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The effect of *Lycium barbarum* on reproductive system and the expression of Crisp-1 protein in experimentally diabetic male rats

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ABSTRACT

Aim: The main purpose of this study is to investigate the effects of *Lycium barbarum* polysaccharides (LBP) on the male reproductive system and Crisp-1 protein expression in experimentally diabetic Wistar Albino male rats.

Material and Method: In this study Wistar Albino male rats (3-4 months old) were randomly selected and divided into four groups; Control, LBP control (only LBP), Diabetic and Treatment (diabetic + LBP) group. For the experimental diabetes model, a single dose of 55 mg/kg STZ was injected intraperitoneally. In the treatment group, the diabetic rats were administered with 200 mg/kg of LBP by gastric gavage for 15 consecutive days.

Results: In histological examinations, increased intertubular connective tissue, congestion, vacuolization and edema were observed in testicular tissues of the diabetic group. The histopathological changes were improved after LBP treatment. Also, the number of total sperm count and sperm motility were significantly increased in the treatment group. Our biochemical analysis results showed that the serum testosterone level were significantly increased and serum MDA level were significantly decreased after treatment with LBP. Compared to the diabetic group, the apoptotic cells were decreased in the treatment group. Crisp-1 protein expression was increased in the treatment group, Crisp-1 possitive vesicle-like structures and apical blebs were also examined in the epididymal tissues.

Conclusion: It is concluded that *Lycium barbarum* polysaccharides have a therapeutic effect on the male reproductive damages of diabetes and also enhances Crisp-1 protein expression in the epididymis.

Keywords: Antioxidant, crisp-1 protein, cysteine-rich secretory protein, diabetes mellitus, infertility, *Lycium barbarum*

INTRODUCTION

Diabetes mellitus (DM) is a major metabolic disorder worldwide. It is characterized by chronic hyperglycemia and imbalances in carbohydrate, fat and protein metabolism that develops as a result of failures related to insulin production (1). Hyperglycemia caused by DM causes tissue damage and disruption of the balance between free radical production and antioxidant defence by triggering oxidative stress (2,3). Oxidative stress which occurs as a result of high production of reactive oxygen species (ROS) is responsible for important complications related to diabetes (4). One of these important complications is a malfunction in the male reproductive system and infertility (5-7).

In fertilization process, important events such as sperm binding and penetration of the zona pellucida and merging with the egg plasma membrane take place through cell connections and are regulated by specific molecules found in both gametes. One of these molecules is the rat epididymal Crisp-1 protein, which is the first member of the Cysteine-Rich Secretory Protein (CRISP) family (8). Crisp-1 is expressed from the epididymal epithelium and released into the lumen where it is associated with the surface of the maturing sperm (9-11). Crisp proteins accompany sperm through the passage of both male and female reproductive systems and have many important functions in the fertilization (12). Crisp-1 protein plays important roles in sperm capacitation, sperm-zona pellucida (ZP) binding, and sperm-egg fusion (13-16). As a result of in vitro fertilization studies, it has been shown that sperm lacking Crisp-1 has a disadvantage in its communication with ZP and its ability to fuse with the egg (17).

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In recent years, natural herbal solutions have been sought in the field of treatment and these studies have attracted attention. *Lycium barbarum*, also known as Goji berry, has been used in traditional Chinese medicine for centuries in herbal treatments and stands out with its antioxidant properties that suppress oxidative stress. Studies have shown many important properties of *Lycium barbarum* polysaccharides (LBP) such as immune system regulator, antitumor, antioxidant, and anti-radiation. For this reason, *Lycium barbarum* stands out as an effective natural material in the treatment of diabetes and male fertility (18-24).

MATERIAL AND METHOD

Experimental Animals and Ethics

The study was carried out with the approval of Abant Izzet Baysal University Clinical Researches Ethics Committee (Date: 28.01.2016, Decision No: 2016/04). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki. The experimental animals were obtained from Abant İzzet Baysal University Experimental Animal Application and Research Center. The animals were kept in a 25±3 °C environment suitable for 12 hours light/dark cycle during the study and were fed with ad libitum food and water. The study was started with 40 healthy male Wistar Albino rats (3-4 months old) but was completed with 31 rats due to deathly complications of diabetes.

Study Design and Groups

Schematic summary of the experimental study design is shown in **Figure 1**. Rats were randomly selected and divided into four groups:

Group 1: Control group (n=6),

Group 2: LBP control group (only LBP administration) (n=6),

Group 3: Diabetic group (STZ) (n=9) and

Group 4: Treatment group (STZ+LBP) (n=10).

Before the experimental study, blood-glucose levels were measured and the rats with normal values (<250 mg/dl) were included in the study. Streptozotocin (STZ) (Sigma Aldrich, CAS no: 18883-66-4) was used to induce experimental diabetes. Rats were fasted 12 hours before STZ injection. Just before the injection was performed, rats were weighed and fasting blood-glucose values were measured using the Accu-Check Go device from the blood obtained by cutting the tail tip. Besides, the amount of STZ solution was calculated according to the body weight of the rats. A single dose of 55 mg/kg STZ dissolved in citrate buffer (0.1 M pH: 4.5) was administered intraperitoneally. In the 24 hours after injection, 30% dextrose solution was added to the water containers of the rats to prevent deaths due to hypoglycemic shock. Diabetes was determined by measuring blood-glucose values from the blood taken from the tail end of the rats 48 hours after STZ injection. Rats with a blood-glucose level of 250 mg/dl and above were considered as diabetic as previously stated (24).

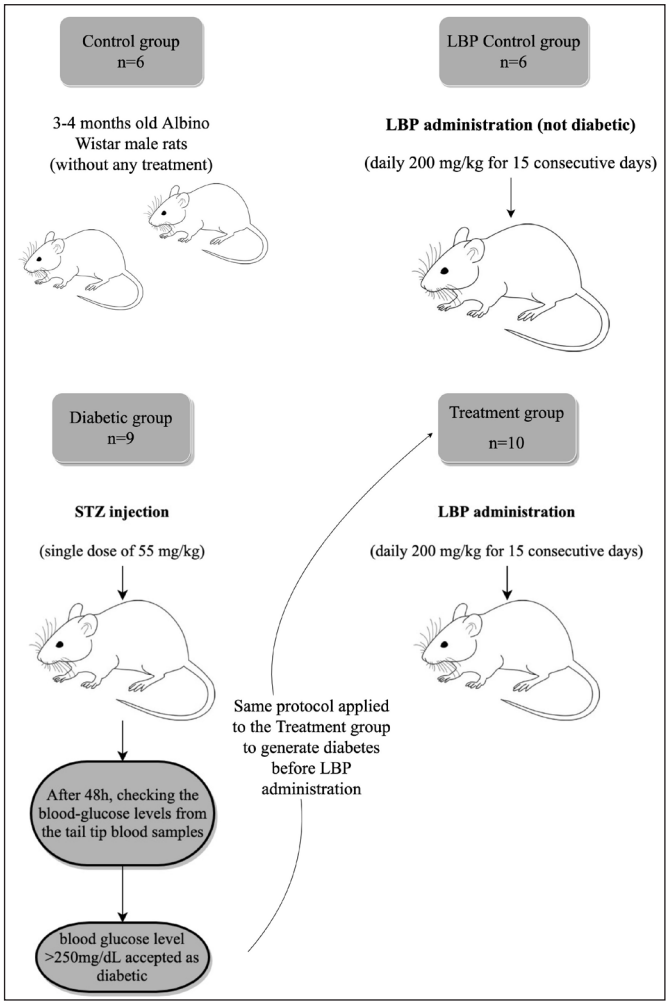

Figure 1. Schematic summary of the study design and experimental groups

After the rats were accepted as diabetic, *Lycium barbarum* polysaccharides (LBP) were administered by gavage of 200 mg/kg once a day for 15 consecutive days in the LBP treatment group. At the end of 15 days, animals were sacrificed after being treated with anaesthetic agents administered at the indicated doses. All tissues were fixed by 10% formaldehyde. Epididymal tissues were used for both histological examination and sperm smear analysis.

Preparation of *Lycium barbarum* Polysaccharides

LBP was purchased commercially and prepared based on previous methods (25). Briefly, dried fruit was pulverized with a blender. To separate the fatty parts; chloroform + methanol (2:1) was used and then refluxed with 80% ethanol at 80°C to separate oligosaccharides. The residue was added four times hot water and extracted four times. Then, the filtrate was combined and concentrated in

rotary at 60°C, and precipitation was performed with 95% ethanol, 100% ethanol and acetone, respectively. The precipitate was collected and dried in vacuum. The extract was freshly prepared and used.

Histological and Immunohistochemical Examination

After the fixation with 10% formaldehyde, the testicular tissues were embedded in paraffin and sectioned into 4-µm thick slices with microtome (Leica). Hematoxylin & Eosin, Masson's Trichrome stainings were used for histological examinations. Caspase-3 primary antibody (Invitrogen, Cat no: PA5-16335) was used for examining apoptotic cells in testicular tissue sections. Also, Crisp-1 primary antibody (Mybiosource, Cat no: MBS2032852) was performed for epidydimal tissue sections to examine Crisp-1 protein expression.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) Assay

TUNEL assay was used to evaluate apoptosis in testicular tissues. The 4-µm thick slices firstly deparafinized and then all the protocol were assessed according to the ApopTag® Peroxidase In Situ Apoptosis Detection Kit manual (cat no: S7100).

Biochemical Analysis

Blood samples were taken from the inferior vena cava and heart from anaesthetized rats for biochemical analysis. Then, sacrification was performed by cutting the aorta. The blood samples were placed in yellow-capped 5 ml gel vacuum serum separator tubes and centrifuged at 4000 rpm for 10 minutes. The serum parts obtained were taken into eppendorf tubes and stored at -80 ° C until the analysis. Testosterone (T) (Elabscience, Catalog no: E-EL-0072), follicle stimulating hormone (FSH) (Elabscience, Catalog no: E-EL-R0391) and oxidative stress parameter malondialdehyde (MDA) (Elabscience, Catalog no: BC0025) levels were examined. In the analysis of these parameters, "Enzyme-Linked Immunosorbent Analysis" (ELISA) method and thiobarbituric acid (TBA) method were used. The kits used in the analyzes were the original Elabscience (Elabscience Biotechnology Co. Ltd; Wuhan; P.R.C.) kits and were used by following the procedure specified by the manufacturer.

Assessment of Sperm Number, Motility and Aniline Blue Staining

For semen analysis, sperms revealed by linting method from cauda epididymis in PBS were counted at 20x magnification under light microscope using Makler sperm counting camera (Makler counting chamber sefi-medical instruments). In semen analysis, number, motility and morphology parameters were examined according to the criteria of the World Health Organization (WHO). Acidic aniline blue staining is used to evaluate sperm chromatin condensation. The sperm smear preparations fixed with 3% glutaraldehyde for 30 minutes and then stained with aniline blue (pH:3.5) for 10 minutes. Washed twice in PBS and left to dry. The preparations were examined under a light microscope at 20x and 100x magnification.

Statistical Analysis

The obtained data were analyzed using SPSS statistical program (IBM Statistics for Mac Os, Version 21.0). Mann Whitney-U test, Kruskal Wallis and One-Way ANOVA tests were used to compare data. Results were evaluated with the significance level as $p \leq 0.05$.

RESULTS

The body weights of the rats weighed before and after the experiment. It was observed that the rats in the treatment group lost weight at the end of the experiment, but there was no significant difference. Also, polyuria was observed in diabetic rats.

STZ-Induced Diabetes Leads to Histopathological Changes and LBP Has Therapeutic Effects on Testicular Tissues

In the testicular tissues of the control group (**Figure 2. A**), there is no structural abnormality; seminiferous tubules and spermatogenic cells preserved their structural integrity. In the testicular tissues of the diabetic group, edema in the intertubular areas (**Figure 2. B**) and cell debris in the lumen of the seminiferous tubule (**Figure 2. C**) were observed. In addition, vacuole formation within the seminiferous tubules, increased connective tissue in the intertubular area and congestion in the vessels were observed (**Figure 2. D,E,F,G**). In the testicular tissues of the treatment group, similar to the control group, general structural integrity and intertubular connective tissue were normal (**Figure 2. H**).

LBP Decreases the Diabetes-Related Apoptosis of Spermatogenic Cells

TUNEL assay method was used to identify apoptosis and the apoptotic cells were counted in five different areas with 20x under the light microscope. A statistically significant difference was observed between the control and the diabetic groups ($p=0.001$, $p \leq 0.05$) and also between the diabetic and treatment groups ($p=0.024$, $p \leq 0.05$) (shown in **Figure 3**).

In the immunohistochemical examinations with light microscopy, increased number of TUNEL positive apoptotic cells in the diabetic group indicates that diabetes leads to apoptosis by damaging spermatogenic cells (shown in **Figure 4**). In the treatment group, decreased number of TUNEL positive cells indicates less spermatogenic cell damage and apoptosis compared to the diabetic group.

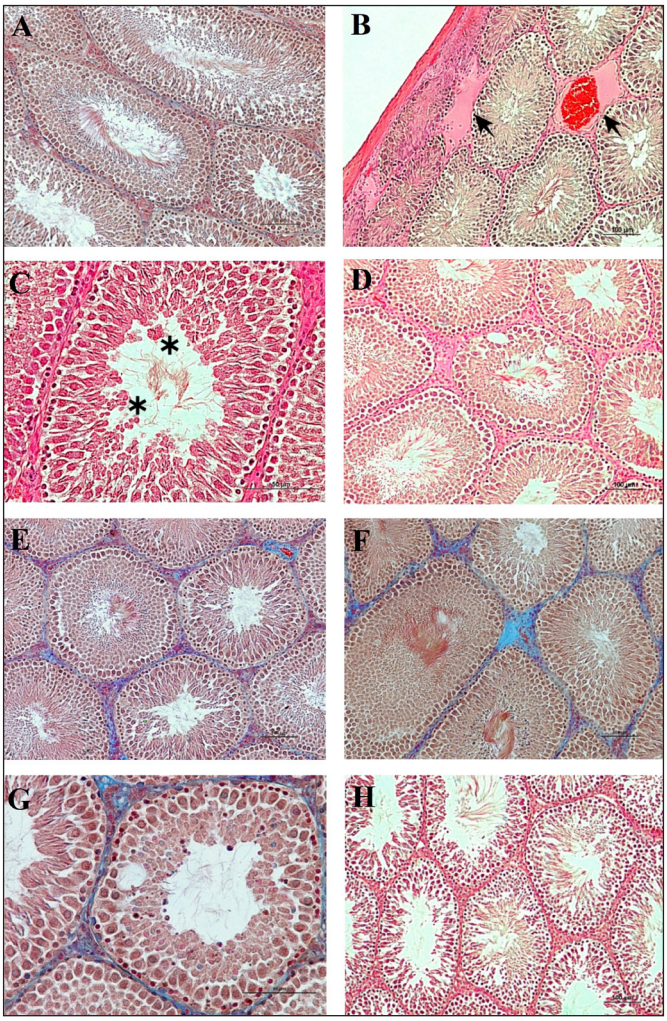

Figure 2. Histological examinations of testicular tissues. A: Control group. Seminiferous tubules and spermatogenic cells preserved their structural integrity. B-C: Diabetic group, edema in the intertubular areas and cell debris in the lumen of the seminiferous tubule were observed. D-G: Diabetic group, vacuole formations within the seminiferous tubules, increased connective tissue in the intertubular area and congestion in the vessels can be seen. H: Treatment group, the seminiferous tubules were similar to the control group. Masson's Trichrome and Haematoxylin & Eosin histological stainings were performed and examined with x200 and x400 magnifications.

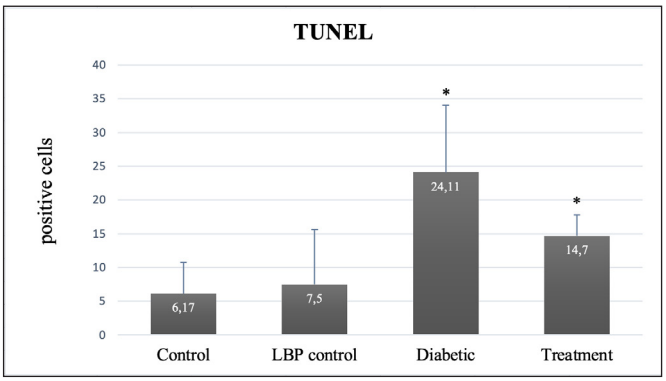

Figure 3. TUNEL immuhistochemical staining. TUNEL positive (apoptotic) cells were counted for each group. A significant difference is found between the diabetic and treatment group (p=0.024, p ≤0.05). Results were evaluated with the significance level as p ≤ 0.05

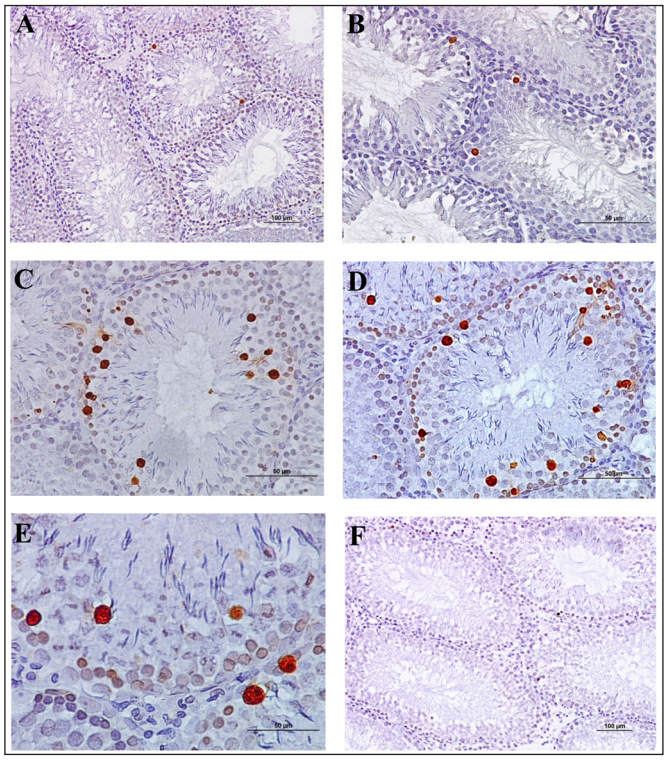

Figure 4. TUNEL apoptotic assay. In the immunohistochemical examinations, increased number of TUNEL positive apoptotic cells in the diabetic group indicates that diabetes lead to apoptosis by damaging spermatogenic cells. A: Control group, B: LBP control group, C-E: Diabetic group, F: Treatment group

Increased Caspase-3 Activation Indicates Apoptotic Cells in Diabetic Testicular Tissue

To determine apoptotic cells in testicular tissues, positively stained cells with Caspase-3 immunolabeling were counted for each group (shown in **Figure 5**). There was a significant difference between the control and diabetes groups (p=0.026, p≤0.05). The increase in the number of caspase-3 positive cells in the diabetes group showed that diabetes-induced apoptosis by increasing caspase-3 activation in testicular tissue (shown in **Figure 6**). The number of positive cells in the treatment group showed a significant decrease compared to the diabetes group (p=0.013, p≤0.05). In addition, there was no significant difference between the control group and the treatment group (p=0.958, p>0.05).

LBP Enhances Fertility-Related Crisp-1 Protein Expression in the Epididymis

In the Crisp-1 immunolabeling, we observed cytoplasmic Crisp-1 staining in epididymal epithelial cells and also stereocilia stained with Crisp-1 antibody (shown in **Figure 7**). Spermatozoa inside the epididymis tubule lumens were also positively stained. In addition, vesicle-like secretory structures protruding towards the lumen stained positively were observed both in the lumen and on the surface of the epithelial cells like apical blebs (shown in **Figure 7D**).

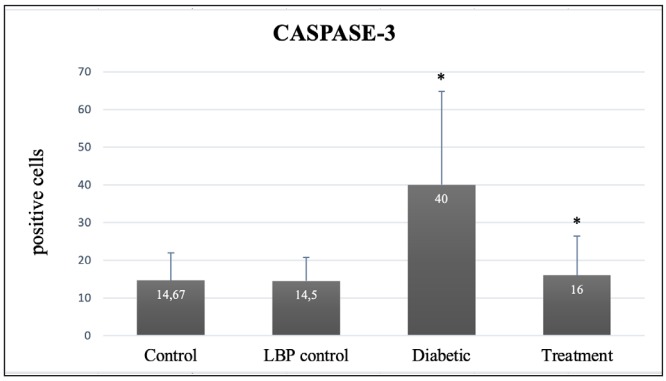

Figure 5. Caspase-3 immunohistochemical staining. Caspase-3 positive cells were counted for each group. A significant difference is found between the diabetic and treatment group (p=0.013, p≤0.05). Results were evaluated with the significance level as p ≤ 0.05

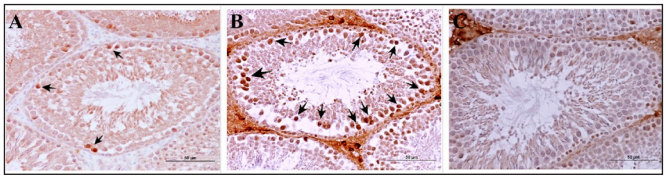

Figure 6. Caspase-3 immunohistochemical staining for apoptotic cells. A: Control group, B: Diabetic group, C: Teratment group (x200).

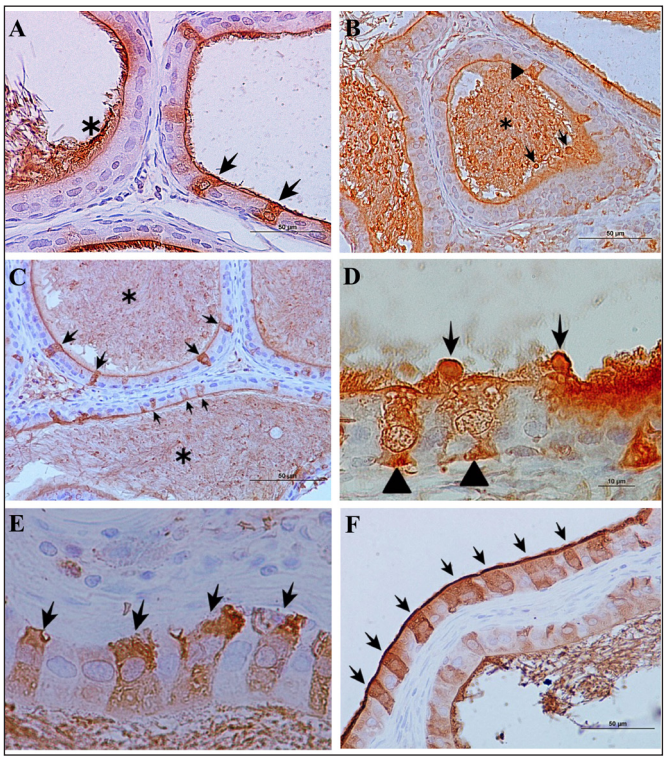

Figure 7. Examination of the Crisp-1 protein expression in the epididymal tissues. A,E,F: The cytoplasmic Crisp-1 staining in epididymal epithelial cells and also stereocilia stained with Crisp-1 antibody. B,C: Spermatozoa inside the epididymis tubule lumens were also positively stained. D: Vesicle-like secretory structures protruding towards the lumen and staining positively were observed both in the lumen and on the surface of the epithelial cells like apical blebs

Crisp-1 positive cell counting was performed for each group (shown in **Figure 8**). There was no significant difference between the control and diabetes groups (p=0.346, p>0.05). There was a statistically significant difference between diabetes and the treatment group (p=0.001, p≤0.05). There was also a statistically significant difference between the control and treatment groups (p=0.013, p≤0.05).

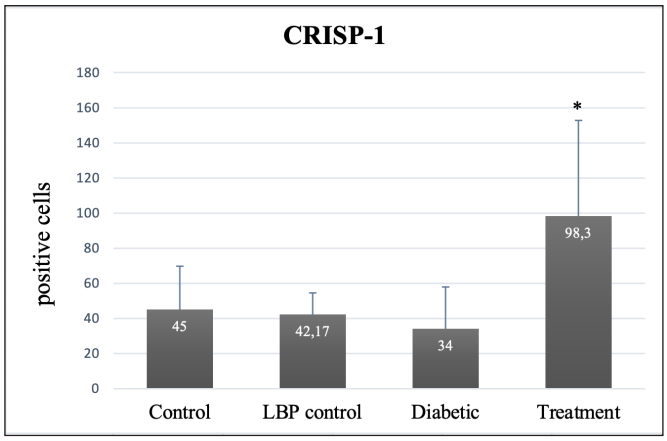

Figure 8. Crisp-1 immunohistochemical staining. Crisp-1 positive cells were counted for each group. A significant increase is found in the treatment group (p=0.001, p≤0.05). Results were evaluated with the significance level as p≤0.05

It was determined that there was a decrease in the number of Crisp-1 positive cells in the epididymis tissue of the diabetes group, but there was no statistically significant difference when compared with the control group. In the treatment group, there was a significant increase in the number of Crisp-1 positive cells.

LBP Treatment Reduces Diabetes-Related Lipid Peroxidation

To examine oxidative stress, we analyzed the MDA (Malondialdehyde) levels among all groups. A statistically significant difference was observed between the diabetes and treatment groups (p=0.050, p≤0.05) (shown in **Figure 9**). In the diabetic group, there was a slight increase in MDA values, as expected, indicating lipid membrane damage. It was observed that MDA significantly decreased in the treatment group, that is, lipid peroxidation decreased. The MDA values of the treatment group were found to be close to the control group values.

LBP Treatment Has Enhancer Effects on Decreased Serum Testosterone Level

Testosterone levels were analyzed to examine the effect of diabetes on male stereoid hormones and reproductive ability. Testosterone levels were expected to decrease significantly (p=0.015, p ≤0.05) in the diabetic group (shown in **Figure 10**), indicating that damage to the male reproductive system and spermatogenesis were

negatively affected. It was observed that testosterone increased significantly (p=0.003, p≤0.05) in the treatment group, that is, positively affected spermatogenesis. In addition, testosterone values of the treatment group were determined to be close to each other, not showing a significant difference with the control group.

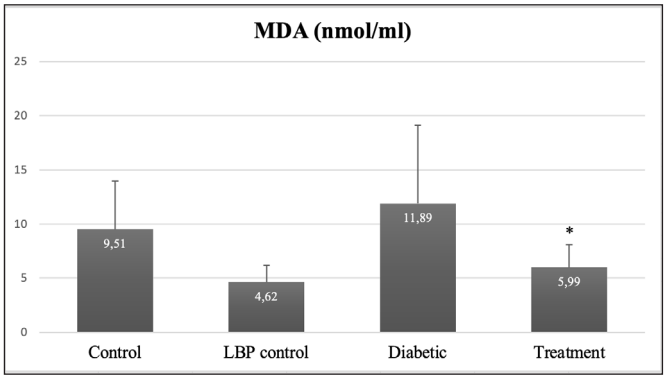

Figure 9. Biochemical analysis of serum MDA levels. A significant difference is found between the diabetic and treatment group (p=0.050, p≤0.05). Results were evaluated with the significance level as p≤0.05

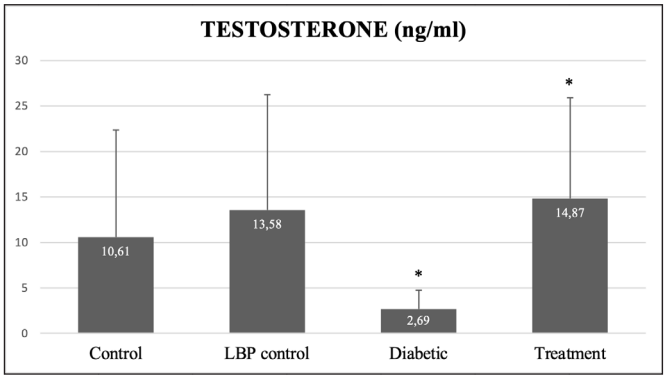

Figure 10. Biochemical analysis of serum Testosterone levels. Testosterone levels decreased significantly (p=0.015, p ≤0.05) in the diabetic group. It was observed that testosterone levels increased significantly (p=0.003, p≤0.05) in the treatment group. Results were evaluated with the significance level as p≤0.05

LBP Treatment Has No Significant Effect on the Serum FSH Level

The obtained serum FSH values of the groups can be seen in the **Figure 11**. Although there was an increased serum FSH level in the LBP control and the LBP treatment groups, compared to the diabetic group, it was not statistically significant (p=0.356, p>0.05).

Total Sperm Count and Motility Increased with LBP Treatment after STZ-Induced Diabetes

The total sperm count and sperm motility values of the groups were performed immediately after sacrification and shown in the **Table**. The values should be multiplied by 105. There was a statistically significant difference for total sperm count between diabetes and treatment groups (p=0.000, p≤0.05). Total sperm count of the diabetic group showed that diabetes caused a decrease in sperm number. Compared to the control and LBP control groups, the sperm motility of the diabetic group was decreased. It was also determined that the sperm motility in the LBP treatment group was increased significantly compared to the diabetic group (p=0.000, p≤0.05). There was no difference between the groups for sperm morphology. In addition, no difference was detected with the Aniline blue staining performed to determine the sperm DNA integrity (shown in **Figure 12**).

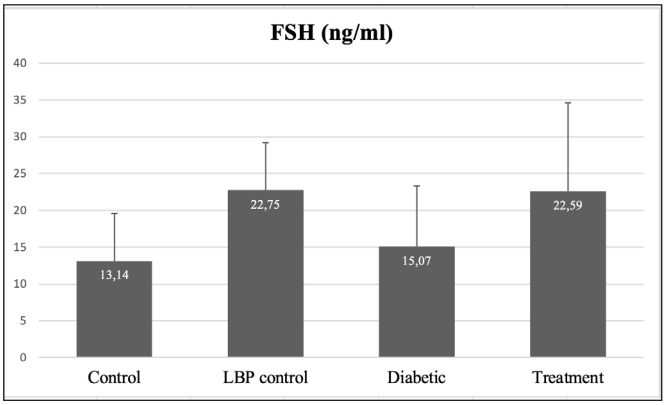

Figure 11. Biochemical analysis of serum FSH levels. There was no statistically significant difference. Results were evaluated with the significance level as p≤0.05

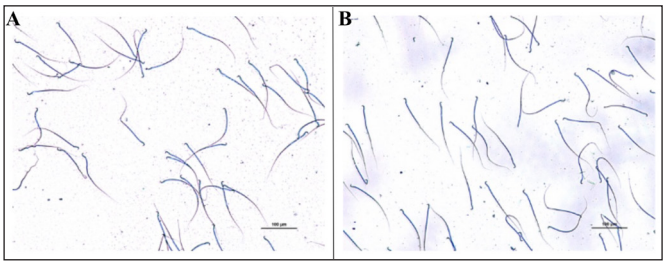

Figure 12. The Aniline blue staining was performed to epididymal sperm smears. A: Control group. B: Diabetic group

Table. Total sperm counts and sperm motility values of the groups. (The values should be multiplied by 105)

Groups	Control	LBP control	Diabetic	Treatment
Total sperm count±SD	106±26.65	165.5±67.21	111.89±50.29	191±103.54
Motile sperm count±SD	18.17±9.15	15.33±19.13	1±1.2	28.20±20.14

DISCUSSION

Diabetes Mellitus (DM) is a very common metabolic disease worldwide. One of its most important complications is spermatogenic dysfunction and infertility (26-28). Oxidative stress increases with diabetes and damages directly testicular and epididymal tissues (29). Crisp-1 protein is one of the molecules that regulates fertilization by playing a role in important events such as capacitation, sperm-egg interaction and gamete fusion. Crisp-1 protein secreted from epididymal epithelial cells and delivered to the lumen binds to the sperm surface during epididymal passage (11,30).

In current study, we demonstrated that the diabetes-related oxidative stress leads to the histopathological and biochemical changes of male reproductive system. In addition, we showed that the LBP, as a natural antioxidant, reduces the harmful effects of diabetes. Also, we showed that the expression of Crisp-1 protein is affected by diabetes-associated oxidative stress, and LBP treatment enhances its expression in the epididymis.

The molecular mechanism of diabetes-related male reproductive system damage and the protective effects of LBP have been elucidated in several studies (19-23). It has been found that LBP had an anti-apoptotic effect by reducing the expression of Caspase-3, which is an indicator of apoptosis, in testicular tissues of diabetic rats (31). Also, it has been reported that LBP could reduce irradiation-induced apoptosis of spermatogenic cells by upregulation of Bcl-2 expression, could increase serum testosterone level and decrease MDA level (22). Consistently, our data showed that the number of apoptotic cells decreased significantly after the LBP treatment. In addition, we showed that the LBP improved serum testosterone level and reduced serum MDA level. The serum FSH level analysis was also performed in our study. Although we showed an increase in the FSH level for the LBP treatment, we could not examine a significant difference between the diabetic and LBP treatment groups. This result could be related with the different mechanisms of hormonal control in the male reproductive system. Treatment of infertility-related hormonal dysfunction requires an understanding of the hormonal basis of spermatogenesis (32). While FSH stimulates spermatogenesis by effecting Sertoli cells, testosterone is mainly secreted from Leydig cells under the control of LH (luteinizing hormone). This can explain why the serum testosterone level was significantly increased but the serum FSH level was not increase at significance level after the LBP treatment. It has been showed that LBP could also improve sperm motility and total density (33). Consistently, our study showed that the total sperm number and sperm motility were improved after LBP treatment. Although we expected a decrease for the sperm motility in STZ-induced diabetic group, it was unexpectedly very low. It might be caused by toxic effect of STZ or a mistake in experimental process during sperm linting etc. Lei et al. (34) reported that the LBP treatment increased the number of HIF-1a positive cells in the testicular tissues of diabetic rats compared with the control group. They also showed that LBP reduce apoptosis, increase sperm motility, and also upregulate PCNA and SIRT-1 expression. Our data was consistent with this study in terms of the histopathological examination, apoptosis and sperm motility. Unlike, we also performed the biochemical analysis for the serum testosterone, MDA and FSH levels. In addition, immunohistochemical examination of the Crisp-1 protein expression in epididymal tissues was performed in our study.

The Crisp-1 protein was first identified in the rat epididymis by Cameo and Blaquier (8). They reported that Crisp-1 is secreted in the epididymis and then it attaches to the sperm surface during the epididymal passage. It has been reported that when Cuasnicu et al. (35) applied polyclonal anti-Crisp-1 to block the Crisp-1 protein, the fusion of sperm with oocyte was inhibited. Busso et al. (36) reported that the epididymal Crisp-1 protein binds to the relevant regions on the egg surface and plays a role in gamete fusion and sperm-ZP interaction. The effect of LBP, which has a known fertility-enhancing effect, on Crisp-1 protein expression was investigated for the first time with our study. We showed that LBP enhances Crisp-1 protein expression in epididymal tissues. Our work is unique in this respect. In our Crisp-1 immunolocalization study, it was observed that stereocilia extending along the epithelium and sperms in the epididymal lumen were stained with Crisp-1 antibody. We also observed that round vesicles that protrude from the epididymal epithelium to the lumen and are free in the epididymal lumen showed Crisp-1 positive staining. Our findings are consistent with the previous studies of Sullivan et al. (37-38). They indicated that there is an apocrine secretion in the apical cytoplasm of principal cells and these apical blebs contain epididymosomes, so we might have observed Crisp-1 protein containing epididymosomes. Epididymosomes were defined as membranous vesicles involved in the transfer of epididymal proteins to sperm and were reported by Sullivan et al. (37-38). Thimon et al. (39) investigated the protein compositions in the epididymosome and determined that it contains many proteins, including the Crisp-1 protein, and reported that the Crisp-1 protein was secreted in the epididymis and then assembled in the epididymosomes.

CONCLUSION

We showed that the Crisp-1 protein is related with the molecular mechanism of diabetes-induced damages of male reproductive system and the protective effects of LBP. However, future comprehensive studies are needed for the Crisp-1 protein to reveal the detailed mechanism of function and the relationship with infertility.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the approval of Abant Izzet Baysal University Clinical Research Ethics Committee (Date: 28.01.2016, Decision No: 2016/04).

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

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