Protective effects of *Lycium barbarum* polysaccharide on male sexual dysfunction and fertility impairments by activating hypothalamic pituitary gonadal axis in streptozotocin-induced type-1 diabetic male mice

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**Abstract.** Diabetes-associated male sexual dysfunction and fertility impairments are both common clinical complications with limited therapeutic options; hence it seriously affects the quality of life of the patients, in particular, the patients of reproductive age. *Lycium barbarum* polysaccharide (LBP) has long being believed to maintain and to promote reproductive functions in the traditional medical practice in China. The current study was to investigate if LBP may contribute to recovery of male sexual dysfunction and fertility impairments in diabetic individuals. The effects of LBP on sexual behaviors and histological changes of testis were studied in the type-1 diabetes male mice induced by intra-peritoneal (i.p.) injection of streptozotocin (STZ). After oral administration of LBP (10, 20 or 40 mg/kg), sildenafil citrate (SC, 5 mg/kg) or saline for 62 consecutive days, the typical abnormal changes in the sperm parameters, in relative weight of reproductive organs, and in morphology of testis were observed in diabetic mice. LBP treatment of the diabetic mice considerably reversed those changes and Johnsen’s testicular score, serum testosterone (T), follicular stimulating hormone (FSH) and luteinizing hormone (LH) level were also increased to different degrees. Moreover, our data have also shown that a marked improvement in sexual behavior and fertility level after administration of LBP (40 mg/kg) compared to the diabetic group. These results suggested that LBP can exert functional recovery of male sexual dysfunction and fertility damages induced by diabetes in male mice, which is likely to be mediated through regulating the hypothalamic-pituitary-gonadal axis endocrine activity.

**Keywords:** *Lycium barbarum* polysaccharide, Sexual dysfunction, Fertility impairments, Diabetic mice, Hypothalamus-pituitary-gonadal axis

**DIABETES MELLITUS** (DM) is a common disease characterized by an increase in blood glucose levels due to the absolute or relative deficiencies in insulin secretion and/or insulin action in the beta cells of pancreas [1]. International Diabetes Federation reported that 382 million people were affected by diabetes worldwide in 2013 and this figure will go up to over 592 million by 2025 [2]. Type-1 diabetes is accompanied by autoimmune destruction of pancreatic beta cells and requires exogenous insulin injection for life. Another important complication of type-1 diabetes is sexual function impairment [3]. International Diabetes Federation and Textbook of Diabetes reported that 28% of type-1 diabetic patients suffered from sexual dysfunction [4]. Thus it is urgent to develop new hypothesis-based therapeutic strategies to alleviate or prevent diabetes-impaired male sexual function and fertility.
growing in European children by 3% annually, with a growing number being diagnosed in early childhood. Sexual dysfunction and fertility impairment are all major secondary complications in both diabetic patients and animals [3-5]. Sexual dysfunction was linked to diabetes which has been perceived since the 10th century when Avicenna had mentioned the “collapse of sexual function” as a specific complication of diabetes [5]. Epidemiological surveys showed that about 90% of diabetic patients have turbulence in sexual function and fertility [6-8]. Increased evidences elaborated a relationship between diabetes and testicular functions in both clinical research and animal models [7, 9-11]. Also, in streptozotocin (STZ) induced diabetic animal models demonstrated sperm count decreased, motility reduction, serum testosterone impairment, sexual dysfunction and varying degrees of testicular lesions [3, 10, 12, 13].

Although the exact mechanisms by which DM alters the sexual function and spermatogenesis leading to infertility are not easy to fully plumb, in vivo evidence cannot be disregarded even though dispersed and indirect [14]. However, current studies suggest that the male reproductive endocrine system is essential for promoting the maturity of sperms and maintaining normal sexual function [15-17]. Studies have reported that testosterone (T), follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels could be used as markers of reproductive functions. The failure of the hypothalamic pituitary gonadal axis maintaining proportionate levels of these endogenous hormones may result in disruption of spermatogenic and sexual function; and ultimately leads to infertility [18]. Systemic metabolic imbalance in long-standing severe hyperglycaemia affects the hypothalamic pituitary testis axis function, disturbs the jurisdiction function of the endocrine system, and influences steroidogenic capacity of Leydig cells [19-21]. Studies have suggested that STZ induced diabetes caused 2 major changes in Leydig cells: 1) a decrease in total Leydig cells number, and 2) an impairment in cells function [21]. It is well known that T secretion is operated by Leydig cells under LH stimulation and is an indispensable steroid hormone for both spermatogenesis and libido in males [21]. Once again we know that the production of spermatozoa is performed inside the seminiferous tubules. T synthesis disorder is caused by changes in Leydig cells, and have been correlated with damage to seminiferous tubules and decrease of libido in males [22, 23]. In addition, sertoli cells, regarded as the nurse cells for germ cells, provide nutritional and physical support for spermatogenesis. FSH secreted by sertoli cells is a major role player in the development of testes, which promotes the combination of androgen binding protein (ABP) with testosterone and maintains the level of testosterone in spermatogenic cells. Both clinical and basic studies have demonstrated that diabetes induces a decrease in the serum LH and FSH levels with raised FSH/LH ratio, and this alteration is responsible for the diabetes-related effects on libido and spermatogenesis [21-24]. A better understanding of the hormonal requirements of spermatogenesis and libido is desirable for the improvement of treatment for male infertility [25, 26]. On this basis, any effective intervention strategies that prevent and reverse the infertility caused by diabetes would be beneficial. Thus, seeking agents that could alleviate diabetes-induced damage to male sexual dysfunction and fertility impairment is an important area of inquiry.

*Lycium barbarum* (Lycium barbarum, Gouqi), abundantly grown in Ningxia as a food and medicine, is well-known in traditional Chinese medicine for centuries to promote health and longevity. One of the Gouqi’s main active ingredient is *Lycium barbarum* polysaccharide (LBP), which have shown multiple pharmacological activities such as hypolipidemic, antioxidant, anti-aging, antitumor, and immunomodulation et al [27, 28]. A wide array of studies confirmed that sexual functionality, sperm quality as well as the serum endocrine hormone levels were significantly improved after administration of LBP in rat in the following reproductive damage models such as corticosterone-induced inhibition of the sexual behavior [29], radiation-induced spermatogenic damage [30] and bisphenol A-induced testis spermatogenic injuries [31]. Based on the above considerations, we hypothesized that LBP might minimize fertility impairment in secondary complications of diabetes. The purpose of this study was to test the protective effects of varying doses of LBP on sexual dysfunction and fertility impairment associated with diabetes as well as to further identify its underlying mechanisms.

**Materials and Methods**

**Animal treatment**

The experimental protocols were conducted in accordance with the Institutional Animal Ethics Committee.
LBP improves the diabetic infertility

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LBP improves the diabetic infertility

Endocrine Journal  Advance Publication

of Ningxia Medical University (Ethical number: 2016-036). Animal care and experimental procedures complied with the regulation and guidelines for the care and use of laboratory animals (CHN publication 1988, amended 2011). One hundred and fifty healthy adult male ICR (Institute of Cancer Research) mice, aged 10-12 weeks and weight in the range of 23-27g, were obtained from and maintained in the animal center of Ningxia Medical University (Certificate no. SYXK Ningxia 2016-0001). All mice were housed in separate standard cages under a temperature controlled environment (22-24 °C) with mean relative humidity (40-70%) under a 12 h day and night cycle for one week. During the whole experimental period, all animals were allowed to have food and water ad libitum.

**Induction of Type-1 diabetic condition**

A diabetic model was established experimentally in mice which had fasted overnight by a single intraperitoneal injection of a sodium citrate buffer (0.1 mol/L of citrate, pH 4.5) of streptozotocin (STZ, Sigma, U.S.A.) at a dosage of 45 mg/kg body weight for 5 consecutive days. Diabetes was confirmed by measuring the blood glucose level using a glucometer (Johnson & Johnson, U.S.A.) with a drop of blood obtained by tail-vein puncture. The mice with blood glucose values above 16.7 mmol/L at 72 hours after the final STZ injection were considered to be diabetic and were then randomized for the experimental groups.

**Drug administration and study design**

*Lycium barbarum* polysaccharide (LBP) was purchased from Ningxia Agricultural and Forestry College, Yinchuan, China and Sildenafil Citrate (SC), as the positive control, was obtained from American Pfizer (100 mg active ingredient per tablet). These reagents were dissolved in the 0.9% (w/v) NaCl solution before use and were administered through an intragastric gavage in a volume of 0.1 mL/10 g body weight. Fig. 1 illustrates LBP and in vivo study treatment plan respectively. All of mice were randomly assigned to the following six groups: (1) Control group (health mice); (2) Diabetic group (untreated diabetic mice); (3) Diabetics with Sildenafil Citrate (5 mg/kg) group; (4) Diabetics with LBP 10 mg/kg group; (5) Diabetics with LBP 20 mg/kg group; (6) Diabetics with LBP 40 mg/kg group. All groups were administrated with drugs or saline once daily for 62 consecutive days by after the successful construction of the diabetic condition. The dosage used in the present study was based on previous study and depended on our preparatory experiments [29, 32].

**Blood glucose levels, body weight and reproductive organs weights**

Every day before the drug administration, all mice were weighed until the end of the animal experiment. Blood glucose levels were monitored on the 0th, 10th, 20th, 40th, and 62th day of the initializing the treatments throughout the study. After the animals were sacrificed at the end of the 10th week, testes, epididymis and seminal vesicles were removed, freed of surrounding connective tissue and their weights (absolute and relative to body weights) were recorded. Right testes were fixed with Bouin’s fluid to prepare for histopathologic analysis as follows.

**Mating behavior tests**

Behavioral estrus was produced in the presence of sexually receptive female mice which had received a subcutaneous injection of diethylstilbestrol (DES, 1 mg/kg body weight) for 3 days before the beginning of the test. The male mice were placed in a rectangular plastic cage (32 cm × 16 cm × 16 cm) under dim red illumination. The training was performed on mice for 30 min after 5 pm each day for 3 consecutive days. The male mice which failed to perform intromission within 5 min were excluded from the study as they were considered sexually inactive [33, 34]. In this step, 6 out of 14 mice in diabetic group, 0 out of 10 mice in control group, 3 out of 13 mice in SC group, and 4 out of 12 mice, 5 out of 13 mice and 2 out of 14 mice in LBP (10, 20, 40 mg/kg) were excluded from the study respectively. Before testing, the selected mouse was allowed 10 min to acclimate to the test environment, then a sexually receptive female mouse was placed in the same cage, and the activity of a pair of animals was video-recorded for approximately 20 min for later analysis (Fig. 2). Sexual behavior parameters are presented in Table 1. The analyst of the animal sexual behavior was blinded from knowledge of the animal’s group assignment.

**Fertility test**

The fertility test was carried out according to methods described by Klari Noormetes [35]. Briefly, all male mice in each group were mated with untreated virgin female mice, aged 8-12 weeks, in the ratio of...
Fig. 1  Picture of *Lycium barbarum* polysaccharide (LBP) and the in vivo study treatment plan respectively

Structure and origin of *Lycium barbarum* polysaccharides (A). *Lycium barbarum* fruits (a), brown-colored LBP (b), and six main monosaccharides present in LBP (c). A diagrammatic sketch showing the treatment methodology for this study (B). Two batches of mice were employed in this study for detection of the different indicators. The red colored mice at day 8 of experiment are used to depict type 1 diabetic mice due to intra-peritoneal (i.p.) injection of streptozotocin (STZ) for 5 consecutive days. The treatments with SC (5 mg/kg), LBP (10, 20 and 40 mg/kg) were maintained daily for 62 consecutive days.
Fig. 2  Different stages of male mice in the sexual behavior experiment

(A) Male mice were allowed 10 min to acclimate to the test environment before the test. (B) After introduction of female mice, male mice approach the female mice. (C) Male mice mount female mice. (D) Female mice take lordosis posture and male mice starts intromission. (E) Male mice autogroom after intromission and performs intromission again. (F) After ejaculation, male mice take a rest before another sexual cycle.

Table 1  Mating behavior tests

<table>
<thead>
<tr>
<th>Sexual behavior parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mounting Frequency (MF)</td>
<td>Number of mounts before ejaculation</td>
</tr>
<tr>
<td>Intromission Frequency (IF)</td>
<td>Number of intromission before ejaculation</td>
</tr>
<tr>
<td>Mounting Latency (ML)</td>
<td>Time from the introduction of female into the cage of the male up to the first mount</td>
</tr>
<tr>
<td>Intromission Latency (IL)</td>
<td>Time from the introduction of the female up to the first intromission by the male</td>
</tr>
<tr>
<td>Post-ejaculatory Interval (PEI)</td>
<td>Time from the first ejaculation up to the next intromission by the male</td>
</tr>
<tr>
<td>Ejaculatory Latency (EL)</td>
<td>Time from the first intromission of a series up to the ejaculation</td>
</tr>
</tbody>
</table>

1: 2. The sexually mature female mice were checked for postcoital plugs each morning. If a plug was observed, it was considered as being at gestation day 0 and the female was taken away from the cage and placed in a separate cage (or replaced with another virgin female mouse). If there was no vaginal plug after three days from either of two female mice, they were removed and replaced with two other virgin females. This was performed three times, so each male mouse had been introduced to six females by the end of the period. Plug-positive mice were cared for under standard conditions until delivery. Fertility rate, mating rate, pregnancy rate and numbers of the litter size were calculated for each male using the following formula:

\[
\text{Fertility rate} \% = \frac{\text{number of pregnant female mice}}{\text{number of mated female mice}} \\
\text{Mating rate} \% = \frac{\text{number of female mice with plug}}{\text{number of mated female mice}} \\
\text{Pregnancy rate} \% = \frac{\text{number of pregnant female mice}}{\text{number of female mice with plug}}
\]

Epididymal sperm preparation and analysis

The mice were sacrificed at the end of the 62-day treatment. The right cauda epididymis was weighed and finely dissected and placed in 1 mL of prepared Ham’s F10 medium for 30 min to allow the spermatozoa to swim into the culture medium freely. Sperm count (10⁶/mL) and motility was assessed using a Makler chamber [34, 36].
**Hormonal analyses**

On the 70th day, blood samples (approximately 1-2 mL blood) were collected from eyes of mice around 0800 h in the morning using a serum separator tube without anticoagulants and then were allowed to clot for 2 h at room temperature before centrifugation for 20 min at 1,000 × g. The serum was separated and stored at −80 °C until used to test for the endocrine hormone parameters. Serum testosterone (T), follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels were determined in aliquots (0.05 mL) by an enzyme-linked immunosorbent assay (ELISA) specific commercial kit (Life Span Bio Sciences, Inc. U.S.A).

**Morphological evaluation by light microscope**

Histological changes of testis tissues were detected by routine histological examination [37]. After fixation in Bouin’s solution for 24 h, the tissues were embedded in paraffin blocks and sectioned at 5 mm before stained with hematoxylin and eosin (H&E). The histopathological damages that included degeneration, desquamation, vacuolation and reduction in germinal cells in seminiferous tubules were evaluated at a constant magnification of 400× with light microscopy. The images were viewed and analyzed by a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan). Johnsen’s testicular score system [38] was used to evaluate the degrees of the histopathological damages in control and various experimental groups. For each mouse, thirty cross-sectioned tubules were randomly selected and evaluated systematically. The degree of testicular damaged was assessed using a 10-level grading scale from 1 (very poor) to 10 (excellent). All assessment was done in a blinded manner by two histopathologists.

**Morphological evaluation under electron microscopy**

The testes were prefixed with a fixative solution for 2 h at 4 °C, and then cut into 1 mm × 1 mm × 1 mm coronal blocks. Each specimen was rinsed with 0.1 M phosphate buffer and then soaked in 2% osmium tetroxide after being dehydrated and embedded with epon. Subsequently, the semithin sections (0.3 μM) were cut and stained with toluidine blue to identify the target area. Ultrathin sections (60-nm-thick) were obtained and stained with 2% lead citrate and 0.4% uranyl acetate. The morphological changes of the testes were then evaluated and imaged using by H-7650 electron microscope (Hitachi, Tokyo, Japan) (20,000 ×) in a blinded manner.

**Data analysis**

Statistical analysis was performed by the SPSS 19.0 statistical software (IBM, USA). The Chi-Squared test was used to compare the mating rate (%), pregnancy rate (%), fertility rate (%); other data were statistically evaluated by the one-way analysis of variance (ANOVA). All values were expressed as the mean ± standard deviation. The significance between groups was determined by the Student paired t-test. Values were considered significant at p < 0.05.

**Results**

**Body weight (BW), reproductive organ weights and blood glucose level**

Significant differences in the body weight among different experimental groups were found after 4 weeks of treatment (Fig. 3A). Among the groups, there was a statistically significant difference in the final BW and reproductive organ (testes, epididymis, seminal vesicles) weights. The diabetic group showed a low weight gain, and decreased weights of absolute and relative reproductive organs (testes, epididymis, seminal vesicles) (p < 0.01 for these measured parameters) when compared to the control group. Both these weights were improved by the administration of LBP (40 mg/kg) compared with the diabetic group (p < 0.01; Table 2). Statistically, blood glucose levels were seen to be significantly higher in the diabetic group compared to the control group (p < 0.01). Administration of LBP (20 and 40 mg/kg) groups showed blood sugar concentration decreased significantly in 20-70 days compared to the diabetic group, but it failed to revert to normal level (Fig. 3B). There was no significant effect in the LBP (10 mg/kg) and the sildenafil treatment groups compared with the diabetic group (p > 0.05).

**Mating behavior test**

The mating behavior records showed a significant decrease in sexual motivation of diabetic mice (Fig. 4). Specifically, there was remarkably decreased in mounting frequency (MF), intromission frequency (IF), significantly extended in mounting latency (ML), intromission latency (IL), post-ejaculatory interval (PEI) and ejaculatory latency (EL) in the diabetic group compared to the control group (p < 0.01 for these measured parameters). The diabetic group treated with LBP (40 mg/kg) showed noticeable reduction in
LBP improves the diabetic infertility

Table 2 Effect of oral administration of LBP for 62 days on weight characteristics and sperm parameters of male mice

<table>
<thead>
<tr>
<th>Weight parameters</th>
<th>Control</th>
<th>DM</th>
<th>DM+SC 10 mg/kg</th>
<th>10 mg/kg LBP</th>
<th>20 mg/kg LBP</th>
<th>40 mg/kg LBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Initial</td>
<td>35.35 ± 1.63</td>
<td>31.03 ± 1.60</td>
<td>30.61 ± 2.22</td>
<td>31.15 ± 1.40</td>
<td>30.51 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>44.45 ± 2.99</td>
<td>27.65 ± 3.11</td>
<td>32.88 ± 4.97</td>
<td>30.35 ± 2.69</td>
<td>30.70 ± 3.21</td>
</tr>
<tr>
<td>Organ weight (mg×100)</td>
<td>Testis</td>
<td>17.36 ± 1.85</td>
<td>7.43 ± 0.76</td>
<td>11.62 ± 2.31</td>
<td>9.54 ± 1.69</td>
<td>11.41 ± 1.78</td>
</tr>
<tr>
<td></td>
<td>Epididymis</td>
<td>6.26 ± 0.78</td>
<td>2.69 ± 0.37</td>
<td>3.37 ± 0.86</td>
<td>3.20 ± 0.39</td>
<td>3.65 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>Seminal vesicle</td>
<td>31.56 ± 7.07</td>
<td>8.36 ± 3.47</td>
<td>11.58 ± 4.97</td>
<td>9.43 ± 2.93</td>
<td>10.78 ± 3.15</td>
</tr>
<tr>
<td>Organ coefficient = genital/body weight×100</td>
<td>Testis</td>
<td>0.39 ± 0.03</td>
<td>0.27 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>0.30 ± 0.03</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Epididymis</td>
<td>0.14 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Seminal vesicle</td>
<td>0.71 ± 0.16</td>
<td>0.29 ± 0.10</td>
<td>0.36 ± 0.15</td>
<td>0.30 ± 0.07</td>
<td>0.32 ± 0.09</td>
</tr>
<tr>
<td>Sperm parameters</td>
<td>Sperm count (×10⁶/mL)</td>
<td>77.40 ± 14.67</td>
<td>17.77 ± 8.08</td>
<td>27.20 ± 5.45</td>
<td>25.94 ± 13.03</td>
<td>31.15 ± 9.87</td>
</tr>
<tr>
<td></td>
<td>Viability (%)</td>
<td>84.11 ± 2.17</td>
<td>46.10 ± 10.46</td>
<td>78.27 ± 8.89</td>
<td>58.54 ± 8.33</td>
<td>58.65 ± 9.58</td>
</tr>
<tr>
<td></td>
<td>Motility (%)</td>
<td>44.34 ± 7.57</td>
<td>27.82 ± 4.73</td>
<td>40.81 ± 8.59</td>
<td>30.29 ± 6.38</td>
<td>30.29 ± 6.86</td>
</tr>
<tr>
<td></td>
<td>Abnormal sperms</td>
<td>20.12 ± 5.12</td>
<td>49.26 ± 8.59</td>
<td>33.69 ± 6.56</td>
<td>47.55 ± 9.26</td>
<td>42.69 ± 7.17</td>
</tr>
</tbody>
</table>

Shows the body weight, reproductive organ weight and reproductive organ coefficient as well as sperm parameters of the control and experimental groups. Each value indicate the mean ± standard deviation (n=8). a = vs control group, b = vs DM group, * p < 0.05, ** p < 0.01.
Fig. 4  Sexual behavior parameters in the control and experimental groups
Each bar indicates mean ± standard deviation (n=8). (A) ML = mounting latency; (B) IL = intromission latency; (C) MF = mounting frequency; (D) IF = intromission frequency; (E) EL= ejaculatory latency; (F) PEI = post-ejaculatory interval. a = vs control group, b = vs DM group, * p < 0.05, ** p < 0.01.
ML and IL ($p < 0.01$, $p < 0.05$, respectively) (Fig. 4 A and B) as well as PEI and EL ($p < 0.05$, $p < 0.01$, respectively) (Fig. 4 E and F), significant increase in MF and IF compared to the diabetic group ($p < 0.05$) (Fig. 4 C and D). As expected, SC was the most effective on the increase of sexual motivation among all treatment groups.

**Testes’ fertility**

The pregnancy rate, mating rate and fertility rate significant lower in female mice mated with diabetic males than in those mated with healthy male mice ($p < 0.01$ for these parameters; Table 3). LBP group (40 mg/kg) showed a significantly increased in mating rate, pregnancy rate, fertility rate and average litter size compared to the diabetic group ($p < 0.01$; $p < 0.05$; $p < 0.01$; $p < 0.01$, respectively). The SC group showed no significant alterations in pregnancy rate and fertility rate ($p > 0.05$), but the mating rate and average litter size increased significantly compared to the diabetic group ($p < 0.05$). However, neither the fertility rate nor average litter size of the LBP (10 mg/kg) group had any significant changes compared to the diabetic group ($p > 0.05$).

**Sperm count, viability, motility and abnormal sperm**

The sperm quality which included sperm viability, sperm count and the normal sperm count, were detected and significantly reduced in the diabetic group when compared with the control group ($p < 0.01$ for these parameters; Table 2). These effects were significantly reversed in the LBP (40 mg/kg) treatment group ($p < 0.01$). In this study, SC treatment group showed a statistical significant increase in sperm motility and viability ($p < 0.01$), but not had any significant difference in the sperm count compared with the diabetic group ($p > 0.05$).

**Hormonal analyses**

This study showed a significant reduction in serum hormone levels in the diabetic group compared with the control group ($p < 0.01$; Fig. 5). The LBP 40 mg/kg treatment group was able to reverse partially the decrease of the serum T, FSH and LH levels and increase of the LH/FSH ratio, and the effects are statistically significant ($p < 0.01$, $p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). The changes of FSH and LH levels in the 10 mg/kg LBP group were not significant compared to the diabetic group ($p > 0.05$). In the SC group, the data showed a significant increase in serum hormone levels compared with the control group ($p < 0.01$). In addition, this study found that there was a high/good correlation between T and LH, T and FSH as well as LH and FSH through correlation analysis (Table 4).

**Histopathological examinations**

Testicular histopathological changes (H&E) and Johnsen’s scores in the six groups are presented in Fig. 6. Control group mice showed regular histological structure of the seminiferous tubules with normal spermatogenesis. The sertoli cells remained well-arranged, and the spermatocytes were arranged compactly and distributed radially in about 5 to 7 layers (Fig. 6A). Whereas in the diabetic group, the amount of the seminiferous tubules were disrupted massively displaying the degenerative and vacuolated pathological structures. The spermatogonia, spermatocytes, and spermatids were significantly damaged with varying degrees of spermatogenetic arrest (Fig. 6B). Also, the Johnsen’s scores decreased significantly in comparison to the control group. Moreover, relatively normal testicular structures and significantly increased Johnsen’s scores were observed in the LBP (40 mg/kg) treatment group (Fig. 6F).

### Table 3 Effect of oral administration of LBP for 62 days on fertility levels of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Males</th>
<th>Females</th>
<th>Females (plug)</th>
<th>Females (pregnancy)</th>
<th>Mating rate (%)</th>
<th>Pregnancy rate (%)</th>
<th>Fertility rate (%)</th>
<th>No. of litters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>54</td>
<td>43</td>
<td>40</td>
<td>79.63</td>
<td>93.02</td>
<td>74.07</td>
<td>14.00 ± 1.73</td>
</tr>
<tr>
<td>DM</td>
<td>9</td>
<td>54</td>
<td>21</td>
<td>14</td>
<td>38.89 $^{***}$</td>
<td>66.67 $^{***}$</td>
<td>25.93 $^{***}$</td>
<td>6.83 ± 1.33 $^{***}$</td>
</tr>
<tr>
<td>DM+SC</td>
<td>9</td>
<td>54</td>
<td>32</td>
<td>21</td>
<td>59.25 $^{*}$</td>
<td>65.63</td>
<td>38.89</td>
<td>8.86 ± 2.27 $^{*}$</td>
</tr>
<tr>
<td>LBP (10 mg/kg)</td>
<td>9</td>
<td>54</td>
<td>23</td>
<td>15</td>
<td>42.59</td>
<td>65.21</td>
<td>27.78</td>
<td>8.00 ± 2.46</td>
</tr>
<tr>
<td>LBP (20 mg/kg)</td>
<td>9</td>
<td>54</td>
<td>29</td>
<td>22</td>
<td>53.70</td>
<td>75.86</td>
<td>40.74</td>
<td>12.14 ± 1.95 $^{b*}$</td>
</tr>
<tr>
<td>LBP (40 mg/kg)</td>
<td>9</td>
<td>54</td>
<td>39</td>
<td>35</td>
<td>72.22 $^{b**}$</td>
<td>89.74 $^{b*}$</td>
<td>64.81 $^{b**}$</td>
<td>13.17 ± 1.60 $^{b**}$</td>
</tr>
</tbody>
</table>

Shows the mating rate; pregnancy rate; fertility rate and number of litters of the control and experimental groups (n=9). No. of letters indicate the mean ± standard deviation. $^{a} =$ vs control group, $^{b} =$ vs DM group, $^{*} p < 0.05$, $^{**} p < 0.01$.  

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**Endocrine Journal Advance Publication**
Fig. 5  Effects of LBP on the serum hormone levels in diabetic mice
Each bar indicates mean ± standard deviation (N=6).  (A) T = testosterone;  (B) LH = luteinizing hormone;  (C) FSH = follicular stimulating hormone;  (D) The ratio of LH/FSH.  a = vs control group, b = vs DM group, * p < 0.05, ** p < 0.01.

Table 4  The Correlation analysis of T, FSH and LH

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>1.00</td>
<td>0.918 (0.01)</td>
<td>0.985 (0.00)</td>
</tr>
<tr>
<td>FSH</td>
<td>1.00</td>
<td>0.956 (0.003)</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Morphological evaluations

As seen in Fig. 7, in the control group, the cytoplasm of spermatogonia and sertoli cells contained abundant organelles such as mitochondria and rough endoplasmic reticulum with a typical homogeneous staining in the matrix (Fig. 7A-a; Fig. 7B-a). In the diabetic group, these cells displayed severe impairment of organelles such as vacuolated mitochondrion and extended endoplasmic reticulum. Nuclear lysis and nuclear membrane invagination accompanied by an ambiguous boundary was also noticeable (Fig. 7A-b; Fig. 7B-b). Compared with the diabetic group, the LBP (40 mg/kg) group spermatogonia and sertoli cells showed various degrees of recovery with relatively normal nuclear membranes and a regular shaped nucleus, while the moderate mitochondrial injury was still visible (Fig. 7A-f; Fig. 7B-f).

Discussion

In this study, LBP exerts beneficial effects on sexual performance and fertility in STZ-induced severely diabetic mice. All the sacrificed mice were dissected, the results showed that LBP significantly increased testis and epididymis weights, prevented the testicular tissue damage induced by diabetes and improved sperm quality. Following these findings, we also explored the possible mode of action of LBP against infertility and suggested that treatment with this natural product for 62 days may influence the hypothalamic-pituitary-testicular axis, which regulated the synthesis and secretion of T, FSH and LH of the pituitary gonadotropic cells.

In our experiment, blood glucose levels decreased during 20-70 days on both LBP (20 and 40 mg/kg)
treatment groups compared with the diabetic group. The differences of the blood glucose levels occurred as early as after 10 days of LBP administration, remained statistically significant at all time-points after 20 days. Although the exact mechanism of the glucose-lowering action is out of scope of this study, it has been suggested that LBP activates PI3K- and p38MAPK-mediated signaling pathways and subsequently improves insulin sensitivity [27]. It was noticed though that the blood glucose level failed to revert to normal in our study and this could be associated, at least in part, with the dosage of LBP. As expected, an increase in the mean body weights was observed and this increase was statistically significant after administration of 40 mg/kg LBP, reflecting benefit of better blood glucose control.

Previous studies showed that the improvement of the reduced testicular weights by the administration of LBP in animals might be attributed to the improvement of lesion of reproductive organs [33, 39, 40]. Recent studies utilized morphology based technique showed histopathological changes in the testis associated with DM [41]. Our study identified these changes with a large number of seminiferous tubule structures were disrupted with numerous vacuoles that spread around the testicular cells and various degrees of spermatogonial arrest. The 20 mg and 40 mg/kg LBP treatment groups showed a significantly higher Johnsen scores.

Fig. 7 The representative ultrastructure morphological photomicrographs in testicular germ cells including Sertoli cells (A) and Spermatogenic cells (B) (×20,000 magnification; Bar = 2 μm)

(a) Control group; (b) Diabetic group; (c) SC group (5 mg/kg); (d-f) LBP groups (10, 20 and 40 mg/kg). N, nucleus; NM, nuclear membrane; ER, granular endoplasmic reticulum; M, mitochondrion; L, lysosomes.
and displayed a varying degrees of recovery and relatively normal testicular morphology.

Functional outcome of the morphological improvement by LBP in the male mice subjected to STZ was further studied. It is well known that long-term hyperglycemia can cause loss of libido and low quality sperm [42-44]. In animal studies, it has also been demonstrated that the ML and IL extended with increased MF, IF and increased PEI and EL in diabetic animals. The administration of LBP (40 mg/kg) resulted in significantly decreased ML, IL, PEI and EL along with increased MF, IF in comparison with the diabetic group. These results clearly demonstrate that LBP has the potency to restore sexual functions which included increase in sexual activity and ability, in sexually impaired diabetic male mice. In addition, spermatogenesis is another critical function of the testes. It has been demonstrated that sperm morphology was significantly affected by hyperglycemia [44, 45]. Furthermore, our study revealed that the epididymis sperm count, motility, and the viability were significantly reduced in diabetic mice which clearly indicated the impairment of spermatogenesis under the hyperglycemic condition. Also, oral administration of LBP (40mg/kg) significantly increased the epididymis sperm count and viability, but decreased the percentage of sperm death in diabetic mice. This clearly indicate the spermatogenic property of the LBP.

Fertility problems due to the impairments of male sexual function and sperm quality caused by diabetes remains an urgent public health concern and have gradually become a focus for research topics in both medical and pharmaceutical fields [46]. Our study showed the administration of 40 mg/kg LBP significantly increased the pregnancy, mating, and fertility rate; and the average litter size which subsequently improved the subfertility status of DM mice. These results clearly demonstrated LBP could effectively improve the potency of the fertility average and reverse the status of subfertility or infertility caused by diabetes. This may be due to the effectiveness in improvement of sperm quality and recovery of sexual capability by the administration of LBP.

During the past two decades, accumulating research into the complex mechanisms of diabetic complications has indicated that diabetes could evoke adverse effects for spermatogenic functions and sexual functions, leading to infertility and this might be mediated through hormonal alterations in the hypothalamic pituitary gonadal axis [47]. The hypothalamic pituitary gonadal axis plays an important role in regulation of the different stages of reproductive activities such as sexual differentiation, sexual maturity, reproductive and sexual behavior, etc [34, 48]. Earlier observations similarly suggested that diabetes in humans leads to significant alteration in endocrine regulation and impairment, which indicates that diabetes has the potential to derange the hypothalamic pituitary gonadal axis [16, 25, 26, 47]. It’s well known that T, FSH and LH are important hormones regulating the reproductive functions of males, and also autoregulate their serum concentrations by acting on the hypothalamic-pituitary axis [23]. Gonadotropin Releasing Hormone (GnRH) of hypothalamic secretion acts directly on the pituitary gland through the hypophysoportal vein system, which in turn may activate the anterior pituitary gland to secrete FSH and LH [16, 23]. In adulthood, the endogenous hormone LH promotes Leydig cells of the testis to produce T, and acts with FSH to keep the seminiferous tubule’s higher content of T that promotes development and maturation of spermatogenic cells. Moreover, the release of the testosterone paracrine mechanism plays a crucial role in regulation of sexual libido and maintenance of secondary sexual characteristics [49]. Our research showed the serum testosterone, LH and FSH levels were markedly decreased in the diabetic group. Moreover, this study finds that there was a high correlation between T and LH, T and FSH, and LH and FSH through correlation analysis. The reduction in these three hormone possibly resulted from a interdependent balance that when one hormone rise or fall and the other one made correspondent changes. All of these changes affect the formation of sperm and change the sexual behaviors [20, 21]. Alternatively, in diabetic mice, degeneration of spermatogenic cells and decomposed sertoli cells cytoplasm were observed, which are the major work place of sexual hormone [50]. Therefore impaired testicular cells are also an important cause of endocrine disorders. However, after treatment with LBP germ cell morphology, they showed various degrees of recovery with relatively normal nuclear membranes. In addition, there was a significant improvement in the levels of T, as well as in FSH and LH levels. Therefore, these protective actions of LBP on spermatogenesis and sexual functions in male mice are probably by regulating hypothalamic pituitary gonadal axis at
multiple levels, maintaining the balance of gonadotropin in the body and avoiding the decrease of serum T induced by diabetes. Thus correcting the hypothalamic pituitary gonadal axis dysfunction, playing a positive role against diabetic testicular dysfunction. Reports indicated Sildenafil, as a first-line medicine for treatment of erectile dysfunction and sexual dysfunction, increased sperm quantity and quality [29, 51]. Our experiment showed similar findings, but the LBP showed a better reversal effect on the fertility impairment than sildenafil which might be caused by more mechanisms of action.

In conclusion, the exact chemical composition of LBP seed remains to be explored, its ability to improve the sperm quality, regulate endocrine hormones, and enhance sex performance has been well established. This study provided evidence that LBP may influence diabetic infertility, at least in part, by its action on regulation of the hypothalamus-pituitary-gonadal axis endocrine activity. Therefore, the results from the present study suggested LBP may potentially be used as fertility-enhancing protective agent for the treatment of diabetic male infertility.

Acknowledgments

The authors gratefully acknowledge the financial support by the Natural Science Foundation of China (Grant No. 81660254) and the major construction programs of Ningxia Medical University (Grant No. XY201405 and XY201519).

Disclosure

The authors declare that there are no conflicts of interest.

References


