Protective Effects of Silymarin against the Toxicity of Bisphenol A (BPA) on Boar Sperm Quality

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ABSTRACT

BPA, a diphenyl compound containing groups, that make it structurally similar to synthetic estrogen and is considered as one of the major endocrine disruptors. Silymarin has extensively been used to prevent and/or alleviate some human disease, especially for the treatment of adverse liver conditions. It has an antioxidative efficacy and cancer preventive efficacy. Therefore, we examined the hypothesis that silymarin can inhibit BPA-induced toxicity in boar sperm duing *in vitro* storage.

Sperm characteristics (motility, viability, membrane integrity and mitochondrion activity) in semen exposed to BPA (10～200 uM) were sharply lowered, while it increase in a dose and time dependent manner due to silymarin addition (50～200 uM) into semen extender in the presence of BPA (100 uM). All of the evaluated characteristics were gradually improved in the groups that were treated with silymarin (50～200 uM) in the presence of BPA (100 uM) in comparison to BPA 100 uM alone group, irrespective of incubation periods (3 and 6 h).

These results demonstrate that silymarin can ameliorate the toxicity of BPA on boar sperm characteristics during *in vitro* storage, suggesting that silymarin indirectly act as an antioxidant.

(Key words : endocrine disruptors, silymarin, bisphenol A, sperm characteristic, mitochodria activity)

INTRODUCTION

Recently, there are increasing concerns about endocrine disruptors which are present in the environment a result of industrial development and their impact on reproductive abilities of animals and possibly humans. BPA, a known endocrine disruptor, is widely used as a monomer of polycarbonate plastics and in a variety of applications such as constituents of epoxy resins in the food and beverage containers, baby bottles and table plates, electrical laminants and dental sealants (Brotons *et al*., 1995; Magure, 1998; Papaconstantinou *et al*., 2000). A variety of endocrine disrupting chemicals are increased in male reproductive disorders such as inhibition of spermatogenesis, cryptorchidism, hypospadias and low sperm counts in mammal (Game *et al*., 2006). The exposure of BPA in rodents is able to adversely affect the reduction in body weight, semen volume, sperm number, reproductive organ weight and testosterone concentration and increase in prostate gland in male (Stoker *et al*., 1999; Takao *et al*., 1999) as well as the mammary gland. In females, exposure to BPA also reduce uterus weight and increase in progesterone receptor and prolactin gene expression (Krishnan *et al*., 1993; Colerangle and Roy, 1997). BPA has a mimicking estrogen action in inducing vaginal cornification, uterine vascular permeability, growth and differentiation of the mammary gland and *c-fos* gene expression in female reproductive tracts (Colerangle and Roy, 1997; Milligan *et al*., 1998; Steinmetz *et al*., 1998). The toxicity of BPA is manifested by lipidation and generation of free radical causing oxidative stress (Siu *et al*., 2006). The prevention of toxicity caused by BPA has been shown by using antioxidants such as vitamine C, n-acetyl-cysteine and melatonin (Siu *et al*., 2006).

Silymarin is one of the most frequently studied bioflavonoid in the class of flavonols. It is a polyphenolic flavonoid anti-

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oxidant isolated from the fruits and seeds of milk thistle. An active constituent of milk thiethele (*Silybum marianum*) is basically a flavolignan, silymarin is a major active constituents among four isomeric fluonoids such as silibinin, isosilibinin, silydianin and silychristin (Kaur and Agarwal, 2007). Silymarin possesses special biological effects, such as an antioxidative activity (Valenzuela *et al*., 1989), anti-inflammatory effects (Manna *et al*., 1999) and an inhibitory action on tumor necrosis factor-a (TNFa) expression (Zi *et al*., 1997). Flavonolic antioxidants such as silymarin and quercertin are reported to quench oxygen derived free radicals as well as substrate-derived free radicals by nonationg a hydrogen atom or an electron to free radical, protect cell constituents against oxidative damage (Fraschi *et al*., 2002). Silymarin has also shown a protective action against oxidative damage and revealed an iron-chelating effect and remakable effect in inhibitin of Cu^{2+}-mediated LDL oxidation (Soose, 1994; Skottova *et al*., 1997; Marco *et al*., 2001). Although there is plenty of information on beneificial and biological properties of sylimarin, it has not been attempted to evaluate the protective effects of silymarin against BPA toxicity in boar semen quality.

Therefore, the present study was conducted to investigate the effects of silymarin against toxicity caused by in boar semen during *in vitro* storage.

MATERIALS AND METHODS

1. Semen Preparation

Sperm-rich fractions (30 to 50 ml) were collected from 1～3 pure breed (Duruc, Yorkshire and Landrace) and 85 % of motile sperm was obtained by the gloved hand method at the local AI center (Wonju). After collection, semen was diluted with Modena extender (Funahashi and Sano, 2005) and transported to the laboratory within 2 h of collection at 17℃. Semen was treated with BPA alone (1, 10 and 100 uM) and silymarin (50～200 uM) in the presence of BPA (100 uM). The semen of each treated group was incubated for 3 and 6 at 37℃ under 5 % CO_2 in high humidified air. All of the treatments were repeated at least 5 times with the semen samples from the different boars. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (USA) and were analytical grade.

2. Sperm Analysis

The analysis of semen quality was evaluated based on the motility, viability, membrane integrity and mitochondria activity. Each evaluation method was evaluated a total out of 200 spermatozoa with two different semen samples at least 5 times at 400 × magnification under an inverted phase contrast microscope (Nikon, Japan).

1) Sperm Motility

Sperm motility was subjectively assessed by visual estimations, which was measured by determining the percentage of spermatozoa showing any movement of the flagellum from wave to progressive motion.

2) Sperm Viability

The MTT [3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium-bromide] assay that depend on the ability of metabolically active cells to reduce the tetrazolium salt to formazan was used to evaluate the sperm viabiltiy (Jang *et al*., 2010). The semen samples were washed twice with Hepes-BSA sol. and adjusted to 30×10^6 spermatozoa/ml. A 100 ul of semen samples plus 10 ul of MTT stock sol. (5 mg MTT/ml of PBS) was transferred in each well of 96-well microplate and incubated at 37℃ for 1 h. After incubation, the optical density (OD) values were measured at 570 nm by microtiter plate reader (Bio-Tek, USA). For determination of sperm viability, relative sperm viability (%) was calculated as OD of treated sample/control OD×100.

3) Hypoosmotic Swelling Test (HOST)

The HOST was based on methods described by Jang *et al*. (2010), modified as indicated below. The semen sample was incubated for 30 min at 37℃ followed by mixing a 50 ul semen sample with 1 ml of a hypoosmotic solution (7.35 g Na-citrate and 13.51 g fructose in 1 liter of distilled water). Viable spermatozoa (positive) had coiled or swollen tails after incubation when observed under inverted phase contrast microscopy.

4) Fluorescent Assay of Mitochondrial Activity

Mitochondrial activity was evaluated by employing the technique developed by Fraser *et al*. (2007), modified as indicated below. The percentage of live spermatozoa with functional mitochondria was assessed using a combination of the fluorescent stains Rhodamine 123 (R 123) and propidium iodide (PI). For this assay, 3 ul of R123 solution was added to 1 ml of semen sample (20×10^6 spermatozoa/ml) and incubated for 15 min at 37℃ in the dark. Subsequently, the semen sample was stained

with 10 ul of PI and incubated for 10 min at 37℃. Following the second incubation, the suspension (10 ul) was placed on slides and examined at 400 magnification under epifluorescence microscopy (Zeiss, Germany) equipped with an excitation/barrier filter of 490/515 nm for R123 and an excitation/barrier filter of 545/590 nm for PI. Sperm cells displaying only green fluorescence at the mid-piece region were considered viable spermatozoa with functional mitochondria.

3. Statistical Analysis

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

RESULTS

The effect of BPA alone and sylimarin (50～200 uM) against BPA (100 uM) on the sperm characteristics (motility, viability, membrane integrity and mitochondria activity) were evaluated for 3 and 6 h incubation periods at 37℃. The motility, viability and membrane integrity treated with BPA significantly decreased in a dose- and time-dependent manner (Fig. 1, $p<0.05$).

As shown in Fig. 2, sperm motility between control and silymarin group (200 uM) was not significantly differ, but both groups were significantly increased than BPA alone (100 uM) group ($p<0.05$). The sperm motility in BPA plus siylmarin group slightly increased in silymarin dose-dependent manner irrespective of incubation periods.

Viability in the sylimarin 100 uM plus BPA 100 uM (72.6%±2.5 and 82.1%±5.0), sylimarin 200 uM plus BPA 100 uM group (73.2%±4.4 and 87.3%±3.0) for 3 and 6 h incubation periods was significantly higher than that of the BPA 100 uM group (58.0%±5.4 and 47.0%±5.1, $p<0.05$).

In the results of spermatozoal membrane integrity evaluated by HOST, no significantly changes was observed for 3 and 6 h incubation periods between the BPA 100 uM and sylimarin 200 uM plus BPA 100 uM groups, but sylimarin 200 uM group (15.7%±0.5 and 15.3%±0.5) and control groups (15.7±2.0 and

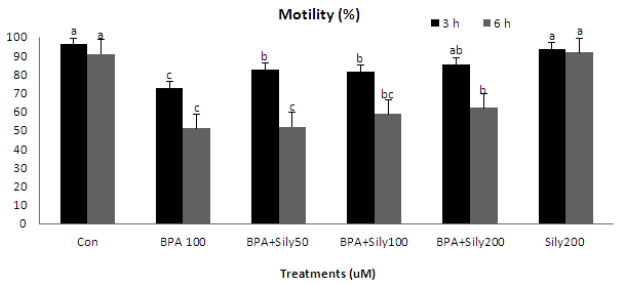

Fig. 2. Effects of silymarin against BPA on boar sperm motility during *in vitro* storage. Boar sperm were incubated for 3 or 6 h in Modena extender with different concentration of silymarin (50~200 uM) in the presence or absence of 100 uM BPA. Con=Control, BPA+Sily50=BPA 100 uM+Silymarin 50 um, BPA+Sily100=BPA 100 uM+Silymarin 100 uM, BPA+Sily200=BPA 100 uM+Silymarin 200 uM. $^{a\sim c}$ Mean values with different superscripts within same incubation periods are significantly differ, $p<0.05$. Data are expressed as means ± SEM of three experiments.

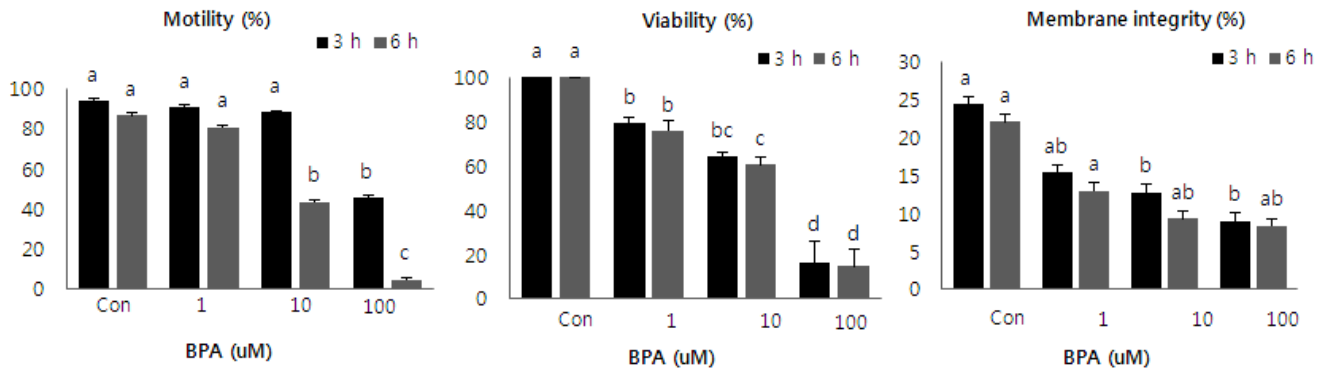

Fig. 1. Evaluation of sperm characteristics (motility, viability and membrane integrity) in boar semen. Boar sperm were incubated for 3 or 6 h in Modena extender with different concentration of BPA (1, 10 and 100 uM). Con=Control. $^{a\sim d}$ Mean values with different superscripts within same incubation periods are significantly differ, $p<0.05$. Data are expressed as are the mean ± SEM of three experiments.

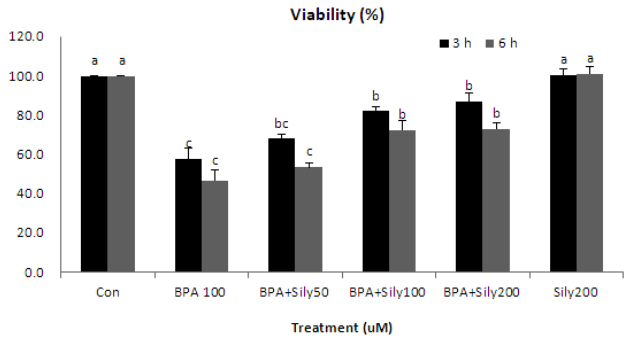

Fig. 3. Effects of silymarin against BPA on boar sperm viability during *in vitro* storage. Boar sperm were incubated for 3 or 6 h in Modena extender with different concentration of silymarin (50~200 uM) in the presence or absence of 100 uM BPA. [a~c] Mean values with different superscripts within same incubation periods are significantly differ, $p<0.05$. Data are expressed as means ± SEM of three experiments.

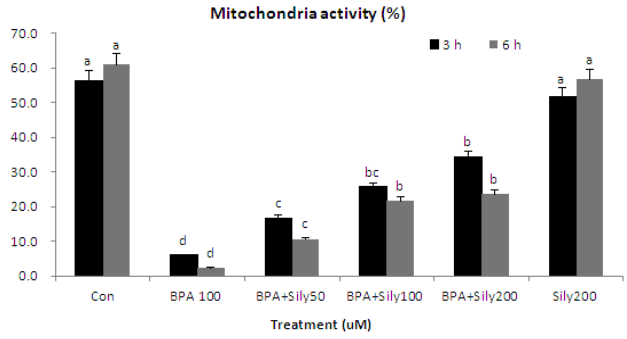

Fig. 5. Effects of silymarin against BPA on boar sperm mitochondria activity during *in vitro* storage. Boar sperm were incubated for 3 or 6 h in Modena extender with different concentration of silymarin (50~200 uM) in the presence or absence of 100 uM BPA. [a~d] Mean values with different superscripts within same incubation periods are significantly differ, $p<0.05$. Data are expressed as means ± SEM of three experiments.

12.7±2.0) was significantly different from BPA 100 uM group (8.3±0.9 and 9.0%±0.8) and silymarin addtion in the presence of BPA for 3 and 6 h incubation periods (Fig. 4, $p<0.05$).

The percentage of spermatozoa with mitochondria activity assessed by Rhodamine staining is shown in Fig. 5. The mitochondria activity in BPA 100 uM group (6.0%±0.8 and 2.3%±0.5) for 3 and 6 h was significantly lower than any other groups (control, 56.3%±2.0 and 61.0%±2.9; silymarin 50 uM plus BPA 100 uM, 16.7%±2.0 and 10.7%±0.5; silymarin 100 uM plus BPA 100 uM, 25.7%±2.5 and 21.7%±2.9; silymarin 200 uM plus BPA 100 uM, 34.3%±0.5 and 23.7%±1.7 and

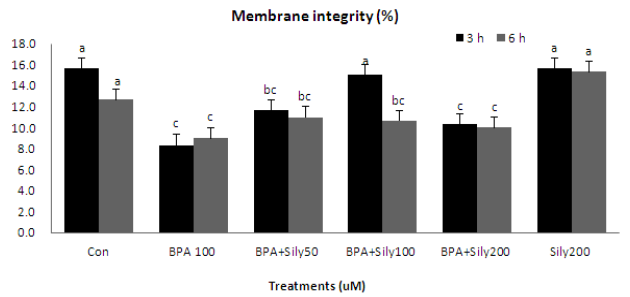

Fig. 4. Effects of silymarin against BPA on boar sperm membrane integrity during *in vitro* storage. Boar sperm were incubated for 3 or 6 h in Modena extender with different concentration of silymarin (50~200 uM) in the presence or absence of 100 uM BPA. [a~c] Mean values with different superscripts within same incubation periods are significantly differ, $p<0.05$. Data are expressed as means ± SEM of three experiments.

silymarin 200 uM, 51.7%±2.4 and 56.7%±2.4, $p<0.05$). The spermatozoal mitochondria activity in silymarin addition in presence of 100 uM BPA significantly increased in dose dependent manner irrespective of incubation periods ($p<0.05$).

DISCUSSION

Silymarin, which has a potent antioxidative feature, is a flavonoid isolated from the fruits and seeds of the milk thistle (*S. marianum*) (Fraschini *et al*., 2002; Zeisel, 2004). In addition, several recent studies have shown the potential hepatopreventive and therapeutic efficacy of silymarin in different animal models and cell culture systems (Kaur and Agarwal, 2007; Polyak *et al*., 2010; Wagoner *et al*., 2010). Its positive effects have been ascribed to the putative anti-oxidative, anti-inflammatory and anti-proliferative properties based on the modulation of specific signaling pathways, transcription factors and gene expression (Katiyar *et al*., 2005).

BPA is widely used in daily life and has the property of being an endocrine distruptor. As a potent endocrine disruptor, BPA has been shown to have both estrogenic and anti-androgenic properties, which provides biological plausibility for direct adverse effects on spermatogenesis (Toyoma *et al*., 2004) in rodents and human and increase proliferation of testicular seminiferous cells, which can lead to low sperm count, abnormal sperm morphology and poor semen quality (Aitken, 2004; Sharpe, 2010). Oxidative stress on sperm by BPA has also been

proposed as a potential mechanism for its adverse effect (Al-Hiyasat *et al*., 2004). The generation of oxidative stress has a toxic effect at high levels on sperm quality and function during *in vitro* storage (Kumaresan *et al*., 2009) and also is implicated in the induction of apoptotic cell death (Juknat *et al*., 2005).

The results of this study indicate that the motility, viability and membrane integrity of boar sperm exposed to BPA irrespective of incubation periods were declined in dose dependent manner, specially BPA 100 uM group at 6 h significantly decreased than any other groups ($p<0.05$). This study agree with the finding of Al-Hiyasat *et al*. (2004) and Kumaresan *et al*. (2009) that oxidative stress on sperm by BPA exposure has a detrimental effects, resulting the decline of sperm motility, viability and membrane integrity.

The BPA exposure of sperm during *in vitro* storage generated oxidative stress and it could be damage the sperm characteristics in mammals. The supplementation of antioxidants into semen extender reduced oxidative damage against ROS on sperm characteristics such as motility, viability, membrane integrity, lipid peroxidation and mitochondria function. Mitochondria are the major sites of intracellular oxidative stress formation, which results in a disruption of electron transport (Halliwall and Gutteridge, 1999). This disruption partly results from the decline of motility and peroxidation of membrane lipids. However, oxidative stress and lipid peroxidation is a damaging process to spermatozoa leading to motility loss and reduced fertilizing ability for many species (Cerolini *et al*., 2000). However, mechanism of the reported adverse effect of BPA on semen quality are not yet completely understood (Thayer *et al*., 2001; Toyama *et al*., 2004).

The present study was conducted to examine whether silymarin acted directly or indirectly as an antioxidant through scavenging oxidative stress or detoxified the sperm extender when boar semen was exposed BPA during *in vitro* storage. The motility, viability, membrane integrity and mitochondria activity in silymarin (200 uM) alone group significantly increased while BPA (100 uM) alone group significantly decreased than any other groups ($p<0.05$). The silymarin supplementation (50～200 uM) into semen extender containing BPA (100 uM) gradually improved on motility, viability and mitochondria activity in dose dependent manner, but membrane integrity did not. The reason for this specific mechanism are unclear in this study.

In relation to the incubation period, especially in cases where the period was greater than 6 h, the sperm characteristics deteriorated in sperm treated with silymarin in the presence of BPA, suggesting the increase of oxidative stress in the semen extender. The results of our studies were consistent with Jou *et al*. (2004), silymarin efficiently prevents mitochondrial ROS formation under basal conditions indicating that as well as at early time points upon secondary oxidative stress induced by H_2O_2 exposure. However, the exact mechanism of BPA or silymarin effects on mitochondrial function, motility and membrane integrity remains unclear. However, these effects may be related to its antioxidative properties.

In conclusion, our results demonstrate that the silymarin supplementation improved the sperm characteristics when sperm were exposed BPA. This study indicates that silymarin has protective features against BPA for the *in vitro* storage of boar semen.

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