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Lycium barbarum polysaccharides: Protective effects against heat-induced damage of rat testes and H_2O_2-induced DNA damage in mouse testicular cells and beneficial effect on sexual behavior and reproductive function of hemicastrated rats

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Abstract

Lycium barbarum, a famous Chinese medicinal herb, has a long history of use as a traditional remedy for male infertility. Polysaccharides are the most important functional constituent in *L. barbarum* fruits. We systematically investigated the effect of *L. barbarum* polysaccharides (LBP) on rat testis damage induced by a physical factor (43 °C heat exposure), on DNA damage of mouse testicular cells induced by a chemical factor (H_2O_2), and on sexual behavior and reproductive function of hemicastrated male rats. The results showed that LBP provided a protective effect against the testicular tissue damage induced by heat exposure. When compared with negative control, a suitable concentration of LBP significantly increased testis and epididymis weights, improved superoxide dismutase (SOD) activity, and raised sexual hormone levels in the damaged rat testes. LBP had a dose-dependent protective effect against DNA oxidative damage of mouse testicular cells induced by H_2O_2. LBP improved the copulatory performance and reproductive function of hemicastrated male rats, such as shortened penis erection latency and mount latency, regulated secretion of sexual hormones and increased hormone levels, raised accessory sexual organ weights, and improved sperm quantity and quality. The present findings support the folk reputation of *L. barbarum* fruits as an aphrodisiac and fertility-facilitating agent, and provide scientific evidence for a basis for the extensive use of *L. barbarum* fruits as a traditional remedy for male infertility in China.
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Introduction

Lycium barbarum L. belongs to the plant family Solanaceae. Red-colored fruits of *L. barbarum* have been used as a traditional Chinese herbal medicine for thousands of years (Gao et al., 2000). The earliest known Chinese medicinal monograph documented medicinal use of *L. barbarum* around 2300 years ago. *L. barbarum* fruits have a large variety of biological activities and pharmacological functions and play an important role in preventing and treating various chronic diseases, such as diabetes, hyperlipidemia, cancer, hepatitis, hypo-immunity function, thrombosis, and male infertility (Gao et al., 2000; Li, 2001). *L. barbarum* polysaccharides (LBP) isolated from the red-colored fruits are the most important functional factor (Qi et al., 2001; Peng et al., 2001b; Wang et al., 2002a; Gan et al., 2003, 2004; Zhang et al., 2005). Five *L. barbarum* polysaccharides (LbGp1–LbGp5) were separated and structurally elucidated (Peng et al., 2001a,b; Peng and Tian, 2001).

There have been some reports on the antifertility effects and fertility-promoting or aphrodisiac effects of medicinal plant extracts (Arletti et al., 1999; Mazaro et al., 2002; Carro-Juárez et al., 2004), but few researchers have reported on the effects of plant polysaccharides on male reproductive function. The pro-

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fertility effect of *L. barbarum* fruits was first described in the sixteenth century by the great Chinese herbalist Li Shizhen, and nowadays *L. barbarum* fruits are included in most fertility-promoting Chinese herbal remedies (Wang et al., 2002b). However, the male fertility-promoting mechanism of action of *L. barbarum* fruits remains largely unclear. In recent decades, some Chinese clinical studies have shown that *L. barbarum* fruits cause a significant improvement in the quality of sperm (Yin and Guo, 1993; Li, 2001). Wang et al. (2002b) reported that LBP could inhibit time- and hyperthermia-induced structural damage in murine seminiferous epithelium, and delay apoptosis in this system, under both normothermic and hyperthemic culture conditions. Although many important findings about biological activities and pharmacological functions of LBP have been obtained, there has been little information concerning the effect of LBP on reproductive system and function.

For the last decade, we have been conducting systematic studies on the biological activities and functions of crude or pure LBP from *L. barbarum* fruits, such as hypoglycemic, hypolipidemic, antifatigue, antioxidant and immune activity effects (Luo et al., 1997, 1999a,b, 2004). The objectives of this study are: (1) to evaluate protective effects of LBP against damage by a physical factor (heat exposure) in rat testes; (2) to assess protective effects of LBP against DNA damage induced by a chemical factor (H_2O_2) in mouse testicular cells; and (3) to investigate stimulating effects of LBP on sexual behavior and reproductive function of hemicastrated male rats.

Materials and methods

Plant materials and chemicals/reagents

Dried fruits of *L. barbarum* were purchased in a local market. They were from Ningxia, a well-known production area for *L. barbarum* in China. Lauryl sarcosine, hydrogen peroxide (H_2O_2), Na_2EDTA, and ethidium bromide (EtBr) were purchased from Sigma Chemical Co. (St. Louis, MO) and Dulbecco's modified Eagle's medium (DMEM), low melting point agarose, and normal melting point agarose were from GIBCO Co. (Grand Island, NY). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were from Jiancheng Bioengineering Institute (Nanjing, China). Testosterone (T) reagent kits, luteinizing hormone (LH) radioimmunoassay kits, follicle stimulating hormone (FSH) and estradiol (E_2) radioimmunoassay kits were obtained from Jiuding Ltd. Co. (Tianjin, China). Testosterone propionate, progesterone, and estradiol benzoate were from Jinyao Amino Acids Ltd. Co. (Tianjin, China). Chloroform, methanol, ethanol, and propanone were from Shanghai Reagents Co. (Shanghai, China). Other chemicals and reagents used were obtained from Sigma. All chemicals and reagents were analytical grade.

Experimental animals

Thirty-six adult male Wistar rats weighing from 180 to 220 g were used for assessing protective effect of LBP on reproductive system (tissues) damaged by physical factors (heat exposure), and 6 adult male Kong Ming mice (20±2 g) for investigating effect of LBP on DNA damage induced by chemical factors (H_2O_2). Ninety-two adult Wistar rats (46 male and 46 female) weighing from 180 to 220 g were used for examining the sexual behavior and reproductive function. They were housed in groups of two in rectangular cages (40×25×20 cm) with wire mesh lids under standardized animal room conditions (12 hr light/dark photoperiod at ~23 °C and ~60% humidity). Food in pellets and tap water were available ad libitum. Animal experiments were performed according to the guidelines for the care and use of laboratory animals established by Wuhan University (Wuhan, China), which are in accordance with the Declaration of Helsinki of the World Medical Association (Wang et al., 2002b).

Preparation of L. barbarum polysaccharides (LBP)

L. barbarum fruits were dried at 60 °C and ground to fine powder. The ground powder samples were refluxed to remove lipids with chloroform:methanol solvent (2:1) (v/v). After filtering, the residues were air-dried, and then refluxed again with 80% ethanol at 80 °C to remove oligosaccharides. The residues were extracted four times in boiled water and filtered. The combined filtrates were concentrated by a rotavapor at 60 °C, and then precipitated using 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried. The dried *L. barbarum* polysaccharides (LBP) obtained were stored in a refrigerator till further use.

Assessment of protective effect of LBP on rat testes damaged by a physical factor (heat exposure)

The treatment of heat exposure followed the method of Miura et al. (2002) with minor modifications. The tested rats were anesthetized with sodium pentobarbital, and then their four legs were tied on vertical holders. Their scrota containing the testes were submerged in warm water (43 °C) for 15 min. Thirty-six male rats were randomly assigned to one of six treatment groups (n=6 for each group), including four experimental groups with four different concentrations of LBP (10, 50, 100, and 200 mg/kg·per day) (43 °C heat exposure) and two control groups, i.e., a negative control group (0.9% saline) (43 °C heat exposure), and a normal control group (0.9% saline) without heat exposure. The rats in the experimental groups were administered through gastric gavage with four different concentrations of LBP dissolved in 0.9% normal saline one time per day for 14 consecutive days, while two control groups received the same volume of 0.9% normal saline without LBP every day. After 15 days, the rats of the negative control and four experimental groups were exposed to heat. Twenty-four hours after heat exposure, blood samples were drawn from the femoral artery of rats for measuring sexual hormones (i.e., T, LH, and FSH) in plasma, and then all tested rats (including normal control group) were sacrificed. The testes and epididymides were removed and weighed (mg/100 g body weight).

The testicular tissues were taken, treated and fixed in 10% polyformaldehyde and paraffin wax, and sliced in 4 μm sections and stained with hematoxylin and eosin (H&E) for morphological observation using an optical microscope at a 100× magnification. Protein content of the testicular tissues was determined by the Lowry method (Lowry et al., 1951). MDA levels and SOD activity of the testicular tissues were examined following the kit instructions. MDA assay was measured by the thiobarbituric acid (TBA) colorimetric method (Ohkawa et al., 1979). SOD assay was based on the ability to inhibit oxidation of oxyamine by the xanthine–xanthine oxidase system. MDA and SOD values were calculated using the optical density (OD) values observed (532 nm for MDA and 550 nm for SOD) and protein content, and expressed as nmol/mg protein and U/mg protein, respectively. The levels of serum T, LH, and FSH were determined with traditional radioimmunoassay (RIA) methods and followed the procedure instructions of the corresponding kits.

Assessment of protective effect of LBP against DNA damage induced by a chemical factor (H_2O_2) in mouse testicular cells

Male mice were sacrificed by cervical dislocation. Separation and preparation of mouse testicular cells followed the method described by Zhang et al. (2001). The isolated testicular cells (10^6–10^7 cells/mL) were randomly divided into three sample groups. All cell samples (1 mL testicular cell suspension +2 mL DMEM culture solution) were incubated at 37 °C. The cells of the LBP+H_2O_2 group were treated with four different concentrations of LBP (50, 100, 200, and 400 μg/mL, respectively), and 1 h later 30 μL of 100 μmol/L H_2O_2 was added and incubated for 25 min. The cells of the normal control group were treated with 30 μL of double distilled water. The cells of the negative control group were treated with 30 μL of 100 μmol/L H_2O_2. DNA damage in mouse testicular cells was estimated using the comet assay as reported by Singh et al. (1988) with minor modification, i.e., single cell gel electrophoresis (SCGE) which was performed under alkaline conditions following the procedure of Zhang et al. (2001). Three slides were prepared for each treatment. Two hundred cells per slide were randomly counted for tail frequency and 25 cells measured for tail length. Comets were observed using an Olympus BX51 fluorescence microscope attached to a solid-state camera (Olympus, Japan). Tail frequency (%) (the percentage of the cells with tail) and tail length (μm) (=maximum total length between comet head and tail−head diameter) were used to assess the DNA damage levels.

Investigation of the stimulating effect of LBP on sexual behavior and reproductive function of hemicastrated rats

The female rats were ovariectomized under anesthesia, and after 2 weeks brought into estrous by subcutaneous injection of estradiol benzoate (200 μm/kg) 48 h before testing, and by subcutaneous injection of progesterone (2 mg/kg) 4 h before testing. They were screened with non-experimental sexually experienced males and only those exhibiting good sexual receptivity (solicitation behavior and lordosis in response to mounting) and no rejection behavior, were used (Arletti et al., 1999). Forty-six screened female rats were used for the mating experiment in this study.

Forty-six sexually vigorous male rats (ejaculation latency shorter than 15 min in at least the last three sessions) were also screened for the mating experiment. They were randomly divided into experimental group ($n=12$), positive control group ($n=12$), negative control group ($n=12$), and normal control group ($n=10$). Except for the rats of normal control group, the rats were anesthetized through intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg), and castrated/orchidectomized via a midline scrotal incision (2 cm) to allow unilateral (right) testis removal. Three days after the surgery, the male rats of the positive control group got a subcutaneous injection of testosterone propionate (2 mg/kg). The male rats of the experimental group received 10 mg/kg of LBP (the dose used was confirmed to be more effective in our preliminary experiment) dissolved in 0.9% normal saline, whereas the male rats of negative control and normal control groups received 10 mg/kg of 0.9% normal saline per day through gastric gavage for 21 consecutive days.

Sexual behavior parameters observed in this study included erection latency, mount latency, and percentage of mount. Fourteen days after the start of gastric gavage, erection latency of the tested male rats was recorded using a BL-420E biological function system (electronic stimulating instrument) (Taimong Co., Chengdu, China). Erection latency was time (in seconds) from first stimulus to penis erection. Twenty-one days after the start of gastric gavage, major sexual behavior parameters of the tested male rats were investigated in a sound-proof dark room under a dim red light. After a 10-min adaptation period in a plastic observation cage, a stimulus-receptive female was introduced to the male by dropping it gently into the cage. The following parameters were recorded within 15 min: mount and intromission latency, time from introduction of the female to the occurrence of the first mount or intromission; ejaculation latency, time from the first intromission to ejaculation; percentage of mount (mounting rats). Tests were terminated immediately after the first postejaculatory intromission; or if intromission did not occur within 15 min of the introduction of the female, or if ejaculation latency exceeded 30 min (Arletti et al., 1999).

Twenty-two days after the start of gastric gavage, blood samples were drawn from the femoral artery of the tested rats for determining serum sexual hormones (T, LH, FSH, and E_2) (see method described above), and then all tested rats were sacrificed by cervical dislocation. The left testes and epididymides of the tested rats were removed to carefully separate and weigh foreskin gland, seminal vesicle-prostate gland, and laevator ani muscle (LAM) and weights are expressed as milligram per 100 gram body weight (mg/100 g). Sperm count and sperm motility from proximal caudal epididymides were investigated. The epididymidimal tissues were mashed and homogenized in a 10-mL tube with 6 mL of normal saline and incubated at 37 °C for 10–15 min to make the sperms fully dissociate and to release the sperms. The sperms were counted

using a haemocytometer under an optical microscope (×200) and expressed as sperm number per milliliter (no.×10^6/mL). The sperm motility (%) was assessed by counting at least 200 sperms and calculated by the formula: (number of motile sperms×100%)/(total number of motile and immotile sperms). All sample analysis of sperms was conducted immediately after the sperm samples were collected.

Statistical analysis

All data are expressed as mean±standard deviation (S.D.). All calculations and statistical analyses were performed with SPSS software for Windows version 10.0 (SPSS Inc., Chicago, IL). The significance of the difference between the means of the control and treated groups was evaluated by Student's *t*-test or χ^2-test. Significance was set as $P<0.05$ or $P<0.01$.

Results

Protective effect of LBP on rat testes against damage by a physical factor (heat exposure)

To evaluate effect of heat treatment on rat testis tissues, the scrota of rats were immersed at 43 °C for 15 min. The weights of testes and epididymides, SOD activity, and serum sexual hormone (T, LH, and FSH) levels in the heat-exposed rats (negative control) were significantly ($P>0.01$ or $P>0.05$) lower while MDA levels were significantly ($P>0.01$) higher than those of the heat-unexposed rats (normal control) (Table 1). LBP had a protective effect on rat testes against damage by heat exposure. When compared with negative control, LBP treatment at three concentrations (10, 50, and 100 mg/kg·per day) significantly ($P>0.01$ or $P>0.05$) increased testis and epididymis weights in the damaged testes, and all four concentrations (10, 50, 100, and 200 mg/kg·per day) of LBP treatments increased SOD activity and reduced MDA levels significantly ($P>0.01$ or $P>0.05$). Also, LBP (10 mg/kg per day) significantly ($P>0.01$) increased T, LH, and FSH levels, in comparison to negative control. Other concentrations (50, 100, and 200 mg/kg·per day) of LBP also increased T, LH, and FSH levels, but mostly not significantly (Table 1).

Histological examination showed normal morphological features in the testis of normal controls. The seminiferous tubule showed successive stages of transformation of spermatogonum into spermatozoa and compact and regular arrangements of cells. Seminiferous epithelium images were made up of spermatogonum, spermatocytes, spermatids, and sperms (Fig. 1A). The testis of heat-exposed rats (negative control) had significant degenerative changes and destruction (Fig. 1B). Seminiferous tubules became irregular (thin and small). The lumen was filled with cellular debris. Both spermatids and sperms disappeared. Few spermatogonum were observed in certain seminiferous tubules. Inside spermatocytes, chromatin agglutination, unclear nuclear membrane, sparse nucleoplasm, and "vacuoles" in nucleus were observed. This indicated that the testis tissues of negative control were significantly damaged by heat exposure. Administration of various doses of LBP partly restored the morphological structure of seminiferous tubules in the damaged testis. The examined results showed better protective effect on the damaged testis in the LBP 10 mg/kg·per day group than other LBP groups (50, 100, and 200 mg/kg·per day). The histological results were consistent with the results of the above biochemical assays. To some extent, the morphological structure of seminiferous tubules for the LBP 10 mg/kg·per day group (Fig. 1C) was close to that of normal control (Fig. 1A), compared with negative control (Fig. 1B). Most seminiferous tubules had intact structure and spermatogona arrangements were basically regular.

LBP protection against DNA damage of mouse testicular cells induced by a chemical factor (H_2O_2)

From comet images by fluorescent microscopy, the undamaged DNA is recognized as a fluorescent core, while the presence of strand breaks in the chain (damaged DNA) causes DNA to migrate and form a tail ("comet") during the electrophoresis (Singh et al., 1988; Zhang et al., 2001). The bigger and more fluorescent the tail, the greater the DNA damage that has been induced. Microscopic examination did not reveal any DNA damage in normal control (untreated by H_2O_2), and the shape of the testicular cells was rounded fluorescent head without a tail (Fig. 2A). Mouse testicular cells in negative control (H_2O_2) exhibited obvious breakage of DNA chains induced by H_2O_2. The damaged DNA fragments formed longer comet tail towards anticathode (Fig. 2B). The results for mouse testicular cells treated with different concentrations of LBP (50,

Table 1
Protective effect of LBP on rat testes damaged by heat exposure (43 °C) ($n=6$ for each group)[a]

Group	Testes (mg/100 g body weight)	Epididymides (mg/100 g body weight)	SOD (U/mg protein)	MDA (nmol/mg protein)	T (nmol/L)	LH (IU/L)	FSH (IU/L)
Normal control[b]	519±122*	192±33**	302.87±67.72**	0.78±0.49**	24.35±3.43**	11.09±0.91*	4.19±1.01*
Negative control (43 °C)[b]	367±56	132±13	206.30±24.55	2.78±1.26	16.09±4.92	8.71±1.48	2.92±0.59
10 mg/kg·per day LBP (43 °C)	549±78**	209±24**	387.41±44.38**	1.11±0.44**	27.05±4.17**	12.60±1.96**	4.71±1.24**
50 mg/kg·per day LBP (43 °C)	526±69*	186±31**	274.41±18.98*	1.24±0.50*	19.51±5.65	10.61±1.62	3.94±0.85
100 mg/kg·per day LBP (43 °C)	493±77*	165±17*	270.08±47.60*	1.39±0.66*	18.41±5.51	10.93±1.84**	3.67±1.42
200 mg/kg·per day LBP (43 °C)	465±86	169±33	272.64±28.22*	1.69±0.90*	17.21±4.61	10.98±1.99*	3.41±0.71

[a] *$P<0.05$ and **$P<0.01$, compared with negative control group. SOD, superoxide dismutase; MDA, malondialdehyde; T, testosterone; LH, luteinizing hormone; and FSH, follicle stimulating hormone.
[b] 0.9% normal saline per day through gastric gavage.

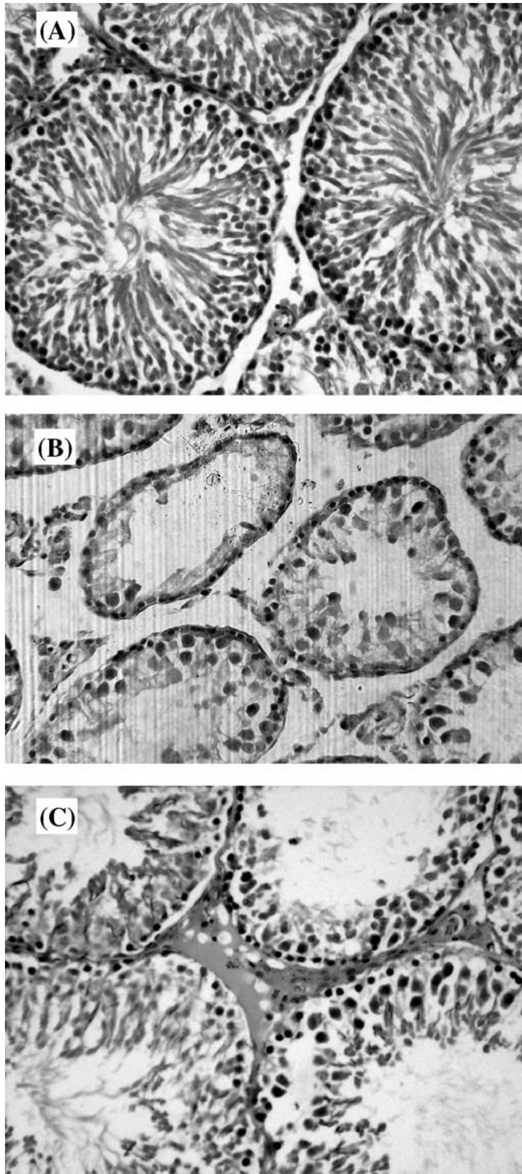

Fig. 1. Typical photomicrographs (×200) of the seminiferous tubules of rat testis (H&E staining). (A) Normal control showing normal morphological features, with all the successive stages of spermatogenesis, and lumen filled with spermatozoa. (B) Negative control (43 °C heat-exposed) showing serious destruction and deterioration of the tubules in the testis, lumen is filled with cellular debris and is devoid of spermatids and sperms. (C) The administration of LBP (10 mg/kg·per day) after 43 °C heat-exposure showing partial recovery effect of LBP on the seminiferous tubules of the damaged testis.

100, 200, and 400 μg/mL) for 1 h and then 30 μL of H_2O_2 (100 μmol/L) for 25 min indicated that DNA damage was significantly attenuated, as compared with negative control. LBP treatment (50 μg/mL) showed that the damaged DNA fragments formed shorter comet tails (Fig. 2C), while cells given LBP treatment at 400 μg/mL exhibited few shorter comet tails (not shown). Mean tail frequency (%) and tail length (μm) showed that, when compared with negative control, pretreatments of LBP (50, 100, 200, and 400 μg/mL) significantly ($P<0.01$) reduced the frequencies of cells with tail and the tail length of the damaged testicular cells (Table 2). The results indicated that LBP had a dose-dependent protective effect on DNA oxidative damage induced by H_2O_2.

Stimulating effect of LBP on sexual behavior and reproductive function of hemicastrated male rats

Compared with negative control (10 mg saline/kg per day), both LBP group (10 mg/kg per day) and positive control (TP, testosterone propionate, 2 mg/kg per day) significantly improved the copulatory performance of hemicastrated male rats, i.e., significantly ($P<0.01$) shortened penis erection latency and mount latency, and clearly ($P<0.05$) improved the percentage of mounting (Table 3). Moreover, the stimulating effect of LBP and TP (positive control) on sexual behavior of the hemicastrated male rats was slightly better than that of normal control rats without castration (10 mg saline/kg per day).

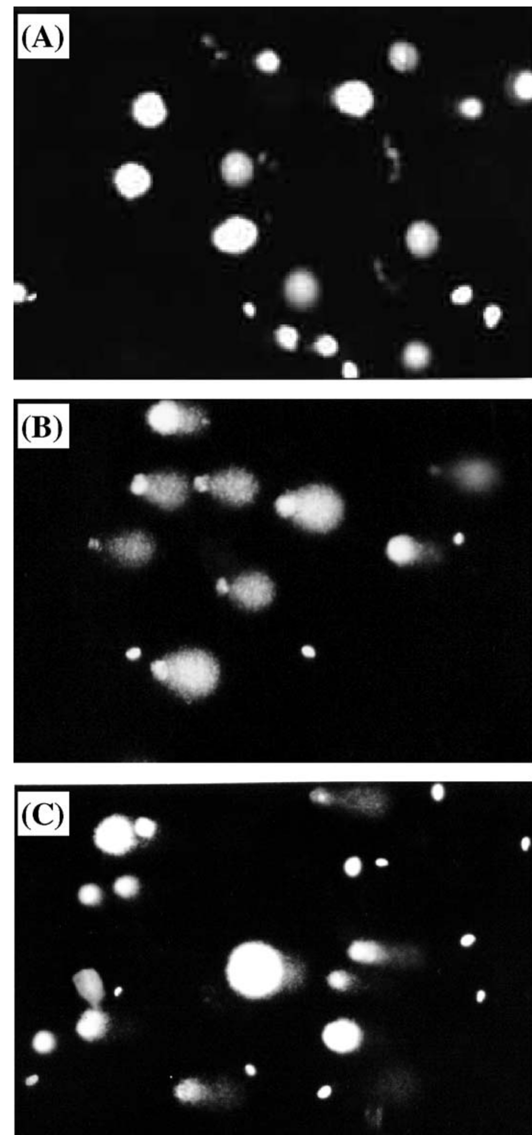

Fig. 2. Examples of comet photomicrographs (×200) of mouse testicular cells. (A) Normal control (H_2O). (B) Negative control (H_2O_2). (C) LBP (50 μg/mL) $+H_2O_2$.

Table 2
Protective effect of LBP on DNA damage induced by H_2O_2 in mouse testicular cells[a]

Group	Tail frequency (%) (n=200)	Tail length (μm) (n=25)
Normal control (distilled water) (30 μL)	5	8.59±2.26
Negative control (H_2O_2) (30 μμL)	96	64.64±15.85
LBP (50 μg/mL)+H_2O_2 (30 μL)	61 *	39.39±15.60 **
LBP (100 μg/mL)+H_2O_2 (30 μ)	55 *	38.63±12.61 **
LBP (200 μg/mL)+H_2O_2 (30 μL)	46 *	34.84±7.84 **
LBP (400 μg/mL)+H_2O_2 (30 μL)	42 *	30.80±11.22 **

[a] Concentration of H_2O_2, 100 μmol/L.
* $P<0.01$ (χ^2 test), compared with negative control group.
** $P<0.01$ (t-test), compared with negative control group.

Serum hormone (T, LH, FSH, and E_2) levels of the tested male rats were also determined in the present study. The order of serum T levels in different groups was: positive control > normal control > LBP group > negative control (Table 3). The differences in T levels between each group and negative control were significant ($P<0.01$). However, serum LH and FSH levels of different groups seemed similar. Additionally, serum E_2 levels of positive control, normal control, and LBP group were significantly ($P<0.01$ or $P<0.05$) lower than that of negative control.

Accessory reproductive organ weights of different groups were measured (Table 4). The differences between each group and negative control were significant ($P<0.01$ or $P<0.05$). The weights of foreskin gland, seminal vesicle-prostate and LAM of LBP group were significantly ($P<0.05$) higher than those of negative control, and clearly higher than (only for foreskin gland) or close to those of normal control (Table 4). The size changes of seminal vesicle-prostate of different groups were compared (Fig. 3). The seminal vesicle-prostate of positive control (Fig. 3A) was biggest, and the seminal vesicle-prostate of negative control (Fig. 3B) was smallest and seemed to shrink. The seminal vesicle-prostate of LBP group (Fig. 3C) was close to that of normal control (Fig. 3D). The results indicated that LBP treatment could significantly increase the weights of foreskin gland, seminal vesicle-prostate and LAM in the hemicastrated male rats. However, the foreskin gland weight of normal control was significantly lower than that of negative control. It might result from orchidectomy. Additionally, both sperm count (concentration) and motility (vitality) of the negative controls were significantly ($P<0.01$) lower than LBP group and other two controls (Table 4).

Discussion

L. barbarum fruits are well known in traditional Chinese herbal medicine and nowadays are widely used as a popular functional food. LBP, a most important functional component in *L. barbarum* fruits, possesses a large variety of bioactivities, such as antiaging, anticancer, immuno-modulating, hypoglycemic, hypolipidemic, antioxidant, antifatigue, and male fertility-facilitating (Gao et al., 2000; Wang et al., 2002a,b; Gan et al., 2003, 2004; Luo et al., 1999a,b, 2004; Zhang et al., 2005). However, the effect of LBP on male reproductive system and function and the related mechanism of action are not well understood.

SOD is a highly specific scavenging enzyme for superoxide radicals ($O_2^{\bullet -}$) and MDA is a reactive end product of lipid peroxidation. SOD activity and MDA level can reflect the degree of damage of testicular tissues induced by ROS (reactive oxygen species, e.g., $O_2^{\bullet -}$ and $OH^{\bullet}$). The ROS-induced injury normally causes decrease of SOD activity and increase of MDA level in testicular tissues. It is well known that a lower scrotal temperature is required for normal spermatogenesis in most mammalian species because testicular germ cells are vulnerable to heat stress and undergo apoptosis in response to increased scrotal temperature (Ikeda et al., 1999; Miura et al., 2002). SOD is most sensitive to heat, and heat exposure leads to the decrease of the SOD activity in rat testicular tissues. SOD depletion in spermatozoa is thought to be associated with male infertility. SOD activity in spermatozoa showed a significant correlation to the number of motile spermatozoa and MDA concentration was significantly related to the number of immotile spermatozoa (Kobayashi et al., 1991). This study showed that after negative control was exposed to heat (43 °C, 15 min), the SOD activity significantly decreased and the MDA level significantly increased in the testicular tissues ($P<0.01$) (Table 1). This indicated that the antioxidant system of the testicular tissues in negative control rats had already been damaged. Excessive free radicals in the testicular tissues resulted in development alteration of testicular reproductive cells and retarded sperm growth. LBP possessed strong antioxidant activity and antiperoxidation effect (Zhang, 1993; Luo et al., 2004). All four LBP treatments in the experimental group significantly

Table 3
Effect of LBP on sexual behavior and serum sexual hormone levels of hemicastrated (HC) male rats

Group	n	Erection latency (second)	Mount latency (min)	Percentage of mount (%)	T (nmol/L)	LH (IU/L)	FSH (IU/L)	E_2 (pg/mL)
Normal control (saline)[a]	10	20.56±4.75**	4.80±1.30**	62.5*	13.87±2.83**	14.57±4.79	5.22±1.68	13.98±3.72**
Negative control (HC+saline)[a]	12	27.75±6.67	8.14±1.95	58.3	2.16±0.60	15.21±3.33	4.89±1.54	19.27±3.03
Positive control (HC+TP)[b]	12	16.77±3.30**	4.45±1.57**	84.6*	18.80±4.82**	14.39±2.96	4.66±1.97	13.20±3.30**
HC+LBP[c]	12	17.23±3.83**	4.64±1.86**	78.6*	6.64±1.64**	14.51±2.54	4.99±1.57	16.41±2.78*

*$P<0.05$ and **$P<0.01$, compared with negative control. T, testosterone; LH, luteinizing hormone; FSH, follicle stimulating hormone; E_2, estradiol.
[a] Saline, 0.9% normal saline (10 mg/kg·per day).
[b] TP, testosterone propionate (2 mg/kg·per day).
[c] LBP, 10 mg/kg·per day.

Table 4
Effect of LBP on weight of accessory sexual organs (reproductive glands) and sperm quantity and quality in hemicastrated (HC) male rats

Group	*n*	Foreskin gland (mg/100 g)	Seminal vesicle-prostate (mg/100 g)	Laevator ani muscle (LAM) (mg/100 g)	Sperm count (no. sperm × 10^6/mL)	Sperm motility (%)
Normal control (saline) [a]	10	9.55 ± 14.21*	520.51 ± 162.75*	61.46 ± 12.80*	13.15 ± 3.36**	63.00 ± 10.69**
Negative control (HC + saline) [a]	12	33.96 ± 10.05	349.97 ± 88.55	43.22 ± 14.57	7.47 ± 2.75	45.17 ± 11.59
Positive control (HC + TP) [b]	12	55.53 ± 17.12**	1032.88 ± 235.16**	81.62 ± 23.94**	13.63 ± 2.29**	64.46 ± 12.67**
HC + LBP [c]	12	48.83 ± 14.36*	509.33 ± 175.10*	60.65 ± 19.90*	12.56 ± 3.74**	63.36 ± 11.91**

*$P<0.05$ and **$P<0.01$, compared with negative control.
[a] Saline, 0.9% normal saline (10 mg/kg·per day).
[b] TP, testosterone propionate (2 mg/kg·per day).
[c] LBP, 10 mg/kg·per day.

($P<0.01$ or $P<0.05$) increased the SOD activity and decreased the MDA level of rat testicular tissues damaged by heat exposure (Table 1). This indicated that LBP could effectively scavenge free radicals, suppress lipid peroxidation, and alleviate the damage to spermatogenic cells induced by heat exposure. The 10 mg/kg·per day treatment was the most effective among four LBP treatments.

Our results were similar to the findings of Lue et al. (2000) who reported that testis weight and testicular sperm counts of the male rats in the heat exposure group (43 °C, 15 min) were decreased to 65.4% and 28.9% of control levels, respectively. In this study, LBP could clearly increase the weights of reproductive organs (testes and epididymides) in heat-exposed rats (Table 1). Histological examination (Fig. 1) showed that LBP could alleviate the harm to the testicular tissue cells caused by heat exposure and partly restore the morphological structure of seminiferous tubules in the damaged testis. The results also indicated that low concentration of LBP (10 mg/kg·per day) had the best protective effect on rat testes damaged by heat exposure, and followed by 50 and 100 mg/kg·per day. However, 200 mg/kg·per day did not have a significant effect (Table 1).

H_2O_2, a strong oxidant and a common chemical in DNA damage, can easily penetrate cell membrane directly into cell nucleus without enzymatic degradation. H_2O_2, one of the main ROS, has been demonstrated to cause lipid peroxidation and DNA damage in cells (Halliwell and Aruoma, 1991). Also,

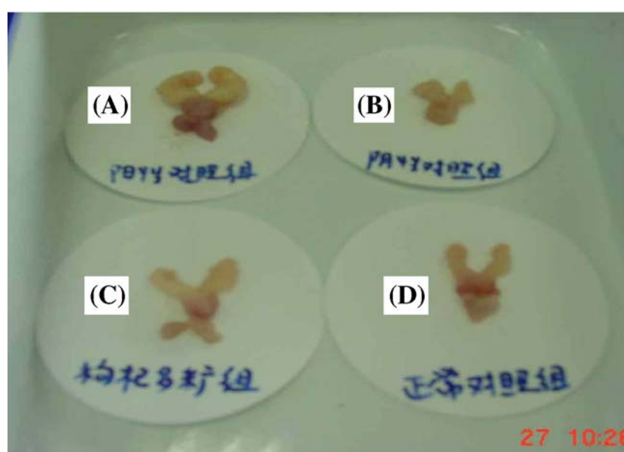

Fig. 3. Size changes of seminal vesicle-prostate glands from positive control (A), negative control (B), LBP treatment (C), and normal control (D).

H_2O_2 reacts with Fe^{2+} or Ca^{2+} to produce the hydroxyl radical ($OH^\bullet$) (e.g., $H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + OH^- + Fe^{3+}$). $OH^\bullet$ has strong oxidation ability. Exposure of DNA to oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases and causing serious DNA damage (Slupphaug et al., 2003).

LBP had significant protective effects against H_2O_2-induced DNA damage in the mouse testicular cells and acted in a dose-dependent manner (Table 2 and Fig. 2). There were two possible antioxidant pathways: (1) LBP directly removed H_2O_2 or scavenged $OH^\bullet$ induced by H_2O_2, suppressed lipid peroxidation of testicular tissues cells, protected cell membrane from oxidative stress, and lowered the DNA damage of the testicular cells to maintain normal structure and functions of the cells; (2) LBP indirectly scavenged the free radicals by activating antioxidant enzyme systems in the testicular tissues to alleviate the DNA damage induced by H_2O_2. In most cells there exist multiple protective mechanisms (antioxidant enzyme systems) designed to prevent ROS-induced injury, such as SOD, glutathione peroxidase (GSH-PX), and catalase (Mennella and Jones, 1980; Sanocka et al., 1997). The present study and a previous study (Wang et al., 2002a,b) indicated that LBP could significantly increase the SOD activity and decrease the MDA level to protect testes and spermatozoa against oxidative damage and lipid peroxidation.

"Function Assessment Procedures and Examination Methods of Health Foods" was published by the Bureau of Health Supervision, Ministry of Health, China (Bureau of Health Supervision, 1998), and reported how to evaluate and judge whether health foods could improve male sexual behavior and function. There are two major assessing experiments, i.e., copulatory experiment and penis erection experiment of castrated animals. If a positive result is obtained in the mating experiment or the erection experiment, the tested health food is considered as confirmed to improve male sexual behavior and function. In this study, a hemicastrated male rat model was established to assess the effect of LBP on male sexual behavior. The results showed that LBP could significantly shorten penis erection latency and mount latency and clearly improve the copulatory performance of hemicastrated male rats, as compared with negative control (Table 3), indicating that LBP can be used as a potential natural aphrodisiac. However, this study did not follow all standard measurements for sexual behavior parameters. Standard measurements for sexual behavior may

include mount latency, intromission latency, ejaculation latency, postejaculatory interval, and additional parameters (Ågmo, 1997). Further assessment of the effect of LBP on male sexual behavior is warranted in the future.

Under normal physiological conditions, serum T, LH, FSH, and E_2 levels in male testes are well balanced (Soderstein et al., 1980; Engelking, 2000). One to three days after castration, major sexual hormone levels decrease and E_2 level increases significantly. T and E_2 are a pair of opposite hormones and their levels in the testes are highly negatively linearly correlated. One of the main physiological functions of testosterone (T) is to promote spermatogenesis. The decrease of T level is an obstacle to spermatogenesis and reduces sperm number and motility. Our investigation showed that the T level in negative control decreased significantly while the E_2 level increased, as compared with normal control (Table 3). When compared with negative control, LBP treatment could significantly ($P<0.01$) increase the T level of hemicastrated male rats. The results suggested that LBP might adjust the secretion of serum sexual hormones and increase sexual hormone levels in the hemicastrated male rats, although the effect of LBP was inferior to that of testosterone propionate. Additionally, the results of sperm count and motility indicated that LBP treatment could significantly ($P<0.01$) improve sperm quantity and quality in the hemicastrated rats (Table 4). This was consistent with the results of Yin and Guo (1993) who conducted a clinical experiment of *L. barbarum* fruits on a cohort of 42 patients with low sperm count and/or low sperm motility. The patients were administered 15 g of *L. barbarum* fruits per day for 4 months. It was found that the sperm quality of 79% patients was significantly improved.

The weights of accessory sexual organs of male animals are usually associated with androgen activity and function. Androgens can stimulate the growth of accessory sexual organs (e.g., foreskin gland, seminal vesicle, and prostate) and increase their weights (Zhang et al., 2002). If certain drugs or natural compounds can increase the weights of accessory sexual organs, they should have similar androgen effect (Cheng et al., 1997). In this study, we determined the weights of foreskin gland, seminal vesicle-prostate, and LAM in LBP treatment, positive control (testosterone propionate), negative control, and normal control (Table 4). Because hemicastration of rats reduced the secretion of sexual hormones, both the T level and the weights of accessory sexual organs in negative control decreased significantly ($P<0.01$), as compared with normal control (without castration). LBP treatment could increase ($P<0.05$) both the T level and the accessory sexual organ weights of hemicastrated rats, as compared with negative control (Tables 3 and 4), and exhibited similar androgen effect. However, the LBP effect was lower than positive control and similar to normal control. This indicated that the adjusting effect and function of LBP did not exceed normal level.

In summary, the present study indicated that LBP showed protective effects against damage to the testicular tissue of male rats induced by a physical factor and on the DNA damage to mouse testicular cells induced by a chemical factor. LBP also improved the sexual behavior and reproductive function of hemicastrated rats. The results effectively support the folk reputation of *L. barbarum* fruits as sexual stimulants and aphrodisiac for traditional remedy of male infertility in China.

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