Protective Effect of *Lycium barbarum* Polysaccharides Against Doxorubicin-induced Testicular Toxicity in Rats

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The present study aimed to investigate whether *Lycium barbarum* polysaccharides (LBP) would protect against doxorubicin (DOX)-induced testicular toxicity. Male Sprague-Dawley rats were treated with distilled water (4 mL/kg) or LBP (200 mg/kg, p.o.) daily for 10 days and followed by saline (0.9 %, 10 mL/kg) or DOX (10 mg/kg) intravenous injection at day 7. Pretreatment with LBP ameliorated DOX-induced reduction in the testicular weights, sperm concentrations and percentage of motile sperms, as well as the increase in abnormal sperm rate. LBP administration to DOX-treated rats successfully reversed the changes in MDA and GHS-Px levels. Compared with the control, pretreatment with LBP significantly increased the plasma testosterone level in the LBP + DOX group. The histopathology examinations further confirmed that LBP effectively attenuated DOX-induced severe degenerative changes of seminiferous tubules. This study illustrated the capability of LBP in attenuating testicular oxidative stress and protecting testis-specific toxicity in DOX-exposed rats.

Keywords: doxorubicin; *Lycium barbarum* polysaccharides; testicular toxicity; antioxidant.

INTRODUCTION

Doxorubicin (DOX), an anthracyclin antibiotic, is a potent antitumor drug in the treatment of both hematological malignancies and solid tumors, used for more than 40 years. However, the clinical use of DOX is circumscribed due to its severe side effects such as cardiotoxicity and nephrotoxicity (reviewed by Minotti et al., 2004). DOX also causes testicular and spermatozoal toxicity characterized by decreased quantity of sperm, decreased percentage of motile sperm, and increased rate of abnormal sperm in association with an increased apoptosis at specific stages of seminiferous epithelial cycle. The biochemical mechanisms of the side effects of DOX are not clearly known yet (reviewed by Carvalho et al., 2009). Oxidative stress and the formation of reactive oxygen species (ROS) are supposed to be the main one (reviewed by Minotti et al., 2004; Menna et al., 2010; Carvalho et al., 2009; Prahalathan et al., 2006). The ROS damage the cell membrane through peroxidation of phospholipids, resulting in the alteration of molecular signal transduction and further damage to mitochondrial and nucleic acid (reviewed by Minotti et al., 2004; Menna et al., 2010; Carvalho et al., 2009). Thus co-administration of DOX with an effective antioxidant may help to alleviate the testicular and spermatozoal toxicity of DOX (Kato et al., 2001).

The fruit of *Lycium barbarum* has been used commonly as antipyretic, aphrodisiac, fertility-facilitating and anti senile agents in Chinese traditional medicine (Potterat, 2010; Chang and So, 2008). The aqueous glycol-conjugates in *Lycium barbarum*, collectively termed *Lycium barbarum* polysaccharides (LBP), are estimated to comprise 5% to 8% of the *Lycium barbarum* fruit and to have a molecular weight range from 24 to 241 kDa (Amagase et al., 2009). LBP was reported to inhibit time- and hyperthermia-induced structural damage and to delay apoptosis in murine seminiferous epithelium *in vitro* (Wang et al., 2002). Furthermore, LBP was also observed to be able to protect testicular tissue against heat-exposure-induced damage and to eliminate DNA oxidative damage of mouse testicular cells induced by H$_2$O$_2$ (Luo et al., 2006). The antioxidant effect of LBP was postulated to be responsible for its protective effect on testicular tissue.

The aim of the present study was to investigate the toxicity of DOX on testis and sperm in male rats, and to evaluate the protective effect of LBP on DOX-induced testicular toxicity. The effect of LBP on DOX-caused oxidative stress was evaluated by the levels of malondialdehyde (MDA, a marker of lipid peroxidation) *in vitro* in testis tissues. The objective was to determine whether there are reductions of lipid peroxidation and increases of antioxidant enzymes that supported the antioxidative effect of LBP responding to DOX-caused oxidative stress. One of the main detoxifying systems for peroxides is GSH. By participating in the glutathione redox cycle, GSH together with GSH-peroxidase (GSH-Px, a three-amino acid-peptide against oxidative scavenging free radicals) convert H$_2$O$_2$ and lipid peroxide to non-toxic products. Finally, the protective effect occurred…

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of LBP on DOX-induced myocardial injury was explored with a histopathological analysis.

MATERIALS AND METHODS

Chemicals. Doxorubicin was obtained from Pfizer Italia S. r.l. (Nerviano, Italy) as a 10 mg/bottle lyophilized powder. It was dissolved in 20 mL of 0.9 % saline for injection. LBP, whose purity was above 60 % (determined by sulfuric acid-anthrone method), was purchased from the Zhejiang Fangge Pharmaceutical Industry Co., Ltd (Qingyuan, China). The MDA and GSH-Px assay kits were purchased from the Nanjing Jiancheng Bio-engineering Institute (Nanjing, China). The rat testosterone ELISA kit was purchased from R&D Systems (Minneapolis, USA).

Animals. Twenty-eight male Sprague-Dawley rats (8 weeks old and 200–250 g weight) were obtained from the Zhejiang Experimental Animal Center. The animals were kept under standard laboratory conditions with a temperature range of 20–25 °C and a humidity range of 50–60 %. The standard diet and water were provided throughout the experimental period. All animal experiments were carried out in accordance with Regulations for the Administration of Affairs Concerning Experimental Animalst promulgated by the State Science and Technology Commission of China, and the guideline for animal experiments of Zhejiang Academy of Medical Sciences.

Experimental protocol. The rats were randomly divided into four groups of eight rats each. The control group rats received distilled water (4 mL/kg) by intragastric irrigation daily for 10 days plus a single intravenous injection of 0.9 % saline (10 mL/kg) at day 7. In the same manner, water was replaced with LBP (200 mg/kg) in the LBP group, or saline replaced with DOX (10 mg/kg) in the DOX group (Xin et al., 2007, 2011). The rats in the LBP + DOX group were treated similarly to the control rats, except that the water and saline were alternated by LBP and DOX, respectively. The general appearance and mortality were observed daily and body weights were recorded on days 1 and 11. On day 11, the rats were killed after being anesthetized with pentobarbital (30 mg/kg, i.v.) and blood samples were collected for testosterone measurement and biochemical analysis. Sperm samples were collected from the cauda epididymes with a pipette and prepared for examination immediately. Both testicles and epididymes were weighed and measured. The sperm count was placed on the slide and covered with a cover-slip. For each sample, approximately 200 sperm cells were examined under an optical microscope (400×). The sperm were classified as motile or immotile. The abnormal sperm rate was assessed by a modified method of Karbalay-Doust et al. (2007). An aliquot of the sperm suspension prepared for the sperm count was placed on the slide and covered with a cover-slip. For each sample, approximately 200 sperm cells were examined under an optical microscope (400×). The sperm were classified as normal, head abnormal or tail abnormal.

Biochemical studies. One gram of testis tissue sample was homogenized in 10 mL of 20 mM Tris-HCl buffer at 4 °C with a homogenizer. Homogenates were centrifuged at 1500 rpm for 15 min. The levels of MDA and the activities of GSH-Px in the supernatants of the tissue homogenate were determined with the MDA assay kit and GSH-Px assay kit according to the manufacturer’s protocols, respectively. In brief, the MDA content was detected by a thiobarbituric acid method and GSH-Px activity was determined via the glutathione assay system.

Histopathological examinations. Formalin-fixed tissue samples were dehydrated through an upgraded ethanol series, embedded in paraffin blocks and sectioned at 3 μm. Ultrathin sections were dewaxed by xylene, hydrated through a degraded ethanol series, and stained with hematoxylin and eosin (H&E). Then they were examined by a pathologist blinded to the treatments under an optical microscopy. The diameter and germative cell layer thickness of the seminiferous tubule (ST) from five different areas of each testis were measured and the average size and thickness of ST were calculated.

Statistical analyses. All the results were expressed as mean ± SEM. Differences between groups were assessed by one-way ANOVA and Tukey’s HSD test. A probability of error (p < 0.05) was selected as the criterion of statistical significance.

RESULTS

Reproductive organ weights and dimensions

At the end of the treatment period, the rats in the control and LBP groups lived normally. However, the rats treated with DOX appeared weak. No death was observed in any group. As shown in Table 1, the mean body weights of the rats between the four groups were similar both at the beginning and prior to the DOX administration. The DOX injection led to a significant decrease (p < 0.05) in body weight, testis weight and epididymis weight. Compared with that in the control group, the testis-to-body weight
ratio and epididymis-to-body weight ratio in the DOX group increased significantly ($p < 0.05$). The body weight and reproductive organ weight of the rats exposed to DOX were preserved by oral administration of LBP. However, only the influence of LBP on testis weight was significant ($p < 0.05$).

**Sperm concentration, motility and abnormal sperm rate**

Table 2 shows the effects of treatment with LBP and/or DOX on epididymal sperm concentrations, sperm motility and abnormal sperm rates. Dosing LBP daily had no significant effect on the sperm quantity and quality. Compared with the control group, the sperm concentrations and sperm motility were significantly decreased, whereas the percentage of abnormal sperm was significantly increased in rats treated with DOX alone ($p < 0.05$). Pretreatment with LBP effectively prevented the DOX-induced decline of sperm quantity and quality in the LBP + DOX group.

**Oxidative stress in testis tissue**

The protective effect of LBP on DOX-induced oxidative stress was evaluated by examination of MDA and GSH-Rx levels in the testis tissue. As shown in Table 3, pretreatment with LBP has no significant effect on the MDA concentration in rats. The DOX treatment significantly increased the MDA levels in testis tissue in the DOX group compared with the control groups ($p < 0.05$), whereas this increment was significantly attenuated by LBP pretreatment. However, there remained a statistical difference in the MDA concentration between the LBP + DOX and control groups. The DOX group was statistically different ($p < 0.05$ vs control group) with regard to GSH-Rx activities in the heart samples. The levels of GSH-Rx increased significantly in the LBP + DOX group compared with that in the DOX group. No significant difference was observed between the LBP + DOX and control groups with regard to the GSH-Rx level.

**Testosterone level**

As shown in Table 3, DOX treatment alone induced a significant decrease in plasma testosterone that was nearly 30% of the control values ($p < 0.05$). Compared with the control group, pretreatment with LBP did not have any effect on the plasma testosterone level, however, the LBP + DOX group had a significant increase ($p < 0.05$).

**Histopathological changes**

The testis tissues from the control (Fig. 1A) and LBP group rats showed no remarkable histopathological findings. However, significant degenerative changes characterized by a depletion of germ cells, irregular seminiferous tubules and a few spermatogonia were detected in germinative cells from the DOX-treated group (Fig. 1B). The thickness of the germinative cell layer and the diameter size of ST in DOX group were significantly smaller than that in the control group.

| Table 2. Effect of LBP (*Lycium barbarum* polysaccharides, 200 mg/kg, p.o.) and/or DOX (doxorubicin, 10 mL/kg, i.v.) treatment on epididymal sperm concentrations, sperm motility and abnormal sperm rates in rats (mean ± SEM, n = 7) |
|-----------------|--------|--------|-----------------|-----------------|
|                  | Control | LBP    | DOX             | LBP + DOX       |
| Epididymal sperm concentration (million g⁻¹) | 351.3 ± 11.1 | 361.1 ± 14.1 | 292.7 ± 18.6* | 336.9 ± 16.9 |
| Sperm motility (%) | 72.1 ± 2.8  | 71.6 ± 2.1  | 46.6 ± 7.9* | 63.5 ± 6.8* |
| Abnormal sperm rate (%) | 6.9 ± 0.5  | 7.1 ± 0.6  | 12.3 ± 1.4* | 8.6 ± 0.7* |
| Head             | 2.7 ± 0.2 | 2.7 ± 0.3 | 5.3 ± 1.0* | 3.4 ± 0.4 |
| Tail             | 4.1 ± 0.4 | 4.4 ± 0.5 | 7.0 ± 1.1* | 5.9 ± 0.7 |
| Total            | 6.9 ± 0.5 | 7.1 ± 0.6 | 12.3 ± 1.4* | 8.6 ± 0.7* |

*Significantly different ($p < 0.05$) from respective values in the control group.

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DISCUSSION

Doxorubicin-based chemotherapy induces toxicities in different organs such as heart, kidney, liver and testis (Carvalho et al., 2009). It was reported that the damage induced by DOX is mainly due to the production of ROS, which results in cell damage by lipid peroxidation, protein cross-linking and DNA fragmentation (Minotti et al., 2004; Carvalho et al., 2009). Although the drug concentration is much lower in the testis than in other tissues due to the existence of the blood–testis barrier, DOX treatment still induces a severe injury to the testis and sperm (Hughes et al., 1998). It was partially attributed to the fact that the structure of the male germ cell membrane is rich in polyunsaturated fatty acids and is thereby especially prone to lipid peroxidation (Lenzi et al., 2002). In the present study, it was confirmed that DOX-induced testicular and spermatozoal toxicity is associated with oxidative damage, and it was found that such toxicity could be alleviated or prevented by LBP, a potent antioxidant.

It was reported that four times treatment with DOX can lead to a dose-dependent decrease in absolute and relative weights of reproductive organs, the number of sperms per cauda epididymis as well as the percentage of motile sperms, and an increase in the rate of morphologically abnormal sperms (Kato et al., 2001). The results in the present study were in accordance with these reports. Sperms are targeted by DOX because of their high mitotic activity (Kato et al., 2001). Decreased sperm quantity, together with a decreased number of seminiferous epithelia reported by others, might be the possible reason for the decrease in the testicular weight, which is mainly dependent on the number of germinal epithelia and germ cells. It is widely accepted that oxidative stress and the production of ROS are involved in DOX damage (Carvalho et al., 2009). Oxidative stress status may play a critical role in making sperm DNA highly susceptible to denaturation, fragmentation and aberration, resulting in the induction of sperm abnormalities. In addition, the antitumor mechanism of DOX, namely directly intercalating into the DNA, may also be related to the damage of sperm DNA and the induction of sperm abnormalities (reviewed by Minotti et al., 2004; Menna et al., 2010; Carvalho et al., 2009). The significant reduction in sperm motility may be ascribed to the peroxidization of DOX on the unsaturated fatty acids in sperm plasma membrane and flagellum, the important machinery for membrane fluidity and sperm motility (Sikka, 1996). Furthermore, DOX treatment can cause a decrease in ATP, an energy source for sperm motility, either by depressing the activities of the testicular tricarboxylic acid cycle enzyme which helps to produce ATP, or by destroying the structure of sperm membrane, which is associated with a rapid loss of intracellular ATP (Prahala-than et al., 2006). In the present study, pretreatment with LBP in DOX-treated rats did not show any significant reduction in the testicular weights, sperm concentrations or percentage of motile sperms, nor any obvious increase in abnormal sperm rate, therefore proving LBP to be effective in attenuating the testicular and spermatozoal damage induced by DOX treatment.

Doxorubicin treatment caused a significant increase in MDA level as well as a decrease in GSH level compared with the control group. These results were in agreement with previous reports (Ateşşahin et al., 2006). The increment in MDA level is direct evidence of lipid peroxidation in the testis. Free radicals, generated by DOX, can act on the unsaturated fatty acids in the plasma membrane and decompose them into aldehyde, especially MDA, resulting in the increment in the MDA level and damage to the plasma membrane.

Figure 1. Representative photomicrographs of testis tissue from control (A), DOX (doxorubicin) (B) and LBP (Lycium barbarum polysaccharides) + DOX (C) groups. Pretreatment with LBP significantly attenuated DOX-induced severe degenerative changes of seminiferous tubules. (HE, × 200; 170 × 43 mm (300 × 300 DPI)). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

Table 3. Effect of LBP (Lycium barbarum polysaccharides, 200 mg/kg, p.o.) and/or DOX (doxorubicin, 10 mL/kg, i.v.) treatment on oxidative stress of testis homogenates and plasma level of testosterone in rats (mean ± SEM, n = 5–7)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LBP</th>
<th>DOX</th>
<th>LBP + DOX</th>
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<tbody>
<tr>
<td>MDA (nmol-mg⁻¹ protein)</td>
<td>27.5 ± 1.3</td>
<td>28.2 ± 3.4</td>
<td>54.6 ± 5.5</td>
<td>39.0 ± 2.6</td>
</tr>
<tr>
<td>GSH-Px (U-mg⁻¹ protein)</td>
<td>208.7 ± 8.6</td>
<td>219.1 ± 11.2</td>
<td>129.3 ± 22.0</td>
<td>190.5 ± 10.3</td>
</tr>
<tr>
<td>Testosterone (nmol-g⁻¹ tissue)</td>
<td>25.65 ± 6.76</td>
<td>28.44 ± 8.49</td>
<td>7.46 ± 5.01</td>
<td>19.45 ± 4.50</td>
</tr>
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*aSignificantly different (p < 0.05) from respective values in the control group.

*bSignificantly different (p < 0.05) from respective values in the DOX group.
The decrease in GSH level is more evidence for the excessive generation of ROS in testes. When ROS begin to accumulate, GSH-Px can deoxidize H₂O₂ and lipid peroxides to nontoxic products meanwhile converting GSH to GSSG. Thus the low GSH level represented high oxidative stress to some degree (Minotti et al., 2004; Granados-Principal et al., 2010). The LBP administration to DOX-treated rats successfully reversed the changes in MDA and GHS-Px levels, indicating the amelioration in oxidative stress and consequently the protection of the testis.

Doxorubicin treatment also showed decreased testosterone levels in rat testis in the present study. Testosterone is secreted by the testis and the reduction of testosterone implies injury to the testicular function (Ateşşahin et al., 2006). The histopathological examinations further confirmed the testicular toxicity of DOX. Pretreatment with LBP significantly increased the plasma testosterone level and attenuated the DOX-induced severe degenerative changes of seminiferous tubules in the LBP + DOX group.

Lycium barbarum is well known as a powerful antioxidant Chinese traditional medicine. LBP, the main active components in Lycium barbarum, possesses a large variety of bioactivities, such as antiaging, anticancer, immuno-modulating and antioxidant (Chang and So, 2008; Potterat, 2010). It has been proved that the scavenging activity of 2,2-diphenyl-1-picrylhydroxyl free radical, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) free radical, superoxide anion or hydroxyl radical for crude polysaccharide (1000 µg/mL) from Lycium barbarum were similar to that of vitamin C and BHA (100 µg/mL). Crude LBP also be proved to have a peak level of ferrous chelating ability at 1000 µg/mL (Lin et al., 2009). Much evidence suggests that LBP exhibits cytoprotective effects via attenuation of oxidative stress. LBP (10 mg/kg) was demonstrated significantly to reduce blood glucose, nitric oxide and MDA in streptozotocin-induced diabetic rats (Wu et al., 2006). Furthermore, another study indicated that LBP (20–50 mg/kg) protects liver and kidney tissue from the oxidative damage of streptozotocin-induced diabetic rats (Li, 2007). Our previous study also proved that LBP (200 mg/kg) elicited a typical protective effect on DOX-induced acute cardiotoxicity via suppressing oxidative stress in rats (Xin et al., 2011).

Because Lycium barbarum is known as a fertility-promoting medicine, the protective effect of LBP on testicular tissue was also investigated by a series of studies. LBP was reported to inhibit time- and hyperthermia-induced structural damage and to delay apoptosis in murine seminiferous epithelium in vitro (Wang et al., 2002). Furthermore, LBP was also observed to be able to protect testicular tissue against heat exposure-induced damage and to eliminate DNA oxidative damage of mouse testicular cells induced by H₂O₂ (Luo et al., 2006). The antioxidant effect of LBP was postulated to be responsible for the protective effect to testicular tissue. The present study also indicated that LBP exhibited an indirect effect in alleviating DOX-induced testis damage by increasing the resistance to oxidative stress-induced injury in rats.

There are three possible mechanisms to explain the protective effects of LBP against DOX-caused oxidative stress in the testes: (1) LBP directly removed ROS, suppressed lipid peroxidation of cells, protected the cell membrane from oxidative stress and maintained normal structure and functions of the cells; (2) LBP indirectly scavenged the free radicals by activating antioxidant enzyme systems in the heart tissues to alleviate DOX-induced oxidative injury; (3) LBP chelated with metal ion by forming a cross-bridge between the carboxyl group in galacturonic acid and divalent ion and to decrease the generation of reactive oxygen species.

Apoptosis is also an important cause in DOX-relative toxicity in various tissues (reviewed by Minotti et al., 2004). The mechanisms were involved in direct mitochondrial damage by ROS or indirect mitochondrial depolarization by pro-apoptotic Bcl-2 family proteins (Jürgensmeier et al., 1998; Minotti et al., 2004). Meanwhile, it has been shown that LBP could induce the increased expression of anti-apoptotic protein Bcl-2 as well as the increased ratio of Bcl-2 to Bax in lens epithelial cells of the whole lens incubated in culture medium and exposed to hydrogen peroxide (Wang et al., 2003). The findings suggested that the antiapoptotic properties of LBP may partially contribute to the testis-protective potential of LBP against DOX-treated testicular damage. However, the surmise needs further investigation.

In conclusion, our study, for the first time, illustrated the capability of LBP in attenuating testicular oxidative stress and protecting testis-specific toxicity in DOX-exposed rats. Together with the fact that LBP did not attenuate the antitumor activity of DOX on carcinoma cells (Xin et al., 2011), these results indicate that LBP merits further investigation as a useful adjunct during the administration of DOX.

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Conflict of Interest
The authors have declared that there is no conflict of interest.


