Histological Alteration and Immunohistochemical Characterization of Sciatic Nerve and Spinal Cord in Streptozotocin-Diabetic Rats

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Abstract:

Diabetes Mellitus (DM) is considered the major origin of diabetic neuropathy (DN). The later is a major chronic diabetes complication characterized by functional and structural alterations in peripheral nerves. Thus, in the present study we investigated the probable histological side effects of DM on the sciatic nerve and spinal cord in streptozotocin (STZ)- diabetic rats. This work included 20 female Wistar rats that were kept in regular conditions in their housing, and then they divided into two main groups; control and diabetic groups (10 animals each). Histological investigation of the sciatic nerve and spinal cord was performed by light microscopy (LM) and transmission electron microscopy (TEM). Moreover, S100 and GFAP immunohistochemical expression were analysed. DN group showed altered histological and ultrastructural features of sciatic nerve and spinal cord in comparison to control one. In addition, a notable reduction in S100 and GFAP immunostaining expression in spinal cord of diabetic rats. The decline of the staining affinity was observed in S100 stain of sciatic nerve of diabetic rats.

Key words: Diabetes Mellitus, Diabetic neuropathy, GFAP, S100, spinal cord and sciatic nerve.

Introduction

Diabetes mellitus (DM) is a common endocrine condition affecting over 100 million individuals worldwide ***(Ismail and Yaheya, 2009, Fan et al., 2022)***. DM is characterized by insufficient insulin production, leading to variations in blood glucose levels. It can consequences from various pathogenic processes, including autoimmune destruction of pancreatic beta cells or abnormalities causing insulin resistance ***(Genuth et al., 2003)***.

The most prevalent chronic diabetes consequence that lowers quality of life is diabetic neuropathy (DN), which producing pain sensation, sensory loss and amputation of limb ***(Calcutt, 2020)***. It affects motor neurons, peripheral nerves and autonomic nervous system ***(Said, 2007, Feldman et al., 2019)***. Diabetic patients frequently experience neuropathic pain ***(Tesfaye et al., 2013, Rosenberger et al., 2020)***, which characterized by hyperalgesia and allodynia. Maladaptive changes in the sensory neurons of the peripheral ***(Reichling and Levine, 2009)*** and central nervous system ***(Latremoliere and Woolf, 2009)*** are the cause of these alterations in the pain response. Moreover, hyperglycemia affectes on the peripheral nerves; including degeneration of intra-epidermal nerve fiber innervation patterns ***(Hulse et al., 2015)*** and hyper-excitability ***(Chen and Levine, 2003)***.

Number of microvascular changes in the peripheral nervous system is happened due to hyperglycemia; including capillary thickening, endothelial cell dysfunction and decreased nerve blood flow ***(Li et al., 2023)***. These alterations can lead to endoneurial ischemia and hypoxia, that can damage nerve fibers ***(Callaghan et al., 2020)***. Furthermore, number of metabolic pathways are activated by hyperglycemia, which can contribute to DN development; including hexosamine pathway, polyol pathway and advanced glycation end product (AGE) pathway ***(Callaghan et al., 2020)***. Also, diabetic hyperglycemia can leads to changes in the interaction between the neuronal and immunological systems ***(Choi et al., 2022)***. It activates astrocytes and microglia in the spinal cord ***(Mika et al., 2009)***. The synaptic communication between neurons is strongly affected by the activation of neuroglia, causing diabetic neuropathic pain ***(Watkins et al., 2007)***.

That is why this study was established to look at how diabetes mellitus affects albino rats' spinal cord and sciatic nerve.

Materials and Methods

1. Animals and housing

Twenty adult females Wistar rats were included, with average weight (200 - 250 gm) and

about 3 – 4 months old. They were purchased from Animal House Laboratory at the Faculty of Pharmacy, Suez Canal University. Then they were housed under standard environmental conditions of temperature (22-24°c), luminosity (12-h light/ dark cycle) and 60% humidity. They fed standard laboratory feed and clean water provided ad labitum. Every animal was given expert care in compliance with the National Institutes of Health's "The guide for the care use of laboratory animals" ***(Nat. Res Council, 2011)***. This was carried out in accordance with guidelines of the animal care and use committee at Faculty of Veterinary Medicine, Suez Canal University **(approval no. 2019036).**

2. Experimental design

- The experimental rats were distributed randomly into the following groups (10 animals each):

Group I: control negative non-diabetic group.

Group II: Positive diabetic group.

3. Induction of type 1 diabetes

After an overnight fasting, a single injection of 50 mg/kg STZ dissolved in citrate buffer (pH 4.5) was injected intraperitonial to 10 rats. In the control group, the same volume of citrate buffer was injected without STZ ***(Ghazipour et al., 2022)***. To induce ketosis, a characteristic feature of type 1 diabetes, the rats were switched from 10% sucrose water to regular water on the second day of the experiment ***(Furman, 2021)***. Gluco-DR glucose strips were used to monitor fasting blood glucose levels following a week of STZ injection. The blood glucose levels after fasting were higher than 250 mg/dl for two successive reading were considered diabetic record ***(Ghazipour et al., 2022)***. Four weeks after induction of type 1 diabetes, the rats were sacrificed for sampling.

4. Collection of samples

Ten rats from each group were sacrificed by cervical dislocation while sedated with 80 mg/kg I.p. of ketamine.The sciatic nerve and L4-L5 spinal segment were collected and immersed in paraformaldehyde 4% for 48 hours.

5. Histological and histochemical procedures

After trimming of the fixed tissue samples, using an ascending ethanol series, dehydration was achieved. The samples were then cleaned in xylene and embedded in paraffin wax. Sections of paraffin blocks were cutting at 5-7 um thick using a leitz 1512 microtome. Tissue sections were mounted on egg-albumin coated slides ***(Suvarna et al., 2018)***. Hematoxylin and Eosin (H&E) stain was used for routine staining procedures. Next, slides were placed in DPX (dibutylphthalate polystrene xylene) mounting medium. for histological investigations. Photomicrographs were captured using an Olympus BX 41 research microscope equipped with a digital AMT

camera and its image capture engine software **(AMT V600.259)**.

Additionally, histochemical procedures aimed to assess the morphology and integrity of the axons and myelin sheaths of spinal cord and sciatic nerve sections; this was done by using the silver staining technique ***(Elsherbiny et al., 2019)***.

6. Ultrastructural visualization of sciatic nerve and spinal cord

One cm of sciatic nerve and spinal cord samples were used for the electrophysiology tests ***(Xiaojing et al., 2015)***. Pre-cooled glutaraldehyde 2.5% in 0.1 M phosphate-buffered saline was used to fix tissue samples for 1-2 hours and post-fixed in 1% osmium tetraoxide solution for 1 hour. Subsequently, they were washed in buffer, dehydrated in ascending grades of alcohol, and then cleared in acetone. Ultrathin sections (80-90 nm) were first stained with lead citrate and then 8% uranyl acetate. Subsequently, they were inspected at 70 kV using a transmission electron microscope **(JEOL JEM 1010)** at Al-Azhar University's Regional Center for Mycology and Biotechnology **(RCMB).**

7. Immunohistochemical examination

Fixed tissue samples from the aforementioned organs at different experimental groups were prepared and placed on positively charged slides. In order to measure astrocyte expression in the spinal cord using immunohistochemistry, glial fibrillary acidic protein (GFAP) immunostaining was carried out ***(Evangelista et al., 2018)***. Moreover, to detect the myelination protein in the sciatic nerve and spinal cord, immunostaining with S100 protein was performed ***(Xiaojing et al., 2015)***. The tissue slides underwent a series of lowering alcohol concentration for rehydration after being deparaffinized in xylene ***(Suvarna et al., 2018)***. Using a microwave oven, the slides were incubated in a citrate buffer (pH 6) in order to accomplish antigen retrieval ***(Suvarna et al., 2018)***. The incubation of the slides with primary antibodies was applied. For GFAP immunostaining, a rabbit anti-GFAP polyclonal antibody **(Diagnostic BioSystems, Cat # PDR028, CA., USA)** at a dilution of 1:100 in PBS was used ***(Evangelista et al., 2018)***. Also, for S100 immunostaining, rabbit anti-S100 polyclonal antibody **(Diagnostic BioSystems, Cat # PDR008, CA., USA)** at dilution of 1: 200 in PBS was used. Then, sections were incubated with secondary antibody anti-rabbit HRP secondary antibody **(Thermo Fisher Scientific, Cat # 31460, UK)** during 30 min. at room temperature. To improve the nuclear staining, the slides were counterstained with Mayer's hematoxylin after being treated for 30 minutes with 3, 3-diaminobenzidine peroxidase enzyme substrate (DAB) as a

chromogen for detection. Ultimately, a microscope examination of the slides was performed to evaluate the target proteins' expression. ***(Suvarna et al., 2018)***.

7.1. Measurement of immunostaining intensity:

Using Image J software, the percentage of immunointenisty was determined on ten immunostained sections **(version 1.33–1.34; National Institutes of Health, Bethesda, MD, USA)**.

8. Statistical analysis

Data were collected as mean ± SE for statistical analysis. Two tailed t teste for independent samples was applied. The statistical program for social science, version 20 **(SPSS software, SPSS Inc. Chicago, USA)** was used for data analysis. The significance level was established at P value < 0.05. ***(Biessels et al., 1999)***.

Results

1. General H&E Staining Results

Inspection of control group showed that the gray matter of lumbar spinal cord containing large basophilic multipolar neurons that distributed throughout the eosinophilic neuropil; each neuron had a central large nucleus with a prominent nucleolus and long cytoplasmic processes **(Fig. 1A1)**. Bundles of myelinated nerve fibers were dispersed in the white matter. Each fiber appeared as an unstained area of myelin sheath surrounding a central dark-stained axon **(Fig. 1A2)**. Additionally, neuroglia cells with their small nuclei were exhibited in the gray and white matters **(Fig. 1A1&A2)**.

The diabetic group showed various degrees of neurodegeneration in the gray matter of lumbar region. . Shrinked neural cells with pyknotic nuclei were characterizing the gray matter. Furthermore, restricted nuclear chromatolysis was evident **(Fig. 1B1)**. Interestingly, perineuronal vacuolation and cavitation of the gray matter were noticed **(Fig. 1B1)**. In addition, signs of demyelination were evident in the diabetic group white matter. This was manifested by spongiosis of white matter which owing to myelin sheath damage and periaxonal vacuolation **(Fig. 1B2)**.

Concerning sciatic nerve, typical histological construction of control rats was seen; each nerve fiber consisted of acidophilic axon enveloped by unstained area of dissolved myelin sheath and an eosinophilic neurilemma. Schwann cells nuclei and small thin walled of blood vessels were also observed **(Fig. 2A)**.

In another hand, the diabetic rats displayed disorganization of myelinated nerve fibers; most of them had obliterated myelin spaces. Others showed atrophy and axon depletion. Furthermore, the blood vessels were more dilated than that of the control group. Prominent decline of the number of schwan cell nuclei was obvious **(Fig. 2B)**.

Histological findings of the Spinal Cord and Sciatic Nerve Stained with Silver Stain Result

In the control group, organization of white matter of spinal cord was characteristic; forming bundles of myelinated nerve fibers. Each one involved central staining axon surrounded with unstained area of myelin sheath **(Fig. 3A)**. While, two main histological changes in the diabetic group were seen as follow; first, impairment of myelin sheath second, vacuolations surrounding the axons **(Fig. 3B)**.

Concerning sciatic nerve, bundles of myelinated nerve fibers tinged with the brown color of silver stain was obvious in the control group. The brown staining axon was surrounded by unstained area of myelin sheath **(Fig. 4A)**. Meanwhile, disintegration of myelinated nerve fibers of the diabetic group was marked. . Limited areas of axonal loss with atrophied nerve fibers wear detected **(Fig. 4B)**.

2. Ultrastructure visualization

2.1. Ultrastructure visualization of spinal cord

Typical organization of white matter in the spinal cord of the control group was noted. **(Fig. 5A)**. Bundles of myelinated nerve fibers were observed; each nerve fiber consisted of a centrally axon surrounded by regular, lamellar distribution of myelin **(Fig. 5A)**.

However, neural disorganization of white matter and disintegration of myelin sheath were observed in the diabetic rats **(Fig. 5B)**. Likewise, intramyelinic vacuoles were prominent (**Fig. 5B)**.

2.1.1. Ultrastructure visualization of sciatic nerve

The control group's sciatic nerves demonstrated that the nerve fibers were bounded by myelin sheath; that consisted of compact and regular myelin lamellae. The characteristic fine collagen fibrils of endoneurium were wedged among the nerve fibers **(Fig. 6 A1 & A2)**.

In diabetic group, sciatic nerves were manifested by widening endoneurium with edema and collagen fibrils. Attractively, invagination of the myelin sheath lamellae was noticeable. Extensive infolding and outfolding with sever compression of the axoplasm; that occasionally contains myelin fragments was marked. . In addition, some of myelinated nerve fibers showed marked thickening of myelin sheath with disappearance of axoplasm **(Fig. 6 B1&B2)**.

2.2. Immunohistochemical Results

2.2.1. S100 Immunohistochemical Staining for Sciatic Nerve.

S100 is a marker for detection of mature Schwan cell; the highest immunostaining affinity was recorded in the control group **(Fig. 7A).** Meanwhile, low staining intensity was in the diabetic group

(Fig. 7B). Morphometric analysis of S100 mean % area immunoreactivity of sciatic nerve showed significant increase of control group compered to diabetic one **(Fig. 7C).**

2.2.2. **S100**

2.2.3. **Immunohistochemical Staining of the Spinal Cord.**

Dense positive brown immunoreactivity on the gray and white matter of the spinal cord was observed by immunostaining affinity of S100, and it was higher in the control group than in the diabetes group **(Fig. 8B)**. When comparing the diabetic group to the control group, the mean percentage area of S100 immunoreactivity revealed a considerable decrease **(Fig. 8C)**.

2.2.4. **GFAP Immunohistochemical Staining of the Spinal Cord**

GFAP is a marker of astrocytes; a strong immunointenisty was observed throughout the gray matter of the control group **(Fig. 9A)**, while remarkable immunostaining decline was in the diabetic group **(Fig. 9B)**. The mean % area of GFAP immunoreactivity on the spinal cord showed a marked increase of control group GFAP affinity in comparison to diabetic group **(Fig. 9C)**.

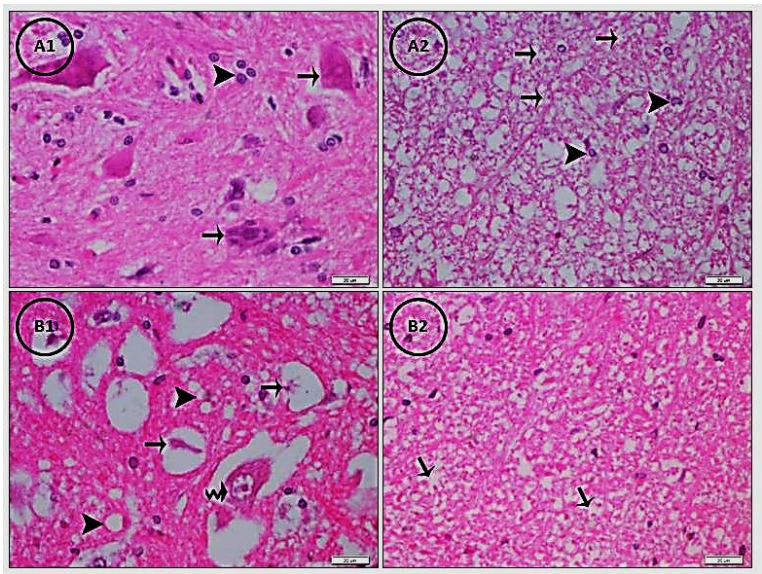

Fig. 1: Photomicrographs of H&E-stained spinal cord sections in experimental groups. (A1) gray matter of control group showing normal multipolar neurons with central nuclei and prominent nucleoli (arrow). Note neuroglia's nuclei (arrow head). (A2) white matter of control group showing bundles of myelinated nerve fibers; unstained areas of myelin sheath surrounding central stained axons were noticed (arrow). Also, neuroglia's nuclei were observed (arrow head). (B1) gray matter of diabetic group showing shrunken cells (arrow) and vacuolated neurophil (arrow head).

Chromatolysis of some neuron's nuclei were observed (curved arrow). (B2) white matter of diabetic group showing spongiosis of white matter and vacuolation around the axon (arrow).

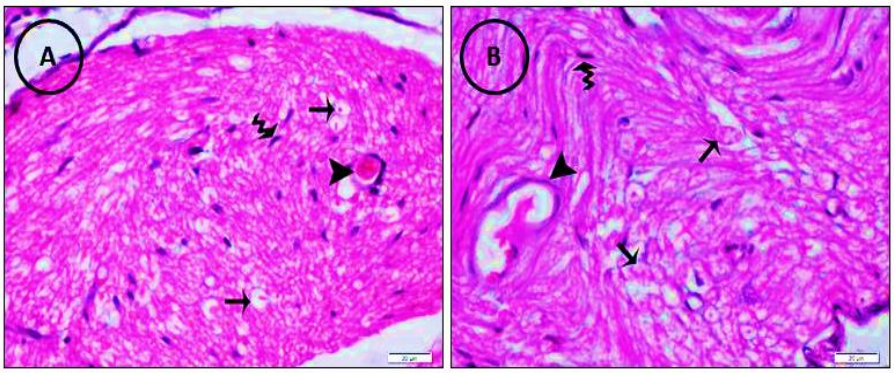

Fig. 2: Photomicrographs of H&E-stained sections of the sciatic nerve in experimental groups. (A) The control group showing bundles of nerve fibers; acidophilic axon surrounded by unstained area of myelin sheath (arrow). Schwan cell's nuclei (curved arrow) with small thin-walled blood vessels (arrow head) were observed. (B) The diabetic group showing disorganization of myelinated nerve fibers with obliteration of most myelin spaces (arrow). Note that the dilated blood vessels (arrow head) and less schwan cell's nuclei (curved arrow).

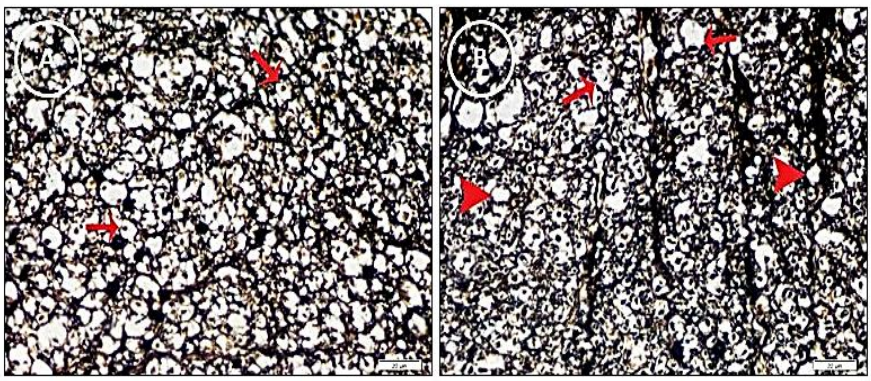

Fig. 3: Photomicrographs of spinal cord stained with silver stain of experimental groups. (A) The control group showing normal white matter. A central axon surrounded with unstained area of myelin sheath was seen (arrow). (B)The diabetic group showing vacuolation around the axon (arrow) and loss of many axons (arrow head).

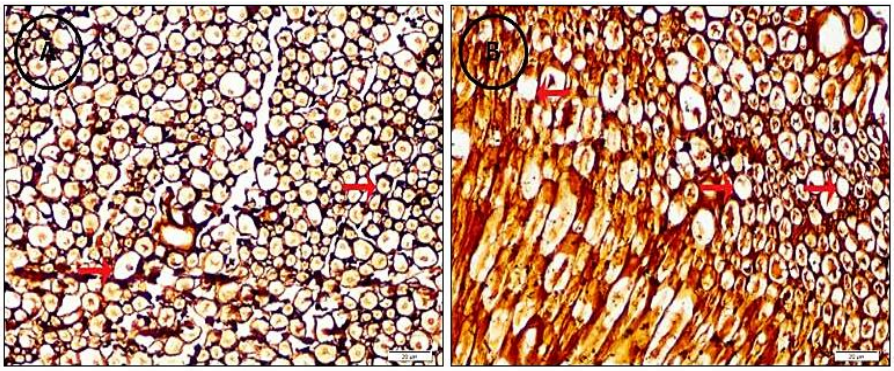

Fig. 4: Photomicrographs of sciatic nerve sections stained with silver stain in experimental groups. (A) The control group showing brown staining axons encircled by unstained areas of myelin sheath (arrow). (B)The diabetic group showing atrophy and fading of axons (arrow).

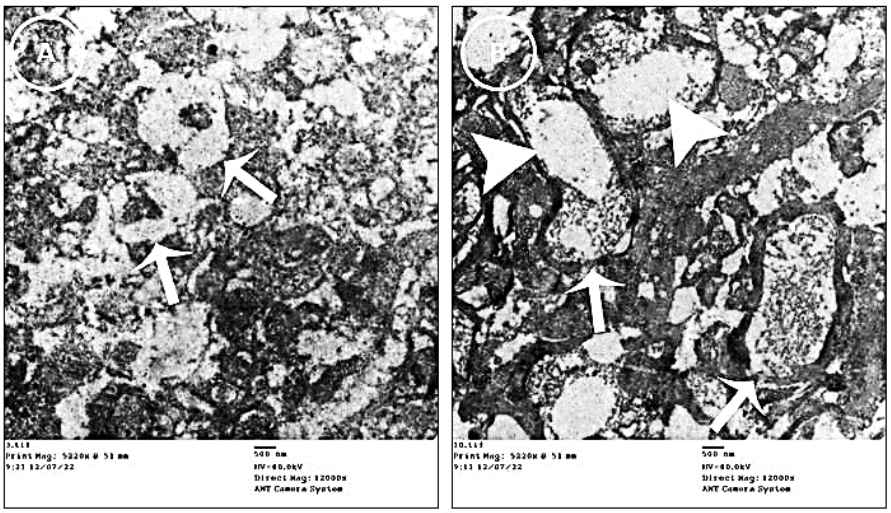

Fig. 5: Transmission electron micrographs displaying of the spinal cord of experimental groups. (A) Control group showing normal bundles of myelinated nerve fibers (arrow). (B) Diabetic group displaying disintegration of myelin sheath (arrow) and intramyelinic vacuoles (arrow head). (X12000).

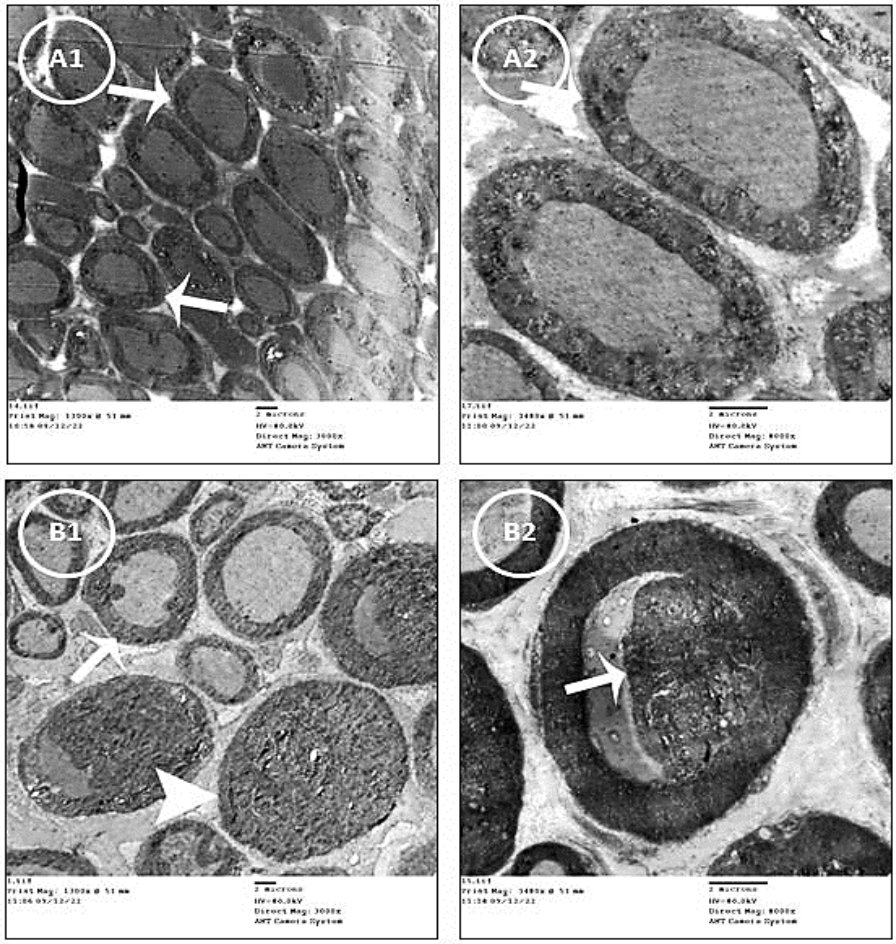

Fig. 6: Transmission electron micrographs of the sciatic nerve of experimental groups. Control group (A1&A2) displaying normal regular myelin sheath (arrow). Diabetic group (B1&B2) showing widely separated myelinated nerve fibers, infolding of myelin sheath (arrow), marked thickening of myelin sheath with no apparent axoplasm (arrow head). (A1& B1) (X 3000). (A2 & B2) (X 8000).

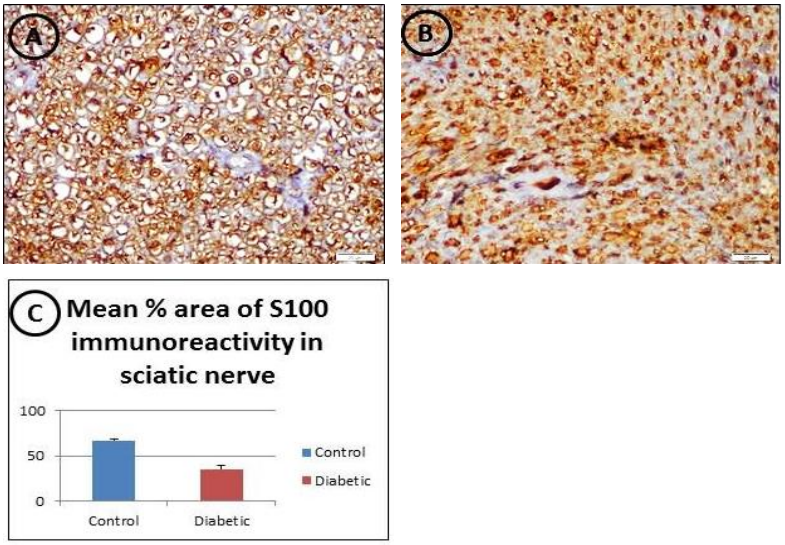

Fig. 7: Photomicrographs of the sciatic nerve immunostained with S100. The control group (A) and the diabetic group (B). Notable reduction of S100 immune expression was in the diabetic group. (C) An illustration of the mean % area of S100 immunoreactivity in the sciatic nerve.

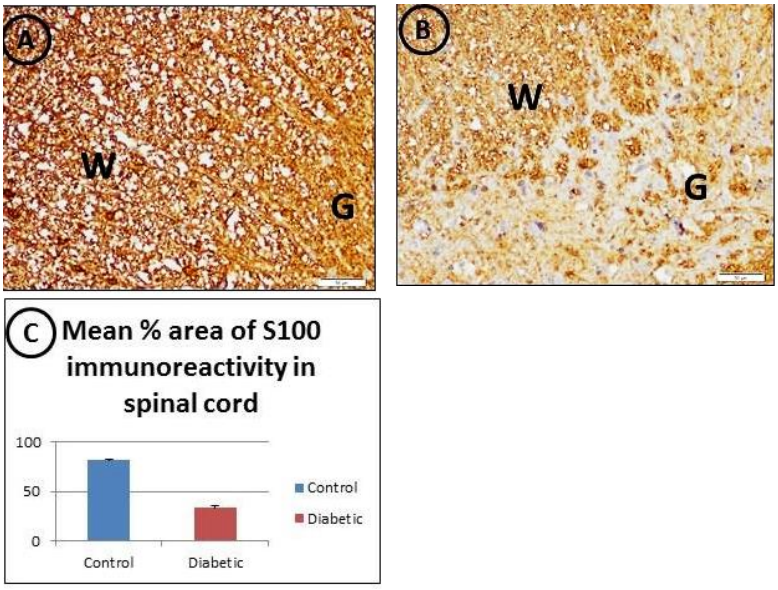

Fig. 8: Photomicrographs of the spinal cord cross-sections immunostained with S100; control group (A) and diabetic group (B) . (C) An illustration of the mean % area of S100 immunoreactivity in the spinal cord. S100 expression was dense positive brownish in the gray (G) and white (W) matter of the control group, while it declined in diabetic group.

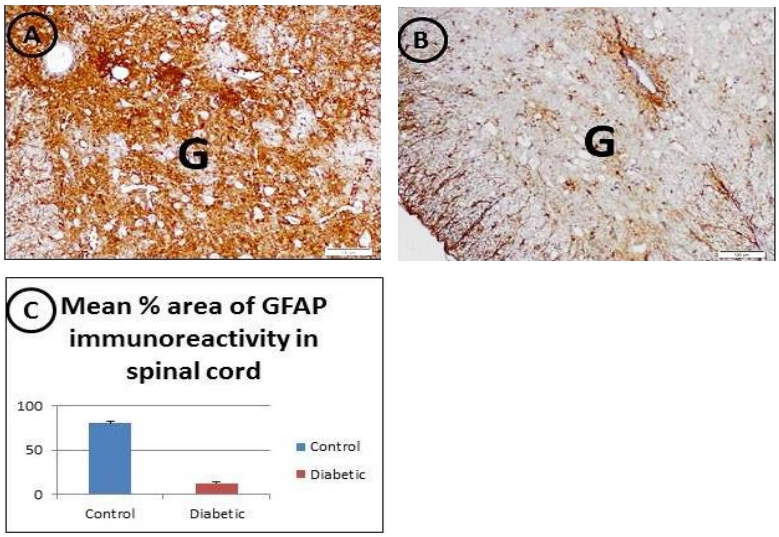

Fig. 9: Photomicrographs of the spinal cord immunostained with GFAP of experimental groups. Control group (A) displaying strong GFAP positive stain in the astrocytes of gray matter (G). Diabetic group (B) showing weak GFAP positive stain in the astrocytes in gray matter (G). (C) An illustration of the mean % area of GFAP immunoreactivity in the spinal cord.

Discussion

The main complication of diabetes was the neuropathy that affecting up to 50% of patients. It was characterized by nerves damage causing many symptoms as pain, numbness, tingling, and weakness ***(Nuha A et al., 2023)***. The mechanisms of diabetic neuropathy development included numerous alterations in the central nervous system. Spinal cord homeostasis was disrupted due to diabetic hyperglycemia. hyperglycemia induced metabolic and enzymatic abnormalities, which disturbed the cell mitochondria function inducing reactive oxygen/nitrogen species (ROS/RNS) overproduction and oxidative stress ***(Premkumar and Pabbidi, 2013, Wang et al., 2014)***.

Moreover, the consequent formation of ROS/RNS induced spinal neuroinflammatory cascades, which had been considered a crucial result of sensory neuropathy ***(Tsuda et al., 2008, Pabreja et al., 2011, Chen et al., 2016)***. In accordance with these concepts, the current study showed that STZ induced diabetic neuropathy as mirrored by marked histopathological changes at L4 & L5 spinal cord; including different degrees of neurodegeneration. This result agreed with the recent studies, which were reported that diabetic neuropathy condition contribute to neuronal damage, especially at L4

& L5 spinal cord ***(Alomar et al., 2021, Elsayed et al., 2023)***.

Histological examination of the sciatic nerve exhibited dramatic changes of diabetic in comparison to non-diabetic rats; such as the occurrence of degenerative alterations to the myelin sheath and axons. This result was in accordance with several studies showed that diabetes type 1 cause histological degenerative alterations to the myelin sheath and axons ***(Han et al., 2016, Evangelista et al., 2018, Alomar et al., 2021)***. Investigators stated that peripheral nerve fibers suffer progressive myelin loss in human patients and in animal models of DN ***(Sugimura and Dyck, 1981, Nowicki et al., 2012)***. In the current study, the ultrastructural results revealed degenerative changes of axons and defective myelination in the sciatic nerves and spinal cord of diabetic rats. Moreover, immune-expression of gene related to myelination (S100) was significantly suppressed in diabetic rats. These results was in agreement with the recent study, which reported that diabetes mellitus cause degenerative changes in the sciatic nerve and decreased the S100 expression in the sciatic nerve of diabetic mouse ***(Yigitturk et al., 2022)***. In addition to providing numerous support functions for axons, Schwan cells (SCs) synthesize myelin, which aids in axon formation, regeneration, and salutatory conduction along axons. Moreover, SCs produce some neurotrophic factors, which provide a microenvironment supporting neural regeneration ***(Frostick et al., 1998, Han et al., 2016)***. SCs dysfunction plays an essential role in the DN pathogenesis ***(Jessen and Mirsky, 2008, Shy, 2009)***. In vitro experiment research, cultured SCs in high-glucose conditions caused reduction of myelination-related genes expression; such as Myelination basic protein (MBP) and S100; indicating dysfunction of SCs ***(Han et al., 2016)***.

In this study, When GFAP immunostaining of the spinal cord was performed on diabetic rats, the gray matter's GFAP immunoreactivity was significantly reduced. Our findings were consistent with several investigations that shown a noteworthy reduction in GFAP positivity throughout the spinal cord, hippocampus, cerebellum, corpus callosum, external capsule, and retina of rats injected with STZ to cause diabetes. ***(Rungger–Brändle et al., 2000, Asnaghi et al., 2003, Coleman et al., 2004, Zainab et al., 2008, Coleman et al., 2010, Hashish, 2015)***. Numerous researches have verified that the olfactory bulb and olfactory epithelium of STZ-diabetic rats showed a reduction in GFAP immunoreactivity. ***(Dennis et al., 2005)***. However, additional research revealed that in both spontaneous non-obese diabetic and STZ induced diabetic animals, there

was an increase in GFAP immunoreactivity in the hippocampus, cerebral cortices, cerebellar cortices, and spinal cord. ***(Baydas et al., 2003a, Baydas et al., 2003b, Evangelista et al., 2018)***.

The deficiency of insulin level was a possible mechanism that decreasing of GFAP immunoreactivity in diabetic animals ***(Zainab et al., 2008)***. several studies had observed that insulin was required to strengthen the GFAP immunointenisty in cell bodies and processes of astrocytes ***(Aizenman et al., 1986, Barber et al., 2000)***. This might be because GFAP mRNA and peptide expression rise in response to insulin***(Toran-Allerand et al., 1991)***, It was predicted that an insulin shortage would lead to a decrease in GFAP expression in astrocytes ***(Zainab et al., 2008)***. Thus, this study was confirmed that diabetes mellitus was changed the functional and metabolic abilities of astrocytes, which caused decreasing on their ability to maintain the supporting of neuron in the spinal cord with resulting diabetic neuropathy ***(Zainab et al., 2008)***.

In conclusion, this study's results demonstrate the various ways that type 1 diabetes affects the sciatic nerve and spinal cord. The histological and immunohistochemical analysis revealed that diabetes mellitus type 1 led to spinal cord and sciatic nerve degeneration; including demyelination, decreased S100 and GFAP expression.

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التغيرات النسيجية والتوصيف النسيجوكيميائى المناعى فى العصب الوركى و النخاع الشوكى فى الجرذان المصابة بالسكرى بالستربتوزوتوسين

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الملخص العربى

يعتبر داء السكري المصدر الرئيسي للاعتلال العصبي السكري. الاعتلال العصبي السكري هو أحد مضاعفات مرض السكري المزمنة الرئيسية التي تتميز بالتغيرات الوظيفية والهيكلية في الأعصاب الطرفية. وهكذا، في هذه الدراسة قمنا بدراسة الآثار الجانبية النسيجية المحتملة لمرض السكري على العصب الوركي والحبل الشوكي في الفئران المصابة بالسكري بالستربتوزوتوسين. شمل هذا العمل 20 أنثى من فئران ويستار تم الاحتفاظ بها في ظروف منتظمة في مساكنها، ومن ثم تم تقسيمها إلى مجموعتين رئيسيتين؛ مجموعات الضابطة والسكري (10 حيوانات لكل منهما). تم إجراء الفحص النسيجي للعصب الوركي والحبل الشوكي بواسطة المجهر الضوئي والمجهر الإلكتروني النافذ. علاوة على ذلك، تم تحليل التعبير المناعي الكيميائي S100 وGFAP. أظهرت مجموعة الجرذان المصابة بالسكرى السمات النسيجية والبنية التحتية المتغيرة للعصب الوركي والحبل الشوكي مقارنة بالمجموعة الضابطة. بالإضافة إلى ذلك، حدث انخفاض ملحوظ في التعبير المناعي S100 وGFAP في الحبل الشوكي لدى الجرذان المصابة بالسكري. ولوحظ انخفاض تقارب التعبير المناعى في صبغة S100 للعصب الوركي لدى الجرذان المصابة بالسكري.

الكلمات المفتاحية: داء السكرى، الاعتلال العصبي السكري، GFAP، S100، الحبل الشوكي، العصب الوركي.