Morphological and Immunohistochemical Analysis of the Effects of Thymoquinone on the Neurovascular Component of Jejunal Submucosa of Diabetic Rat Model

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Abstract: Diabetes Mellitus is characterized by chronic hyperglycemia and causes morphological and functional changes in the gastrointestinal tract. This study was conducted to assess the effects of STZ-induced diabetes on the morphology of neurovascular component of the submucosa of rat's jejunum and its possible reversibility by Thymoquinone (TQ), the active ingredient of *Nigella sativa*. Thirty male Wistar rats were used, 10 in each of three groups; group I: control, group II: STZ-induced diabetes and group III: STZ-induced diabetes treated with TQ. The establishment of diabetes mellitus was confirmed by fasting blood glucose level>200mg/dl. Histological and anti-glial fibrillary acidic protein (AGFAP)-immunohistochemical methodologies were employed in formalin fixed paraffin-embedded sections from the jejunum of all rats. Cytomorphometric measures were done using image-analysis systems. We find that STZ-induced diabetes is associated with altered cellular organization of the villous mucosal lining, altered pattern of goblet cell secretion and deformed submucosal nerve plexus. Statistical analysis reveals significant increase in the following measures in diabetic rats compared to control and TQ-treated rats (p=0.000): i) thickness of brush border membrane of the villi, ii) surface areas of lumina of villous goblet cells, iii) thickness of the walls of submucosal arterioles and capillaries and iv) area% of AGFAP-immunostaining. Thymoquinone is found to have a retrieving action among these changes and might relief the diabetes-induced diarrhea through: i) counterbalancing the secretion pattern of the goblet cells, ii) improving the submucosal vasculature and ii) restoring the neural action of submucosal glia cells. We therefore plan further studies in terms of biochemistry and molecular biology to carefully examine whether these alterations in these aspects may actually exist.

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1. Introduction

Diabetes mellitus (DM) is one of the most common lifestyle chronic diseases characterized by alterations in the cellular metabolism. Its complications are mainly due to the damage, dysfunction and failure of several organs resulting in neuropathy and outlying vasculopathy ***(Zhao et al., 2003).***

The gastrointestinal tract is one of the most intensely attacked areas by diabetic neuropathy that lead to many symptoms including gastroparesis, enteropathy, esophageal motor dysfunction, diarrhoea or chronic constipation ***(Guo et al., 2004)***. Many researchers have detected morphological consequences of diabetic neuropathy in the intestinal wall, such as increased total surface area of small intestine, hypertrophy ***(Zhao et al., 2003)*** and hyperplasia of the intestinal mucosa ***(Lorenz-Meyer et al., 1977)***, and increased number of goblet cells ***(Mantle et al., 1988)***. Several theories have been proposed to explain this neuropathy as; impaired metabolism of fatty acids, reduction in the blood supply to the nerves, oxidative stress and advanced products of non enzymatic glycation ***(De Freitas et al., 2008).***

A traditional component of food that can reduce appetite, glucose absorption in intestine, hepatic gluconeogenesis, blood glucose level and body weight may prove to be useful for prevention and control of DM ***(Mathur et al., 2011).*** The flowering plant, Nigella sativa is a member of the Ranonuculaceae family native to southwest Asia. It is also known as black cumin and has been shown to be rich in fatty acids ***(Lutterodt et al., 2011)***, phospholipids ***(Ramadan and Morsel, 2002)*** and contains a number of active ingredients like Thymoquinone (TQ), hydro-Thymoquinone and nigellone ***(Ghosheh et al., 1999; Tiruppur Venkatachallam 2010)***. In particular TQ has been reported to have a hypoglycemic ***(El-Dakhakhny et al., 2002)***, anti–inflammatory ***(Mansour and Tornhamre 2004; Vaillancourt et al., 2011; Isik et al., 2011)*** and anti-cancer ***(Banerjee et al., 2010)*** properties. It also alleviates allergic asthma ***(El***

Gazzar et al., 2006; Boskabady et al., 2010; Nikakhlagh et al., 2010).

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed by numerous cell types of the central nervous system including astrocytes ***(Jacque et al.,1978)*** and ependymal cells ***(Roessmann et al.,1980)***. Mounting evidence suggests that they are the counterparts in the enteric nervous system of the astrocytes. Similar to astrocytes in the central nervous system, the enteric glia cells (EGCs) in the small intestine possess a densely integrated array of intermediate filaments rich in GFAP ***(Jessen and Mirsky 1983; Liu et al., 2010).***

Though, great attention was thrown on the effects of natural food components on enteric nervous system, limited studies concentrated on their effects on the diabetic enteropathy. In this way our aim in this study was to verify the effects of administration of Thymoquinone on the morphological and GFAP-immunohistochemical alterations in the neurovascular component of the jejunal submucosa in the Streptozotocin-induced diabetic Wistar rats.

2. Material and Methods

Animals

The present study was achieved on 30 adult male, Wistar strains of albino rats weighing 250 - 300g at the beginning of experiment. Rats were kept in specific pathogen-free conditions at room temperature and allowed free access to normal rat chow diet and water *ad libitum*. All rats were kept under observation for one week prior to the experiments to permit the animals to adjust to the environment. They were housed in a facility with 12-12 h light-dark cycle and divided randomly into three main groups: age-matched controls (n = 10), streptozotocin (STZ)-induced diabetics (n = 10) and STZ-induced diabetics followed by TQ administration (n = 10). The control rats were further subdivided equally into two subgroups; 5 rats were received balanced standard diet and the others received balanced diet together with the vehicle (0.5 ml corn oil) orally daily using an insulin syringe.

Experimental protocol

Hyperglycemia was induced by single intraperitoneal injection of streptozotocin (Sigma Chemical Company), St. Louis, MO, USA in a dose of 60mg/kg body weight dissolved in 0.01 ml citrate buffer, pH 4.5. Induction of diabetes was monitored for 28 days by whole blood glucose estimation. Blood samples were obtained from the cut tip of the tail (tail veins) for assay of blood glucose level in the two experimental groups using Accu – Check glucometer (Roche Diagnostics, Mannheim, Germany). Only animals with blood glucose levels >200mg/dl were considered as diabetic ***(Sen et al., 2011)***. From this time on, one group of hyperglycemic rats received 50 mg/kg of TQ dissolved in corn oil once daily orally using insulin syringe for two weeks ***(Arslana et al., 2005)***. Animals were sacrificed at the end of the experiment after intraperitoneal injection of thiopental sodium at a dose of 40 mg/kg ***(Abo Gazia and Hasan, 2012)***. Experiments were performed with the permission of the ethics committee of Taibah University (approval number ET.254). All experiments were conducted in accordance with the principles of laboratory animal care [National Institute of Health (NIH) Publication No. 85-23, revised 1996].

Tissue Sampling

A midline incision was made on the ventral surface (abdomen); approximately 10 cm section of the jejunum was selected. Within a short time, the residual contents in the lumen were cleared using saline. Specimens were fixed in 10% formalin for one day, processed and embedded in paraffin blocks.

Histological staining

Using the previously described methods of (***Bancroft and Gamble, 2008),*** deparaffinized sections 5 mm thick were stained with hematoxylin and eosin (H&E) and periodic acid shift (PAS) stains. PAS stain permitted highlighting carbohydrate contents of basement membranes, intestinal villous brush border and goblet cells ***(Swelim, 2005)***.

Immunohistochemical staining

A 3 μm sections were cut into slides coated with 3-aminopropyltriethoxy-silane for immunohistochemical staining using primary polyclonal anti-mouse antibodies against Glial fibrillary acidic protein (Anti-GFAP mouse monoclonal antibody, catalog No ab10062, Abcam, UK). Briefly, endogenous peroxidase will be quenched with 3% H_2O_2/methanol and non-specific binding will be reduced by incubation with 5% bovine serum albumin. Antigen retrieval will be performed by first pre-treating sections in a microwave oven or incubating them in 0.1% trypsin for 10 minutes at 37.0°C. After being sealed in the buffer containing normal bovine serum albumin and incubated with 50 ml of rabbit anti-human anti-antibodies for the studied parameters, the sections will be incubated with biotinylated goat anti-rabbit IgG for 30 min. For the negative control, the primary antibody will be omitted. Sections will be then incubated in DAB reagent and counterstained with hematoxylin and cover slipped using Protex mounting media (DAB-Stock Stain box; Boster Biotechnology).

Cytomorphometric and immunohistochemical evaluation

Cytomorphometric and immnohistochemical evaluation were carried out using the Image Analyzer (Digital camera CH-9435 DFC 290 coupled to photomicroscope; Leica Qwin standard, Wetzlar, Germany) at the Research Unit, Faculty of Medicine, Taibah University. Five randomly chosen high power fields from all intestinal sections were evaluated as follows: (i) Sections stained with H&E were examined to assess the general histological features (original magnification x100), (ii) Sections stained with PAS were examined to assess the thickness of the walls of submucosal arterioles, the thickness of the basement membrane (BM) of submucosal capillaries, the thickness of the brush border membrane (BBM) of jejunal villi and the surface area of the lumina of villous goblet cells (original magnification x1000; frame area = 786,432.0 μ^2) and (iii) Immunostained sections were examined to evaluate the condition of the submucosal nerve plexuses through measuring the percentage of anti-GFAP (AGFAP) immunostained area within the jejunal submucosa (original magnification x400).

Statistical analysis of Data

The data obtained were analyzed using the Statistical Product and Service Solutions Version 13 for Windows (SPSS, Chicago, Illinois, USA). Comparison between groups was carried out using one-way analysis of variance and least significant difference test (LSD). Data were expressed as mean ± standard error of means (SE). The results were considered significant when the *P* value was less than 0.05.

3. Results

Clinical criteria

Diabetes mellitus was observed in animals of group II and group III by increased blood glucose levels up to 28 days.

Histological analysis

Light microscopic examination of 226ejuna sections of control groups (subgroup I and subgroup II) reveals its wall is formed of mucosa, submucosa and muscularis externa. The lining mucosa is studded with innumerable finger like villi. At their bases, simple tubular invaginations or pits (intestinal glands, or crypts of Lieberkuhn) can be distinguished extending to the muscularis mucosa. The epithelium of the villi is continuous with that of the crypts forming a continuous sheet. The core of the villi is formed of connective tissue (lamina propria) and cells of immune system. The simple columnar epithelium that lines the crypts and covers the villi is distinguished into the absorptive cells and goblet cells. The goblet cells are recognized by their empty-looking cytoplasm and basally located nuclei (Fig. 1a). Their content is PAS+ due to the glycoprotein nature; also the brush border is seen as PAS+ strip over the villi (Fig. 2a). The submucosa is formed of connective tissue layer that contains fibroblasts, blood vessels, and a nerve fiber plexus (Meissner's plexus) (Figs. 1b, 3a). The muscularis externa consists of an inner circular coat and an outer longitudinal coat arranged in a helicoidal pattern. A prominent nerve fiber plexus; Auerbach's plexus, is found between these two muscle layers (Fig. 1b).

Light microscopic examination of jejunal sections of diabetic group reveals altered cellular organization of the mucosal lining especially at the tips of the villi, with either expanded or atrophied warped villi. There are malformed and misplaced intestinal villi with numerous goblet cells in nearly all the examined sections (Fig. 1c). PAS stained sections reveal thickened villous brush border membrane and altered pattern of goblet cells. Their lumina are distended with PAS+ material and pouring its contents into the villous surface (Fig.2b). The significant striking observation in diabetic submucosa is the presence of thick walled blood vessels with variable degree of mural thickening, luminal narrowing and accumulation of PAS+ material in their walls (Fig. 3b).

In comparable with diabetic group, sections from TQ-treated diabetic rat show well formed villi with restored cellular organization (Fig. 1d), thinning of the brush border membrane, fewer and smaller goblet cells (Fig. 2c) and average-sized submucosal blood vessels (Fig. 3c).

Immunohistochemical analysis

Examination of the AGFAP-immunostained jejunal sections of control rats show the immunorective enteric glia network constituting the submucosal plexus with prominent ganglion cells and interpolated processes. Some AGFAP+ cells extend in between and inside the cores of the villi and crypts (Fig. 4a). In the diabetic sections, the submucous plexus appears malformed with apparent decrease in the AGFAP+ enteric glia cells and less prominent processes. AGFAP+ enteric glia cells are scattered in the submucosa with slight expression (Fig. 4b). TQ-treated diabetic sections reveal enhanced AGFAP expression in the well formed submucosal plexus with AGFAP+ glia cells extending in between the villi and crypts (Fig. 4c).

Cytomorphometric analysis

Values of the means and SE of the cytomorphometric measures (thickness of the villous brush border membrane, luminal surface area of the goblet cells, thickness of the wall of submucosal blood vessels and area% of AGFAP immunostaining) are shown in Table (1) and Fig (5). No significant differences are detected between the subgroups of control rats, thus they are taken together as a single

group. One way analysis of variance reveal significant differences between the 3 studied groups in all analyzed measures.

The mean thickness of brush border membrane of jejunal villi and the mean surface area of the luminal villous goblet cells are significantly higher in the diabetic rats when compared to the control and TQ-treated ones. However, the values of BBM and surface areas in the control and TQ-treated groups reveal no significant difference (p=0.058 & p=0.696 respectively).

There is significant difference in the mean thickness of the wall of the submucosal arterioles of the diabetic rats when compared to the control or TQ-treated ones. The mean thickness of the wall of the submucosal arterioles and capillaries in TQ-treated is nearly comparable to that of the control group although the difference between both groups is significant (p=0.039&0.025) in the arterioles and capillaries respectively.

Regarding the AGFAP-immunostained areas, LSD test demonstrates that the mean area% of immunostaining is significantly lower in the diabetic rats compared to the control and TQ-treated ones. On the other hand, no significant difference is detected between the control and the TQ-treated groups (p=0.281) (Fig. 1). STZ- induced diabetes produces 75% reduction (from 3.2% to 0.8%) in the AGFAP+ immunostained area in relation to controls. Subsequent treatment with TQ leads to rise in AGFAP+ immunostained area by 70% (from 0.8% to 2.7%) in relation to diabetic rats.

Table (1): Cytomorphometric measures in the submucosa of jejunum in the animals from control, diabetic and TQ-treated groups. The results are expressed as mean ± SE. *=p<0.05 when all groups are compared.

Variables in the jejunum	Control rats N=10	Diabetic rats N=10	Thymoquinone-treated diabetic rats N=10	*P value*
Percentage of AGFAP-immunostained area in the submucosa (mean± SE)	3.2±0.5	0.8±0.3	2.7±0.3	0.000*
Thickness of the wall of the submucosal arterioles (mean± SE)	55.6±2.8	114.7±2	64.3±3.5	0.000*
Thickness of basement membrane of the submucosal capillaries (mean± SE)	12.7±0.5	18.5±0.8	14.8±0.5	0.000*
Thickness of the brush border of intestinal villi (mean± SE)	24.3±1.4	83.9±2.8	29.3±0.4	0.000*
Surface area of the lumina of villous goblet cells (mean± SE)	24586.4±1012.2	46786.2±2064.9	25383.6±911.9	0.000*

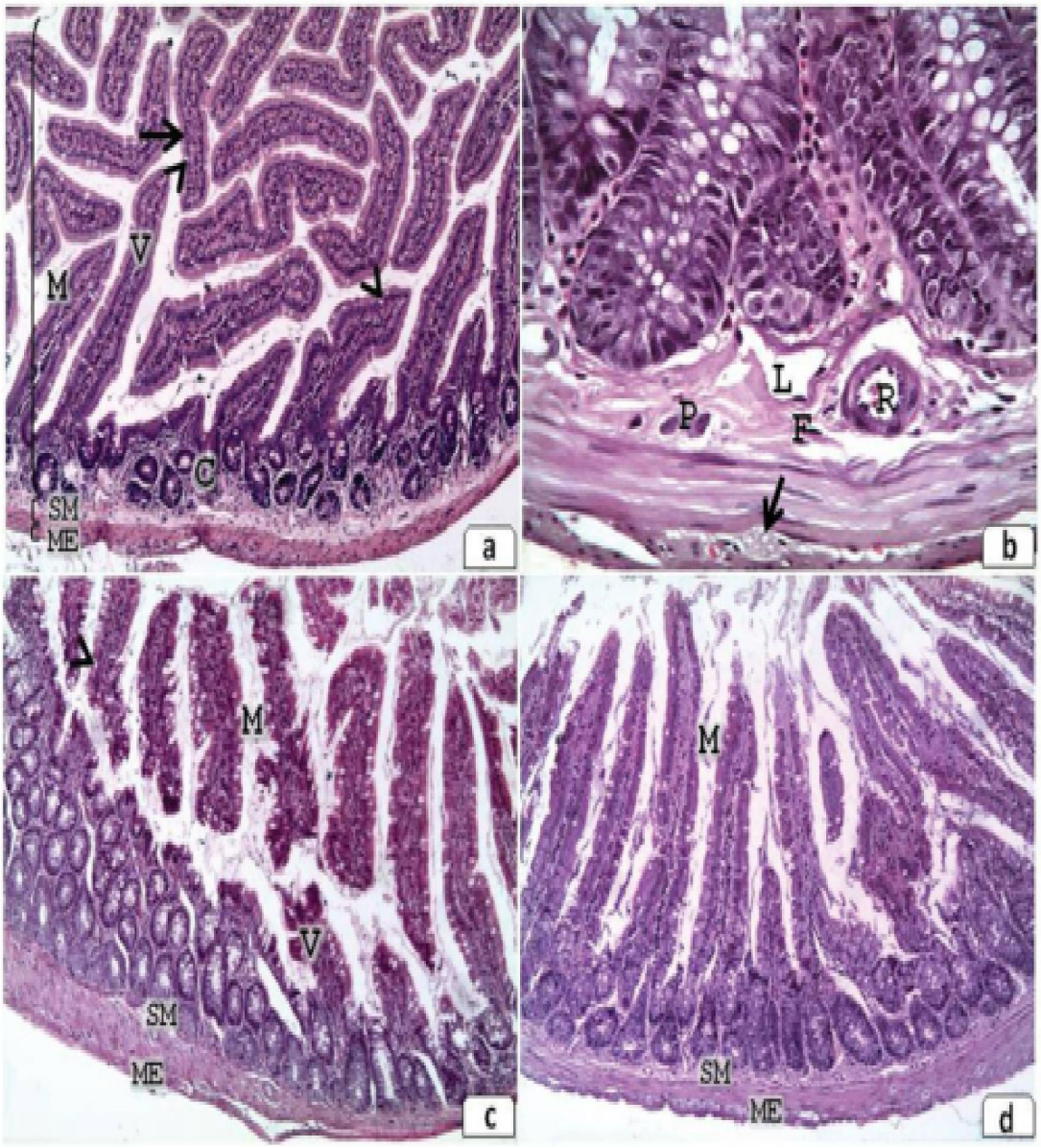

Fig. (1): A Light photomicrographs of a cross sections of rats' jejunum show the wall is formed of mucosa (M), submucosa (SM) and muscularis externa (ME). a) Section from control rats showing the mucosal lining is packed with leaf like villi (V) and simple tubular intestinal glands or crypts of leiberkuhn (C) extend to the muscularis mucosa. The simple columnar epithelium that covers the villi is distinguished into absorptive cells (arrows) and goblet cells (arrow heads) with empty-looking cytoplasm and basally located nuclei. b) A higher magnification of the submucosa of control rats representing a connective tissue layer containing fibroblasts (F), arteriole (R), lymph vessels (L) and nerve fiber plexus (P). The muscularis externa is seen formed of inner and outer coats with nerve fiber plexus in between (arrow). c) Sections from diabetic rat showing altered cellular organization of mucosal lining especially at the tips of the villi (V), with either expanded or atrophied warped villi. Villous epithelium is misplaced with apparently numerous goblet cells (arrow heads). d) Section from thymoquinone-treated diabetic rat showing well formed leaf like villi with restored mucosal cellular organization compared to diabetic rats. [a, c, d (H&E, x100), b (H&E, x400)].

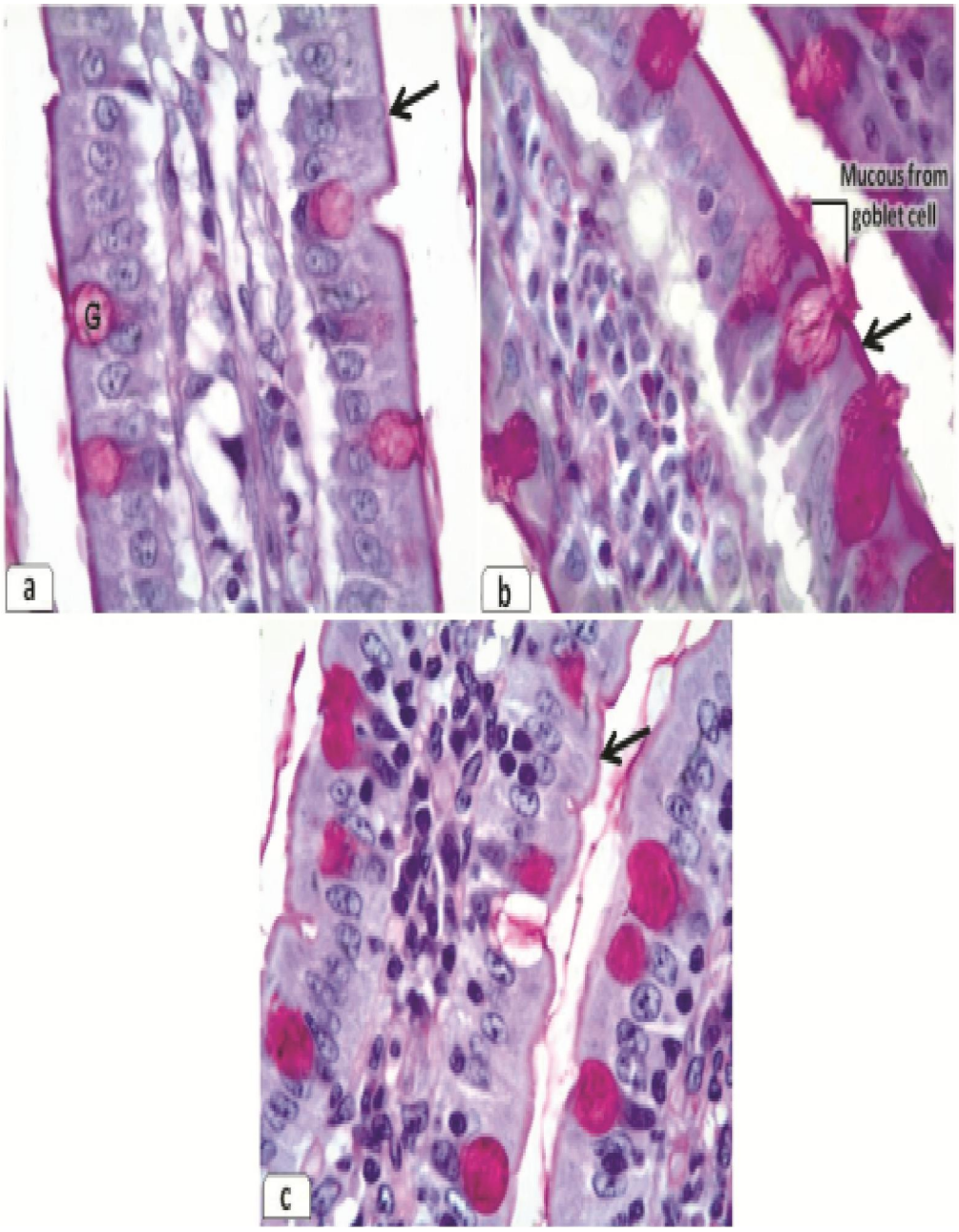

Fig. (2): A Light photomicrographs of cross sections of rats' jejunum stained with PAS. a) Jejunal villi of control rats showing the proper thickness of the villous brush border (arrow) and few goblet cells (G) with average sized lumen filled with PAS+ material. b) Jejunal villi from diabetic rat showing thickened villous brush border membrane (arrow) and numerous widened goblet cells distended with PAS+ material and pouring its contents to the surface. c) Jejunal section from thymoquinone-treated diabetic rat showing thinning of the villous brush border membrane (arrow) and many appropriate-sized goblet cells filled with PAS+ material compared to control rats (PAS, original magnification, 1000).

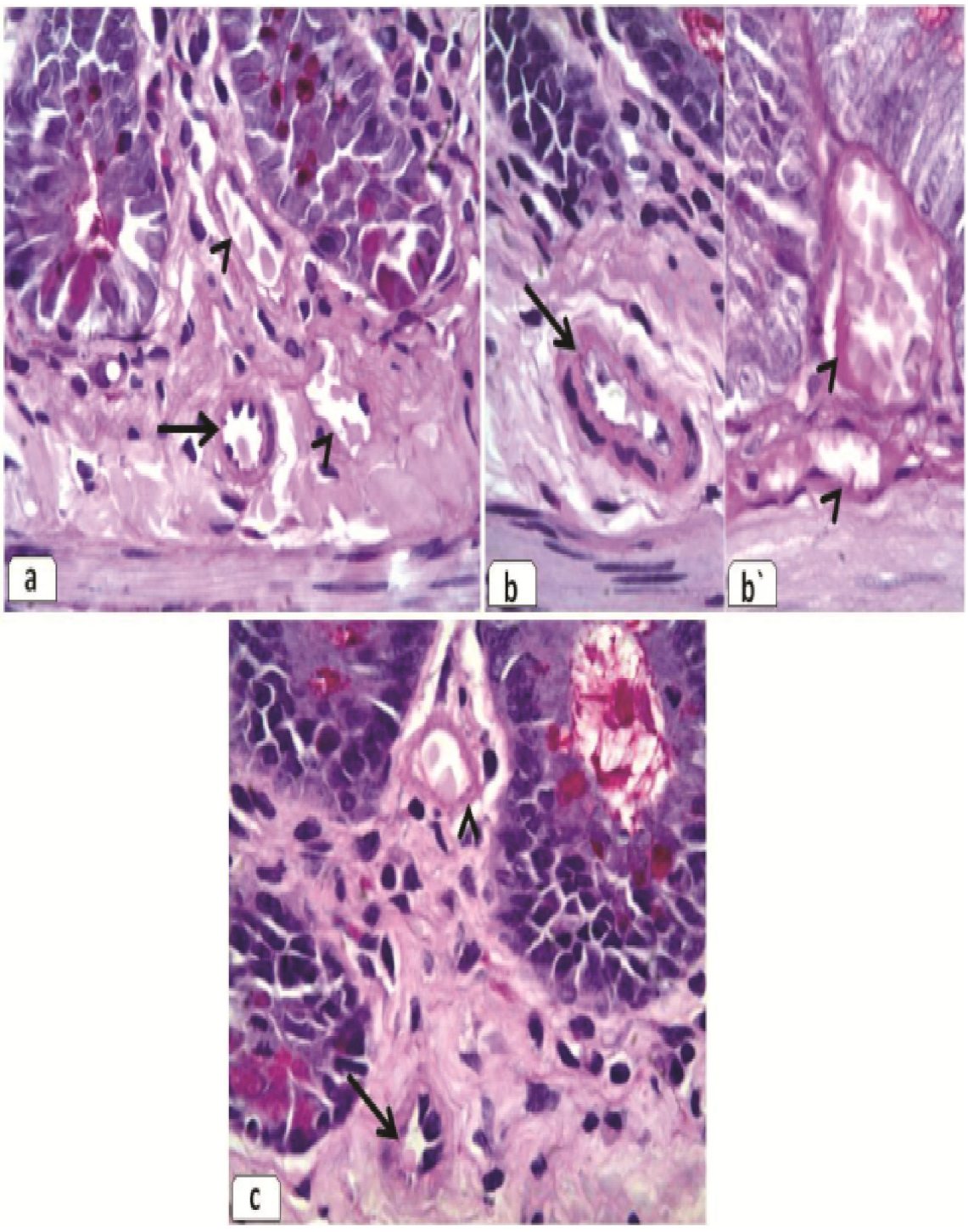

Fig.(3): A Light photomicrographs of cross sections of submucosa of rats' jejunum stained with PAS. a) Section from control rats shows the submucosal arteriole (arrow) with thin wall and wide lumen. The capillaries are seen with thin basement membrane (arrow heads). b, b`) Sections from diabetic rat shows prominent thick walled submucosal arteriole (arrow) with mural thickening and luminal narrowing with accumulation of PAS+ material. The submucosal capillaries (arrow heads) are seen with thick PAS+ basement membrane. c) Section from thymoquinone-treated diabetic rat shows submucosal arteriole (arrow) and capillary (arrow head) with less deposition of PAS+ material compared with untreated group (PAS, original magnification, 1000)

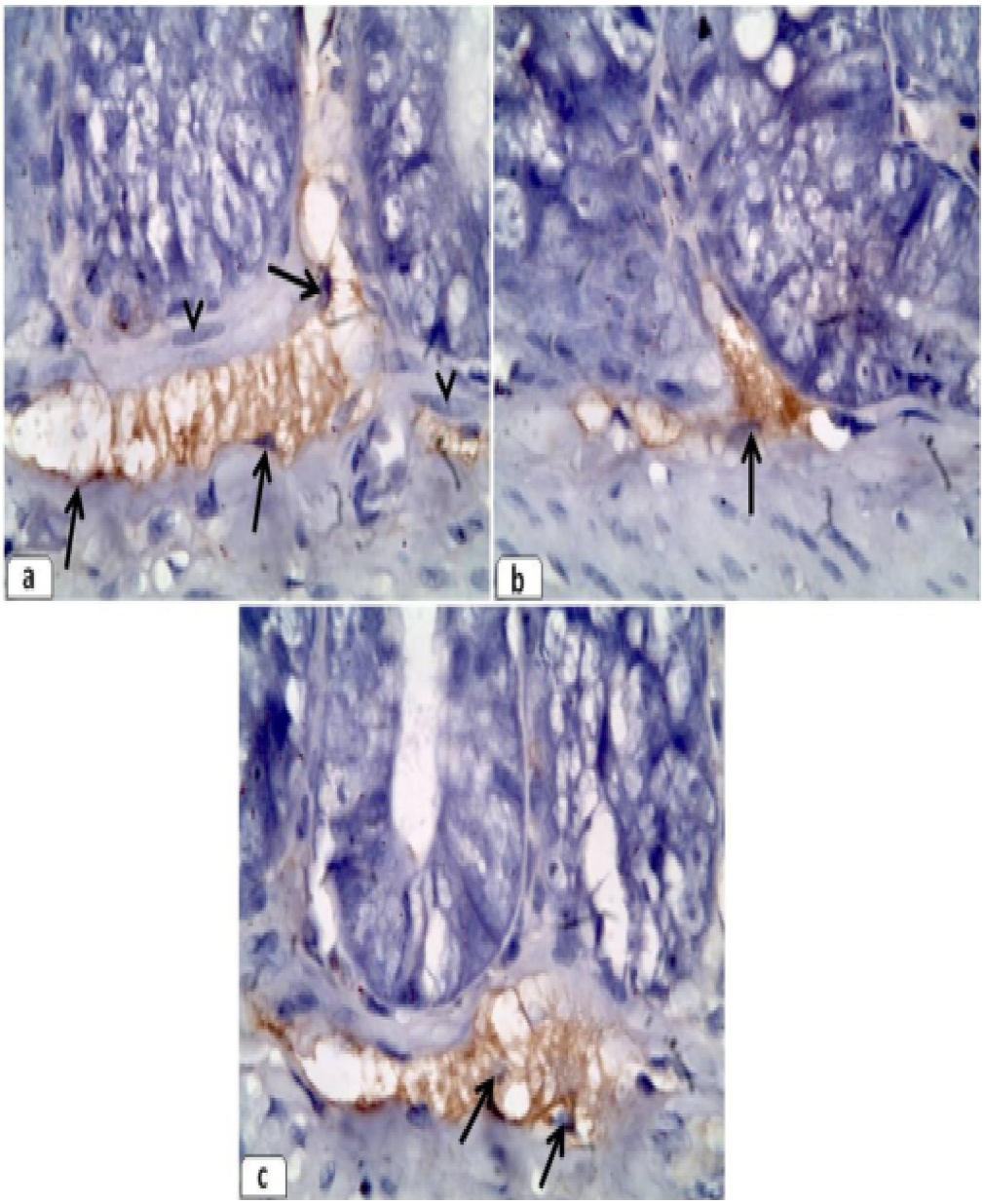

Fig. 4: A Light photomicrographs of AGFAP immunostained sections of submucosa of rats' jejunum. a) Section of control rats showing AGFAP+ (arrows) subpopulations of enteric glia cells placed in the submucosal plexus and extending between the epithelial crypts. Glia cells are multipolar-shaped with long thin processes connecting each other and it could easily be distinguished from unstained fibroblasts (arrow heads). b) Section from diabetic rat showing malformed submucosal plexus with fewer AGFAP+ enteric glia cells and less prominent processes (arrow) compared to control rats. c) Section from thymoquinone-treated diabetic rat showing well formed submucosal plexus with AGFAP+ glia cells (arrows) and long interpolated processes (anti-GFAP, original magnification-1000).

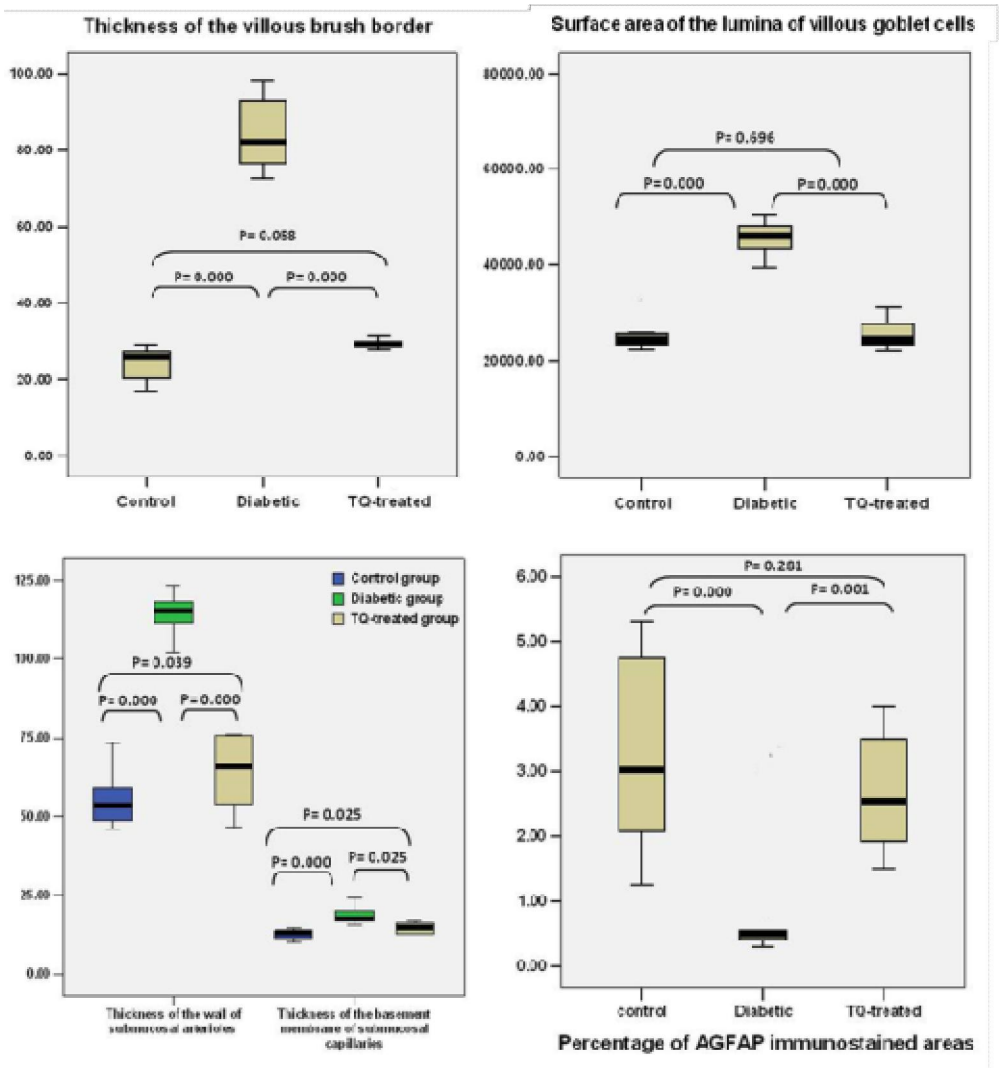

Fig. 5: Boxplot represents the cytomorphometric measures (in _m) in the submucosa of jejunum in the animals from control, diabetic and TQ-treated groups.

4. Discussion

Diabetes mellitus is one of the most common lifestyle diseases and diabetic enteropathy is one of its significant manifestations. Because the intestinal submucosa is responsible for structuring the small intestine ***(Zeng et al., 2003; Yu et al., 2004)***, the morphological changes in this region could impair the functionality of small intestine as a whole. This study was conducted to assess the effects of STZ-induced diabetes on the morphology of neurovascular component of the submucosa of rat's jejunum and its possible reversibility by TQ.

In the present study, STZ-induced DM causes altered cellular organization of the villous mucosal lining with a significant increase in the thickness of the brush border membrane that could be due to accumulation of glycoproteins. Administration of TQ causes restoration of the normal mucosal architecture and reduction of the thickness of the BBM towards the control state.

The increased thickness of brush border membrane after development of DM is matched with the report of ***Maeda et al. (2003)*** in the renal BBM. As BBM is responsible for initiation of intracellular signaling and maintenance of cellular structural integrity ***(Wang and Adler, 2008)***, the detected increase in PAS+ glycoproteins in BBM may provide a possible mechanism for the observed alterations in transport properties in DM as proved by ***Keelan et al. (1991)***. From other point of view, the alteration of BBM together with the cellular disorganization of the epithelial lining could impair the absorptive role of the small intestine in diabetic rats leading to excessive loss and diarrhea ***(Omotoso et al., 2012)***. In this way, the improving effect of TQ on the mucosal lining of small intestine might be due to adjustment

of the glycoprotein composition of BBM through reducing the accumulation of advanced glycation end-products and by decreasing liver glucose production via gluconeogenesis ***(Fararh et al., 2004)***.

Goblet cells (GC) are specialized cells for secretion of the intestinal mucus, a complex glycoprotein gel that covers the surface of epithelial villi and contributes significantly in lubrication and cell protection ***(Vaarala et al., 2008)***. The present study demonstrates a significant increase in the surface area of GC per villous in the jejunum of diabetic rats and the cells appear distended with PAS+ glycoproteins. These alterations in the morphology of GC could represent a predisposing factor for development of diarrhea during diabetes. On the other hand after TQ intake, the distension and the surface area of GC decline significantly. The increase in surface area of GC are consistent with the finding of ***Mantle et al.(1988)*** who reported the increased number of GC in diabetic rats. Additionally, the significant decrease in the surface area of GC in TQ-treated group, suggests a possible reduction in the rate of synthesis of mucus, and reveals the possible retrieving effect of TQ on the diabetes intestinal complications.

This study proves a significant increase in thickness of the walls of submucosal arterioles and capillaries of the diabetic rats and shows an obvious accumulation of PAS+ glycoproteins in their walls compared to the age-matched control and TQ-treated groups. The exact pathogenesis of these microangiopathic changes in DM is still unclear and controversial. ***Kolbe et al. (1990)*** attributed these changes to the imbalance between degradation of basement membrane and synthesis of its components. However, ***Boyd et al. (1990)*** relate it to the increased susceptibility to infections that accompany the defect in glucose metabolism and insulin imbalance.

The submucosal microvasculature as well as the microvasculature of the intestinal villi plays an important role in the process of digestion, absorption and mucosal barrier protection ***(Matheson et al., 2000).***The changes of microvasculature are reported to be the cause of diarrhea and enteritis ***(Lun, 1997; Laroux and Grisham, 2001).*** Comparable diabetic microangiopathic alterations were explored by ***Bódi et al. (2012)*** in the ileum and colon of diabetic rat and previously by ***De Las Casas and Finley (1999)*** in a duodenal biopsy from a patient with long-standing insulin-dependent diabetes mellitus with chronic diarrhoea.

Studying the morphological changes of the submucosal nervous tissue proves that diabetes induce 75% reduction in the area% of AGFAP+ immunostaining together with reduction in AGFAP+ glia cells and less prominent processes in relation to controls. Subsequent treatment with TQ raises the area% of AGFAP+ immunostaining by 70% in relation to diabetic rats.

The enteric nervous system is a division of the autonomic nervous system in the gastrointestinal tract that can mediate independent reflexes (motility, absorption and secretion) ***(Wade, 2002)***. Diabetic individuals with autonomic neuropathy have slower transit across the intestinal tract ***(Öztürk et al., 1996; Nakahara et al., 2002).*** This slower transit would allow the bacterial overgrowth that might explain the development of diarrhea in some diabetic individuals. The damage to the enteric neurons in the present animal model is in consistent with many previous studies ***(He 2001; Furlan et al., 2002)***. Also the reduction of enteric innervation with the onset of the diabetic neuropathy has been documented ***(Zanoni et al., 2003; Shotton et al., 2004)***.

The changes of AGFAP expression in glial cells of diabetic rat jejunum could be the consequence of multiple factors; i) nonviable extracellular conditions such as hyperosmolarity, low nutrient availability, or increased oxidative stress ***(Liu et al., 2010),*** ii) lack of insulin regulatory effect on the enteric glia cells as that was detected in mouse brain astrocytes ***(Toran-Allerand et al., 1991; Liu et al., 2010)*** and iii)Decreased insulin growth factor that affects the glia cell differentiation ***(Fernandez-Galaz et al.,1997)***. In addition, the DM-induced reduction in the number of myenteric neurons in several intestinal segments ***(Hernandes et al., 2000)*** could be reflected on the submucous plexus. This was supported by the existence link between both plexuses as described by ***Meedeniya et al. (1998) and Portbury et al. (1995)***.

The increase in AGFAP expression observed in our study after TQ administration reflects the adequate and effective action of TQ on protecting enteric glia cells. This might be due to the hypoglycemic effect of TQ as described by ***Kaleem et al. (2006) and Kanter (2008)***, who explained this effect through decreasing the oxidative stress and by providing a pancreatic ß-cell protection and regeneration/proliferation by down-regulation of immunological inflammatory activity towards ß-cells mediated by NO.

The main conclusion is that streptozotocin-induced diabetes in Wistar rats is associated with morphological changes in the neurovascular component of jejunal submucosa and that Thymoquinone could have a retrieving action among these changes and might relief the diabetes-induced diarrhea through: i) decreasing the mucous secretion of the goblet cells, ii) improving the submucosal vasculature and ii) restoring the neural action of submucosal glia cells.

We therefore plan further studies in terms of biochemistry and molecular biology to carefully examine whether these alterations in these aspects may actually exist.

Conflict of interest

The authors declare that there is no conflict of interest related to this study.

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