growth and differentiation of the mammary gland and c-fos gene expression in the female reproductive tracts. Our results indicate that at least high dose of single or high dose combination of NP and BPA cause detrimental effect on porcine embryonic development.

As conclusion, NP, DBP and BPA as endocrine disrupters have detrimental effects on sperm characteristics partially through the increasement of lipid peroxidation and free radical formation. In addition, these chemicals cause similar detrimental effect on the development of porcine IVF embryo at morulae and blastocysts stages.

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