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Treatment with *Leiurus quinquestratus* scorpion venom ameliorates the histopathological changes of type-2 diabetic rats' splenic tissues.

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Abstract

Type 2 diabetes mellitus (T2-DM) is a chronic metabolic disorder characterized by tissue resistance to insulin action. *Leiurus quinquestratus* venom (LQV) contains a variety of bioactive components that can be used for the treatment of various diseases. This study aimed to determine the ameliorative role of LQV on the histopathological changes in splenic tissues of T2-DM rats. Forty male Sprague Dawley rats were divided into 4 groups ($n = 10$) as follows; Gp1 (Control group) was fed on a normal balanced diet. Gp2 (Diabetic group) was fed on a high-fat diet (HFD) for 12 weeks and then injected with streptozotocin (STZ) (30 mg/kg) i.p. Diabetic Gp3 and Gp4 groups had been injected with metformin (Met) (150 mg/kg) or LQV (1/10 of LD_{50}), respectively. After two months of treatment, the total body weight relative splenic weight changes, and blood glucose. Also, histopathological and immunohistochemical investigations (CD-3 immunoreactive antibody) in splenic tissues were examined. The results showed that induction of T2-DM in rats led to a significant decrease in the total body weight (-38.56%), relative spleen weight (0.23 ± 0.03), and increase in the level of blood glucose (382.56 ± 2.77 mg/dL). In addition, several histopathological and immunohistochemical changes were observed in splenic diabetic tissues. The treatment of T2-DM rats with LQV led to an improvement in the total body weight of rats (4.07%), relative spleen weight (0.40 ± 0.02), decrease in the blood glucose levels (115.47 ± 1.07 mg/dL), ameliorated the histopathological and immunohistochemical changes occurred in splenic tissues of T2-DM rats.

Keywords: Diabetes mellitus, Metformin, *Leiurus quinquestratus* venom, Spleen, CD3, Immunoreactive, Antibody, Glucose level.

Introduction

Diabetes mellitus (DM) is a multifaceted endocrine and metabolic condition that may result in tissue damage-causing consequences (1). A faulty insulin secretory response causes DM by impairing glucose utilization and causing hyperglycemia, which in turn causes chronic inflammation, hyperinsulinemia,

insulin resistance, or oxidative stress (2). About 90% of instances of diabetes mellitus are type 2 diabetes mellitus (T2-DM), which is brought on by the inability of tissues that are sensitive to insulin to react to it adequately (3). Natural components attenuate high-fat diet-induced tissue dysfunctions and improve obesity complications and T2-DM in rats (4,

5). The long-term effects of T2-DM include the progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with a risk of foot ulcers. The chronic complications of DM are generally divided into microvascular and macrovascular categories (6). There are numerous species of scorpions known. The Buthidae family includes most deadly scorpion species for humans, including *Leiurus quinquestratus* (7). Scorpion venom components have been studied as therapeutics and further applications in drug product development have been reported (8). This venom is soluble in water and composed of oligopeptides, nucleotides, amino acids, and other organic compounds. Research on the possible medicinal benefits of various scorpion venom compounds is growing because these compounds could be good starting points for the creation of novel medications with anti-leishmanial, anti-malarial, and anti-arthritic properties (9). A previous study reported the effects of *Anderoconus Crassicauda* venom in the treatment of diabetes mellitus type 1 in animal models (10). Wan *et al.* (2017) reported that the active polypeptide of scorpion venom shortens wound healing with a stronger anti-inflammation and antibacterial effect and could be an effective topical drug for the treatment of diabetic ulcers (11). It's interesting to note that a previous study found that the whole-body extract of *Scorpio maurus palmatus* scorpion significantly increased the number of β -cells and the size of pancreatic islets in diabetic mice (12). *L. quinquestratus* (LQ) is one of the most dangerous scorpions around the world and represents a health hazard in Egypt. *L. quinquestratus*' venom (LQV) is composed of neurotoxic peptides, enzyme inhibitors, mucopolysaccharides, serotonin, hyaluronidase, phospholipase, and histamine. The pharmacological activities of LQV have been evaluated for its antibacterial and anticancer effects (9, 13). The present study aimed to determine the protective effect of the treatment with LQV on the splenic tissues of T2-DM rats.

Materials and Methods

Chemicals

Metformin hydrochloride was purchased as tablets 500 mg from MINAPHARM Pharmaceuticals Company (10th of Ramadan City, Egypt). It was dissolved in distilled water and given by oral gavage. at dose 15 mg/Kg B. Wt. Streptozotocin (STZ) was purchased from Sigma-Aldrich, USA.

Venom preparation

One hundred *L. quinquestratus* scorpions were collected from Aswan, Egypt by professional hunters. Scorpions were collected in plastic containers. Scorpions were transferred to Invertebrate Division, Zoology Department, Faculty of Science, Tanta University, Egypt. Then scorpion specimens were authenticated and identified by a specialist in animal taxonomy. According to the methodology of (14), scorpions were milked using electrical stimulation (12-17V) of their telsons, and then venom was lyophilized in Corporate Serum and Vaccine (VACSERA). Different concentrations were prepared from the lyophilized venom. Sublethal doses were prepared according to (15).

Experimental animals

Adult white male rats (Sprague Dawley) weighing between (120 \pm 5 g) and 8 weeks of age were purchased from Animal Husbandry, Alexandria University; rats were transported to the Faculty of Science, Tanta University. The rats were given free access to a normal diet and water and housed in cages at room temperature (25°C) with a fixed 12 h light/dark cycle. All animal experiments were approved by the guidelines of the Institutional Animal Ethical Committee (IAEC) that were approved at the Zoology Department, Faculty of Science, Tanta University (Protocol number: IACUC-SCI-TU-0228).

Balanced and high-fat diets

The balanced diet (BD) and the high-fat diet (HFD) were the 2 types of diets adopted. The BD was purchased from Helwan University's Animal Research and Service Center in Helwan, Egypt. 10% protein, 10% fat, 74.4% carbs, 3.5% mineral mixture, 0.1% methionine, 1% vitamin mixture and 1% fiber made up the BD used to feed healthy control rats (16).

A mixture of 64 g of normal chow, 32 g of animal-sourced saturated fat, 300 IU of vitamin D3, and 15% and 12% of cholesterol powder was used to create the HFD diet. Before being kept in a refrigerator at 4 °C, the mixture was rolled into handball size. To shield lipids from oxidative processes, HFD was made every other day (17).

Induction of T2-DM in rats

Rats were fasted overnight and then injected i.p with a single dose of STZ as 30 mg/kg (18). Because of the ability of STZ to induce fatal hypoglycemia because of massive pancreatic insulin release, the rats were provided with 10% glucose solution after 6 h of STZ administration for the next 48 hours to prevent hypoglycemia (19). Seven days following the STZ injection, blood was drawn from the tail vein, and glucose concentration was measured using a portable glucometer (One Touch Select, Life scan, Inc., CA, USA). Animals with an FBS concentration >200 mg/dl were included in this study as diabetic rats.

Experimental design

The rats were divided into 4 groups ($n = 10$) as follows; Gp1 was fed on a normal rat chow diet for 12 weeks administered orally with dist. H₂O. From Gp2 to Gp4, all rats were fed on a high-fat diet (HFD) for 12 weeks and then injected with STZ (30 mg/kg). Gp2 left as T2-DM rats, Gp3 and Gp4 were treated with Met (150 mg/kg) or LQV (1/10 of LD₅₀) for 2 months, respectively. All groups of rats were weighted, and the percentage of the relative spleen weight was calculated. At the end of the experiment, rats were anesthetized with isoflurane and then sacrificed. Spleen tissues were collected and sectioned in buffered formalin for histopathological and immunohistochemical investigations.

Determination of blood glucose level

Serum glucose levels in the different groups after different treatments were determined according to the enzymatic colorimetric method using bio-diagnostics liquizyme glucose reagent (20).

Histological and immunohistochemical studies

Tissue specimens of the spleen were harvested and fixed in 10% formalin. Paraffin blocks were prepared after completing the tissue processing in different grades of alcohol and xylene. Sections (5µm) were prepared from paraffin blocks using microtomes, stained with hematoxylin and eosin, and observed under a light microscope (Optica light microscope, B-350) to examine gross cellular damage (21). The splenic tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µl thickness. The immunocytochemical reaction was performed according to the Avidine Biotine technique that was described by Hsu *et al.* (1981). The intensity of splenic T-cells was calculated by quantification of immunohistochemistry staining using Image J software (22).

Statistical analysis

The IBM SPSS® software package, version 16.0, USA, was used to enter data into the computer and analyze it. S.E. was used for the data analysis together with percentages and numbers. When the data were evenly distributed, the F-test (ANOVA) and Post Hoc test were employed to compare the groups (LSD), according to significance achieved at $p < 0.05$.

Results

Treatment of T2-DM rats with Met or LQV improves the percentages of body weight changes and the relative spleen weight.

The results showed that the group of diabetic rats injected with STZ showed a significant decrease in the % b.wt change ($P < 0.05$) to -38.56% when compared to the negative control group. The % b.wt change of diabetic rats that were treated with Met was -20.74%, while this percentage was 4.07% in the diabetic rats that were treated with LQV (Table 1). The results showed that as compared to the negative control group, there was a significant decrease in the percentages of relative spleen weight in the diabetic group and diabetic group that were treated with Met, however, treatment of T2-DM rats with LQV restored the relative spleen weight ($P < 0.05$) (Table 1).

Table 1. Weights of different groups during the treatment period from W-12 to W-20 and their relative spleen weight (%).

Groups	B.wt (W-12)	B.wt (W-20)	% of b.wt change	R.S.W (%)
Ctrl.	166.0 ± 4.82 ^b	180.3 ± 3.06 ^c	8.61%	0.42 ± 0.13 ^a
Diabetic	309.3 ± 3.59 ^{a,b}	190.5 ± 5.85 ^d	-38.56%	0.23 ± 0.03 ^b
D/Met	298.5 ± 3.11 ^a	236.5 ± 3.11 ^b	-20.74%	0.30 ± 0.03 ^{a,b}
D/LQV	292.1 ± 2.63 ^a	304.0 ± 4.79 ^a	4.07%	0.40 ± 0.02 ^b

The values represented mean ± SD. **B.wt:** Body weight; **R.S.W:** Relative spleen weight; **Ctrl:** Control group; **D:** Diabetic; **Met:** Metformin; **LQV:** *Leiurus quinquestriatus* venom. p-value < 0.05 was considered to be statistically significant. The means that do not share the same letter are significantly different (Tukey's test).

Treatment of T2-DM rats with Met or LQV restores blood glucose levels close to normal values:

As compared to the control group, the T2-DM group showed a significant increase in the blood glucose level ($P < 0.05$). Treatment of T2-DM rats with Met for 2 months led to a significant decrease in the blood glucose level ($P < 0.05$). Treatment of T2-DM rats with LQV showed a highly significant decrease in the blood glucose level when compared to the T2-DM group alone ($P < 0.05$) (Figure 1).

Histological investigation of the splenic tissues

The microscopic examination in the spleen tissues of the normal control rats Gp1 (Ctrl) stained with H & E observed the normal structure of splenocytes with a normal appearance of periarteriolar lymphoid sheaths of white pulp (WP), red pulp (RP), and marginal zone (MZ) between the red and white pulp (Figure 2A). The histological structures of the spleen sections of T2-DM rats Gp2 (Diabetic) injected with a single dose of STZ (30 mg/kg) showed disarrangement and interference of RP and WP between them. The dilated and congested blood vessels (BV), the appearance of dense fibers (F), and vacuolated spaces were also noticed (Figure 2B). The histological structures of the spleen sections of the T2-DM rats Gp3 (D/Met) treated with Met (150 mg/kg) daily for 8 weeks showed partial restoration of the normal structure of spleen representing in the normal form of most WP, RP, and reduction of accumulation of F (Figure 2C). The histological structures of the spleen sections of the

T2-DM rats Gp4 (D/LQV) treated with S.V (0.0075 mg/kg) daily for 8 weeks showed an obvious recovery and improvement of RP, MZ, WP, and complete disappearance of the F (Figure 2D).

Immunohistochemical investigations of spleen for CD3 (T-cells)

The splenic tissues of normal rats Gp1 stained with CD3 marker expressed normal strong immunoreactivity to T cells in the white pulp of splenic parenchyma (Figure 3A). In Gp2 (Diabetic) STZ-induced rats stained with CD3 marker expressed a marked depletion of immunoreactivity to T cells in the white pulp of splenic parenchyma (Figure 3B). The T2-DM rats Gp3 (D/Met) treated with Met (150 mg/kg) daily for 8 weeks expressed an improvement in the expression of immunoreactivity to T cells in the splenic parenchyma (Figure 3C). The T2-DM rats Gp4 (D/LQV) treated with LQV (0.0075 mg/kg) daily for 8 weeks stained with CD3 marker expressed an obvious recovery and increment of immunoreactivity to T cells in the WP of splenic parenchyma (Figure 3D).

Image analysis of splenocytes T-cells

T2-DM rats Gp2 (Diabetic) recorded a significant decrease in the area percentage of splenic T-cells as compared to Gp1 (Ctrl) rats ($p \leq 0.001$) and significant increase in splenic T-cells of Gp3 (D/Met) rats given Met (150 mg/kg) daily for 12 weeks and LQV (0.0075 mg/kg) daily for 8 weeks as compared to STZ-induced diabetic rats group ($p \leq 0.001$) (Figure 4).

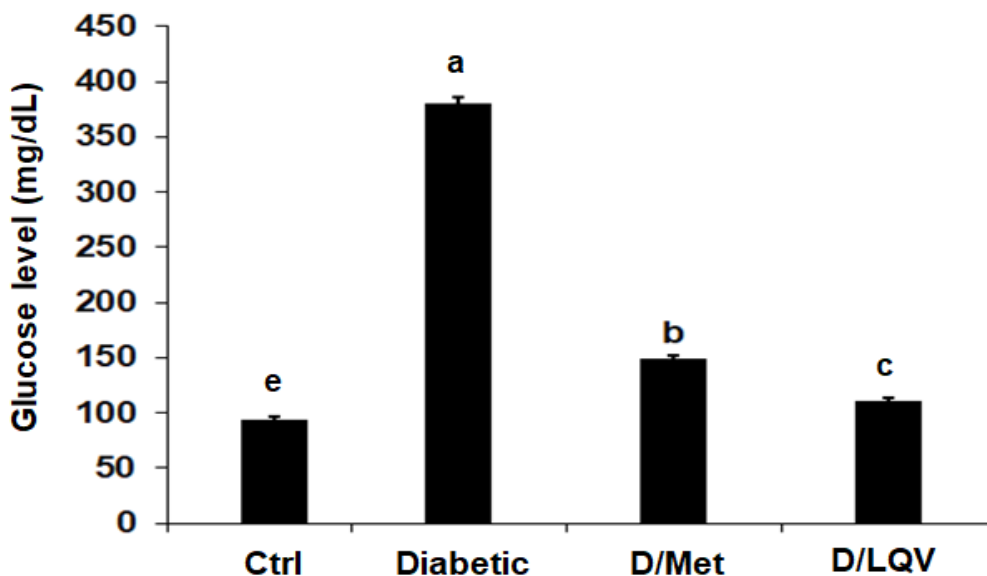
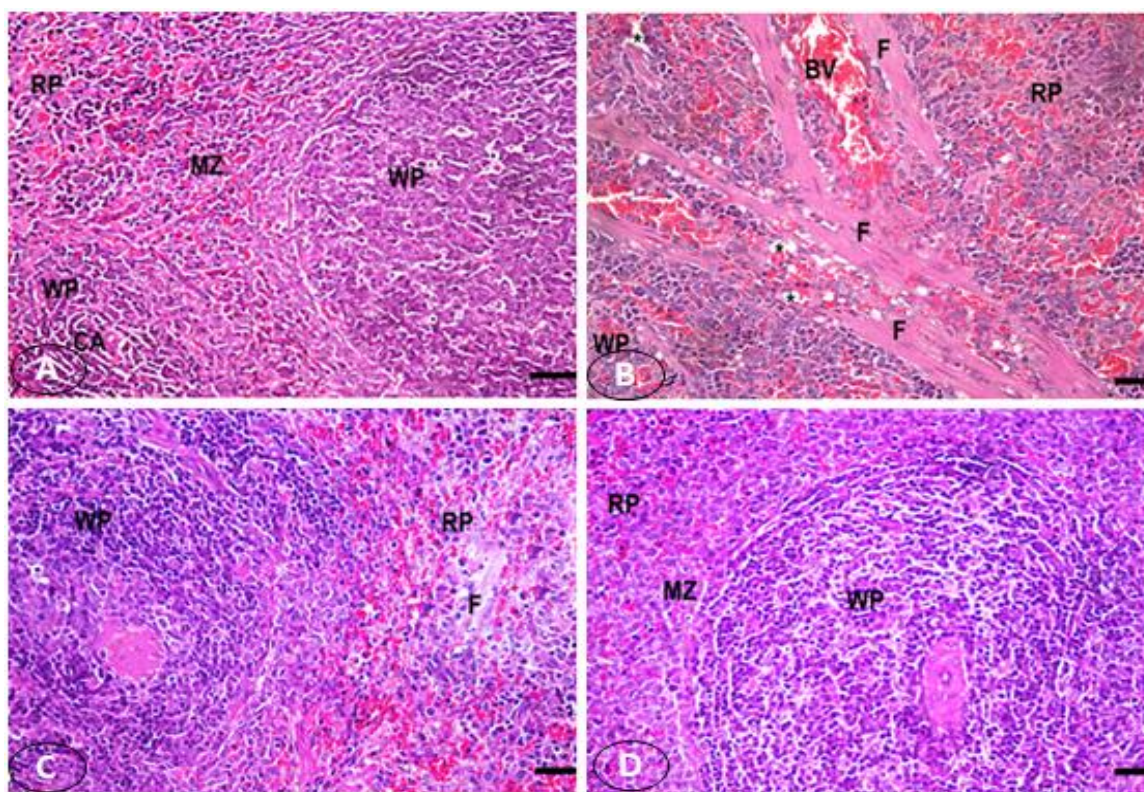
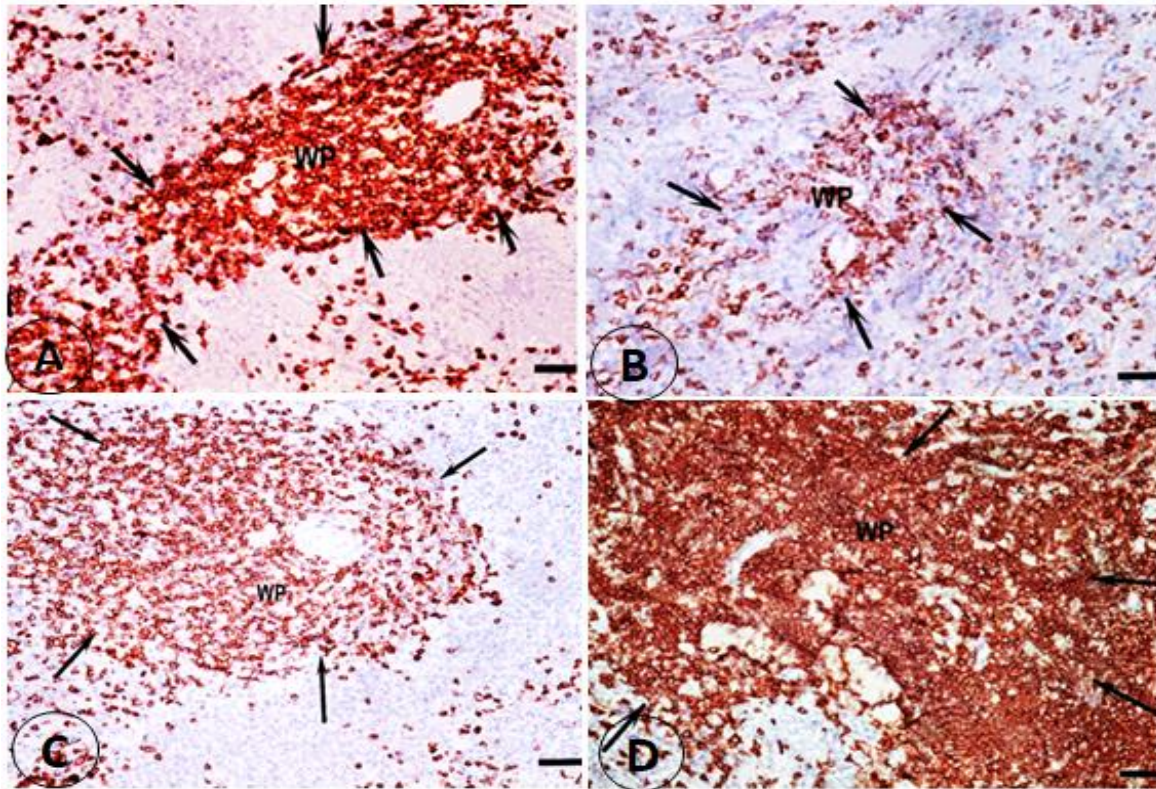


Figure 1. Glucose level in the different groups under the study. The values represented mean \pm SD. Ctrl: Control group; D: Diabetic; Met: Metformin; LQV: *Leiurus quinquestriatus* venom. P -value < 0.05 was statistically significant. The means that do not share the same letter are significantly different (Tukey's test).



Figures (2 A-D). Photomicrographs of splenic sections of Gp1 (Ctrl) rats showing normal white pulp (WP), red pulp (RP), sinusoid (S), central arterioles (CA), and marginal zone (MZ) (Fig.1 A). Photomicrographs of splenic sections of Gp2 (Diabetic) STZ-induced rats showing the disorganization of the white pulp (WP), red pulp (RP), a dilatation & congestion of the blood vessels (BV), and accumulation of the dense branched fibers (F) (Fig.1 B). Photomicrographs of splenic sections of Gp3 (D/Met) rats treated with Met (150 mg/kg) daily for 8 weeks showing partial restoration of the normal structure of spleen representing in the normal form of most white pulp (WP), red pulp (RP) and a reduction of the accumulated fibers (F) (Fig.1C). Photomicrographs of splenic sections of the Gp4 (D/S.V) rats treated with S.V (0.0075 mg/kg) daily for 8 weeks showing an obvious recovery of the normal structure of spleen with normal appearance of white pulp (WP), red pulp (RP), marginal zone (MZ) and completely disappearance of the fibers (F) (Fig.1 D). H&E, scale bar = 6.25 μ m.



Figures (3 A-D). Photomicrographs of splenic sections of Gp1 (Ctrl) rats expressing the normal strong immunoreactivity to T cells (arrows) in the white pulp (WP) of splenic parenchyma (Fig.2 A). Photomicrographs of splenic sections of Gp2 (Diabetic) rats expressing a marked decrement of immunoreactivity to T cells (arrows) in the white pulp (WP) of splenic parenchyma (Fig2 B). Photomicrographs of splenic sections of Gp3 (D/Met) rats received Met (150 mg/kg) daily for 8 weeks expressing an improvement in the expression of immunoreactivity to T cells (arrows) in the white pulp (WP) of splenic parenchyma (Fig.2 C). Photomicrographs of splenic sections of Gp 6 (D/S.V) rats received S.V (0.0075 mg/kg) daily for 8 weeks expressing an obvious recovery and increment of immunoreactivity to T cells (arrows) in the white pulp (WP) of splenic parenchyma (Fig2. D). CD3 immunostain, Bars = 6.25 μ m.

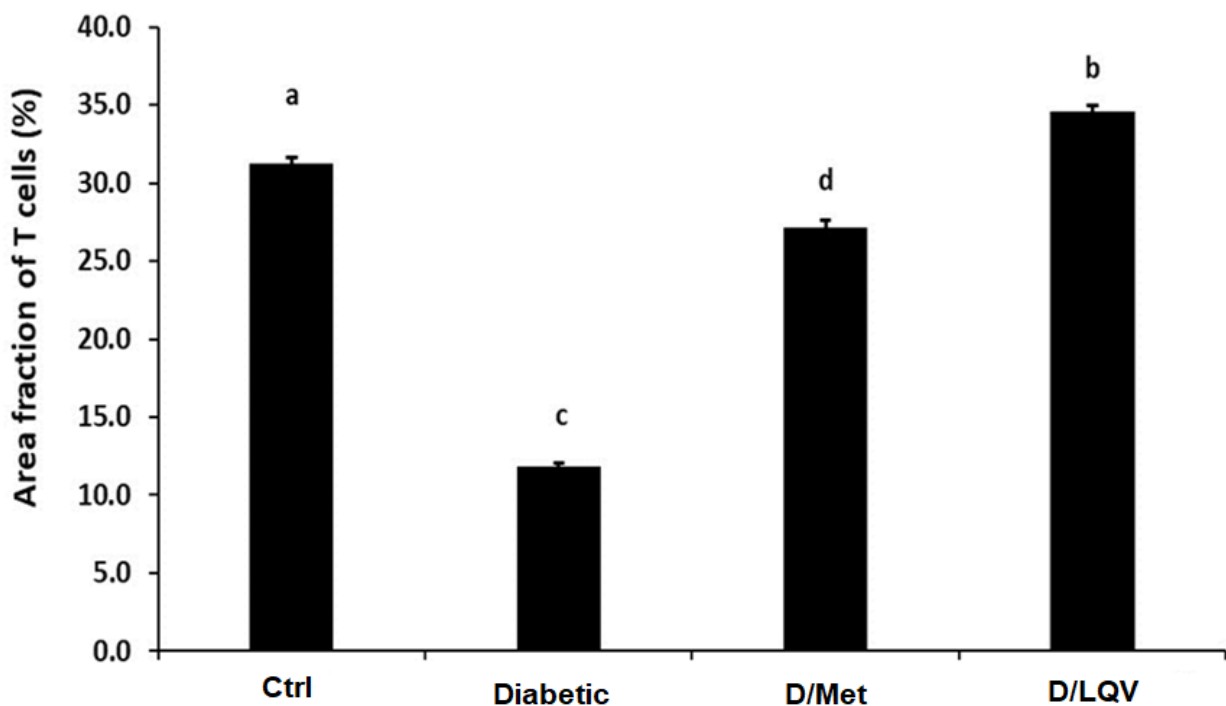


Figure 4. Morphometric measurements showing changes in the mean of area fraction of T cells (%) in all rat's groups. The values represented mean \pm SD. Ctrl: Control group; D: Diabetic; Met: Metformin; LQV: *Leiurus quinquestriatus* venom. P -value $<$ 0.05 was statistically significant. The means that do not share the same letter are significantly different (Tukey's test).

Discussion

Due to impaired glucose metabolism, DM represents a serious public health problem that leads to chronic inflammation and oxidative stress. The therapeutic potential of venom-derived drugs is evident today. The success of venom-derived compounds is linked to their increased bioactivity, specificity, and stability (23). LQV are rich bioactive peptide libraries that offer promising molecules that may lead to the discovery and development of new drugs for the treatment of various diseases (24). The current study evaluated the protective role of the treatment with LQV on the splenic tissues of T2-DM rats. The results showed that the group of T2-DM rats showed a significant decrease in the % b.wt change when compared to the negative control group. The % b.wt change of T2-DM rats that were treated with Met or LQV was significantly improved when compared to the diabetic rats. Treatment of diabetic rats with S.V. improved rat's body weight changes due to the improvement of carbohydrates and fats metabolic disorders induced by diabetes. Met induces weight loss by activating lipolysis by inhibiting adipogenesis. It also inhibits carbohydrate absorption and bile salt uptake through stimulation of GLP-1 which inhibits energy production (25). Met decreases blood glucose through increasing the activity of the insulin receptor and its substrate which increases the uptake of glucose in the liver cell (26). A previous study reported the therapeutic potential of scorpion venom-derived peptides in the improvement of T1-DM status (23). In the present study, the diabetic rats' groups recorded a marked atrophy in the weight of their spleen tissues but after taking Met or LQV daily for 8 weeks; the increment in the weight of the rat's spleen tissues was recorded and returned approximately to normal weight. Moreover, the present study showed that normal control, non-diabetic rat given Met, and non-diabetic rat given LQV had normal-appearing red and white pulps with normal nuclei and a marginal zone separating the red pulp (RP) and white pulp (WP) when their spleen sections were stained with H&E.

Previous studies have shown that WP plays an active role in iron transport in cytotoxic defense, and in scavenging free radicals via enhancement of glutathione (27, 28). Therefore, WP can reduce the effects of oxygen radicals by increasing glutathione. The T2-DM rats demonstrated a disarrangement and interference of red pulp and white pulps between them and reduced the WP, the dilated and congested blood vessels, and the appearance of dense fibers and vacuolated spaces was also noticed. Similar results were recorded in diabetic rats by Ebaid *et al.* (2015) (29). Hashish and Kamal, (2015) illustrated the atrophy of diabetic splenic mice and the diffusion of Wp (30). Also, with dilated blood arteries, mature lymphocytes in the periphery of the spleen were significantly decreased. In diabetic guinea pigs, similarly, Kumar *et al.* (2013) found that the RP and WP were frequently vacuolated with nuclei degeneration (31).

The T2-DM rats that were given Met daily for 8 weeks showed partial restoration of the normal structure of the spleen representing the normal form of most white pulp, and red pulp, and reduction of accumulation of fibers. The T2-DM rats that were given LQV daily for 8 weeks noticed a marked improvement and recovery of approximately normal RP, marginal zone, and WP. Scorpion's venom and toxins contain a variety of peptides and other useful molecules which can be used for the treatment of various diseases (32). The spleen represents a large lymphatic tissue passed by re-circulating lymphocytes, which can promptly elicit specific T or B lymphocyte-mediated immune reactions. It has T-cell and B-cell zones and enables the production of antigen-specific immune responses that shield the body from bacterial, viral, and fungal infections that spread through blood. Immune responses that are harmful to the host can be controlled at the spleen. Diabetes lowers immune response capabilities, including immune cell function reduction and immune organ atrophy (29). The components of inflammation appear to be significantly impacted by LQV and its metabolites, insulin synthesis,

secretion, and action, and all of these may influence the pathogenesis of T2-DM (33).

The IHC observations in the present study exhibited normal strong immunoreactivity to T-cells by using CD3 immunostain in the white pulp of splenic parenchyma and B-cells by using CD20 immunostain in the lymphatic follicles between periarteriolar lymphoid sheath (PALS) and marginal zone of the spleen sections of normal control rat. T2-DM rats expressed a marked decrement of immunoreactivity to splenic T and B cells these results agreed with Hashish and Kamal, (2015) who found that diabetes caused a decrease in the body weight and degeneration of splenocytes in diabetic albino rats treated with STZ (30). In accordance, Koulmanda *et al.* (2003) reported that STZ was directly toxic for lymphocytes, inducing apoptosis *in vitro*, and was responsible for early depletion of blood and spleen T and B cells *in vivo* (34). Moreover, Long and Buckner (2011) reported that STZ-induced diabetes is associated with a higher frequency of T regulatory cells in the blood and secondary lymphoid organs and reduced numbers of T-cells in the blood. Immunity to allogeneic insulinoma cells could be reduced by diabetes-induced immune suppression (35). While T2-DM rats those given Met or S.V daily for 8 weeks expressed an obvious recovery and increment of immunoreactivity to T and B-cells.

In conclusion, LQV treatment significantly ameliorates the histopathological changes of the splenic tissues in the T2-DM rats.

Conflicts of Interest:

The authors affirm that there are no conflicts of interest.

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