Int J Clin Exp Pathol 2016;9(4):4761-4778
www.ijcep.com /ISSN:1936-2625/IJCEP0023630

Original Article

Overexpression of ADAMTS-13 and neuronal nitric oxide synthase relates with neuropathology in streptozotocin-induced type 1 diabetic rats

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Received January 9, 2016; Accepted March 25, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Hyperglycemia plays a critical role in the development and progression of diabetic encephalopathy. A few studies have focused on a disintegrin and metalloprotease with thrombospondin type I repeats-13 (ADAMTS-13) expression in the central nervous system (CNS), and its function continues to remain unclear. The purpose of this study was to compare the expression of ADAMTS-13, neuron specific enolase (NSE), neurofilament (NF), neuronal nitric oxide synthase (nNOS) and glial fibrillary acidic protein (GFAP) in brain tissues of experimental streptozotocin (STZ)-induced diabetic rats. Expression of ADAMTS-13 ($P < 0.001$), nNOS ($P < 0.001$), NSE ($P < 0.001$) and NF ($P < 0.001$) expressions in the brain tissue markedly increased while GFAP decreased ($P < 0.001$) in diabetic animals versus controls. The most prominent finding of our study was that ADAMTS-13 expression increased significantly, suggesting that it may play an important function/s in the regulation and protection of the blood-brain barrier integrity and central nervous system microenvironment in diabetes. The results also suggested that nitric oxide production may increase due to increased nNOS expression and this also might contribute to neuropathology related with diabetes. Furthermore, increased expression of ADAMTS-13, NSE, NF and decreased expression of GFAP may give an idea of the disease progress and thus may have a critical diagnostic significance. To the best of the authors' knowledge, this is the first report on ADAMTS-13 expression in the CNS of STZ-induced diabetic animals.

Keywords: ADAMTS-13, neuropathology, nitric oxide, diabetes mellitus, neurofilament, neuron specific enolase

Introduction

Diabetes mellitus (DM) is a metabolic disease that is characterized by dysfunctions in the glucose metabolism [1, 2]. Hyperglycemia causes brain edema, increases size of brain infarct [1, 3] through triggering of neuronal cell death and also induces some vascular complications [4, 5]. The most severe complication of diabetes is diabetic encephalopathy [6-8]. Although the research about the diabetic encephalopathy has been intensive and comprehensive, the cause of pathophysiology of degeneration is still unknown.

ADAMTS13 is a large zinc-containing metalloprotease enzyme that is involved in blood clotting [9]. It is mainly synthesized in hepatic stellate cells of the liver [10], but is also expressed in endothelial cells [11, 12] and the cells of many other organs including brain [11, 13-15]. ADAMTS-13 plays a role in the regulation of inflammation and prevention of microvascular thrombus formation by decreasing thrombotic activity through destruction of the Ultra Large von Willebrand Factor (UL-vWF) multimers into less active forms [16-18]. UL-vWF is essential in the induction of platelet adhesion after vascular injury [19, 20]. Moreover, it was also discussed that severe ADAMTS-13 expression in the brain and cerebellum decreases infarct risk by preventing thrombus formation [15]. These studies show that ADAMTS-13 has essential roles in the homeostasis of brain and in thrombosis as well as in the regulation of inflammatory processes.

Demyelination is also a major complication of diabetic patients [21-26]. It is thought that

hyperglycemia triggers disruption of the blood-brain barrier (BBB) and increases in its permeability [27-31]. Impairment of tight junctions, which plays a role in the transmembrane protein interactions between the BBB and the endothelial cells, also increases the permeability of the BBB in diseases with myelin disorders such as multiple sclerosis and idiopathic inflammatory demyelination [32]. In a previous study of Dincel and Kul, in Border Disease that is characterized by demyelination, it was mentioned that there might be a positive correlation between ADAMTS-13 and demyelination [11].

Depending on the level of expression, nitric oxide (NO) has dual effects in the CNS. High level of NO that is produced by nNOS and other types of NOS was shown to be responsible for the irregularities in cerebral blood flow [33-36]. It is also known that high-level NO that is produced by neuronal and glial cells (astrocytes, microglia and oligodendrocytes) causes degeneration and apoptosis in CNS [35, 37, 38]. This situation is explained by cytochrome c release that is caused by the loss of the mitochondrial membrane potential [39-42]. It is also known that apoptosis plays an important role in the pathogenesis of DM and some common neurodegenerative diseases [1, 2, 43, 44].

The principle objective of this research is to investigate whether there is a correlation between degeneration of central nervous system (CNS) of streptozotocin (STZ)-diabetic rats and expression of ADAMTS-13. The second objective is to examine whether there is a relationship between severities of degeneration seen in the disease with nNOS expression. Moreover, we also focused on how astroglial activation plays role in this disease, and severity of the degeneration was assessed by analysis of the neuron-specific enolase (NSE) and neurofilament (NF) expressions.

Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) of Turkey. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments at Ataturk University (Permit Number: 390/19.03.2014).

Experimental animals

20 male Wistar albino rats weighing 250-300 were randomly allotted to two experimental groups (n = 10 per group). Animals were housed in a well-ventilated and air-conditioned area provided with independently adjustable light-dark cycle (12 h light/12 h dark cycle) and temperature regulation systems. Temperature was maintained at 22 ± 2°C and humidity was kept at 45%-70%. The rooms and animal cages were cleaned daily and the animals were provided with fresh food and water *ad libitum* on a daily basis.

Induction of STZ model of diabetes

Type 1 diabetes was induced in the rats (diabetic group) by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg body weight) dissolved in 0.1 mm sodium citrate, pH 4.5, while the normal control rats (nondiabetic group) were injected with the buffer only. The development of hyperglycemia in rats was confirmed by blood glucose evaluation. Blood glucose was determined by using an automatic glucometer (ACCU-CHEK Active, Roche Diagnostics Ltd, Germany). Plasma glucose level of the animals higher than 250 mg/dl on day 3 after STZ injection was considered hyperglycaemic [45]. These animals were selected for studies.

Necropsy and histopathologic examination

Animals were sacrificed at the end of 20 days of experiment period by decapitation and brains were quickly removed and processed for histopathology and immunohistochemistry analyses. Brain sections were fixed in 10% neutral buffered formaldehyde for 48 hours and washed under tap water overnight. Following routine tissue preparation procedures, tissue samples were dehydrated through graded series of alcohol and xylene and embedded in paraffin blocks. Paraffin serial sections were cut at a thickness of 4-5 μm and mounted on glass slides. Brains were sectioned at a 5μm thickness, stained with H&E and examined under a light microscope (Olympus BX51 and DP25 digital camera, Japan).

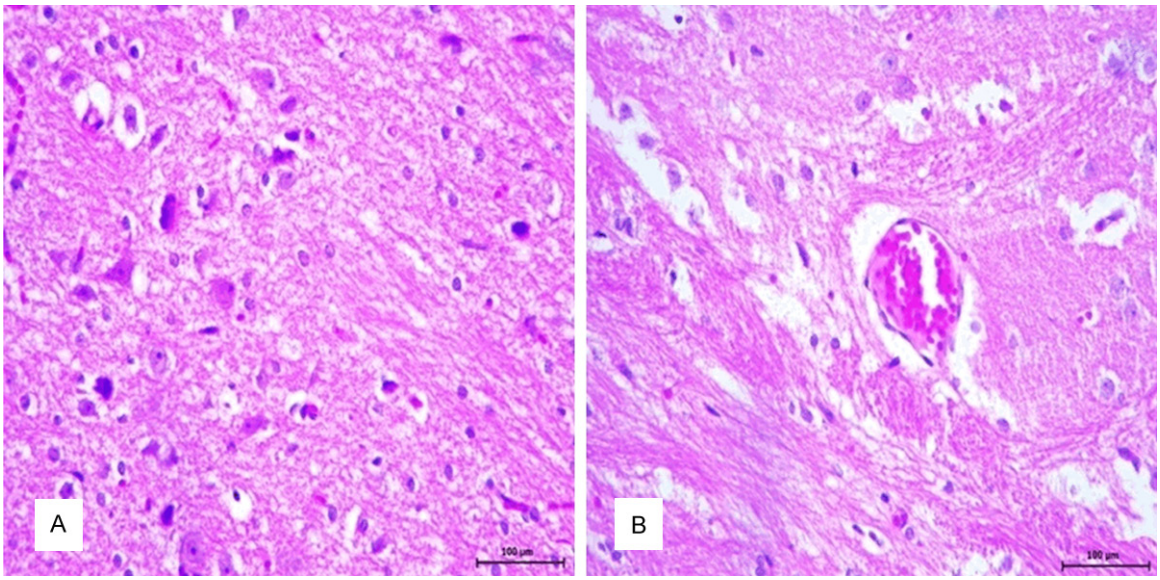

Figure 1. Neuronal necrosis areas were observed. H&E Bar, 100 μm (A) Neuronal necrosis areas and hyperaemia were observed. H&E Bar, 100 μm (B).

Antibodies

Commercial anti-mouse antibodies against ADAMTS-13 (Abcam, Cambridge, UK) diluted to 1:100, nNOS (Santa Cruz Biotechnology, USA) diluted to 1:100, GFAP (Thermo Scientific, USA) diluted to 1:100, NSE (Thermo Scientific, USA) diluted to 1:100 and undiluted NF (Thermo Scientific, USA) were used in the present study.

Immunoperoxidase examinations

Immunohistochemistry was performed to investigate ADAMTS-13, nNOS, NSE, NF and GFAP expressions. Commercial antibodies were visualized on 4- to 5-μm-thick paraffin sections using an indirect streptavidin/biotin immunoperoxidase kit (HRP; Thermo Scientific, USA). All steps were carried out following the procedure described by Dincel and Atmaca, 2015 [46]. Accordingly, tissue sections were placed on adhesive slides, deparaffinized for 5 minutes in each of three xylene series, and rehydrated in a graded alcohol series and distilled water. Antigen retrieval was accomplished by boiling sections on glass slides in citrate buffer (pH 6.0; Thermo Scientific, USA) for 20 min. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in absolute methanol for 7 minutes at room temperature. Sections were rinsed three times with phosphate-buffered saline (pH 7.4) for 5 min between each step of the test. Sections were incubated in blocking serum for 5 min to prevent non-specific binding. Thereafter, tissue sections were incubated with the primary antibody (ADAMTS-13, nNOS, NSE, NF and GFAP) for 60 min in a humidified chamber at room temperature. Sections were treated with a biotin-labeled secondary antibody for 15 min and with the streptavidin-peroxidase enzyme for 15 min at room temperature. Finally, sections were incubated in aminoethyl carbazole chromogen (Thermo Scientific, USA) for 5-10 min to induce the color reaction. Mayer's hematoxylin was applied as a counterstain for 30 sec. Thereafter, sections were mounted with water-based mounting medium (Thermo Scientific, USA). As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted. Sections were immediately analyzed. Immunostaining was evaluated using a binocular microscope and photographed under a 20X objective.

Quantitative histomorphometric analysis and statistics

The density of positive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK), connected to a Lecia DM4000 B microscope (Leica Microsystems Imaging Solutions,

Table 1. Immunoperoxidase test results and statistical data

ANIMALS	N	ADAMTS-13		P <	nNOS		P <	NSE		P <	NF		P <	GFAP		P <
		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd	
Control animals	10	2.074	0.141	0.001	1.540	0.074	0.001	2.298	0.146	0.001	5.227	0.287	0.001	4.912	0.125	0.001
STZ-treated animals	10	3.192	0.236		2.287	0.118		3.101	0.150		7.250	0.458		3.519	0.118	

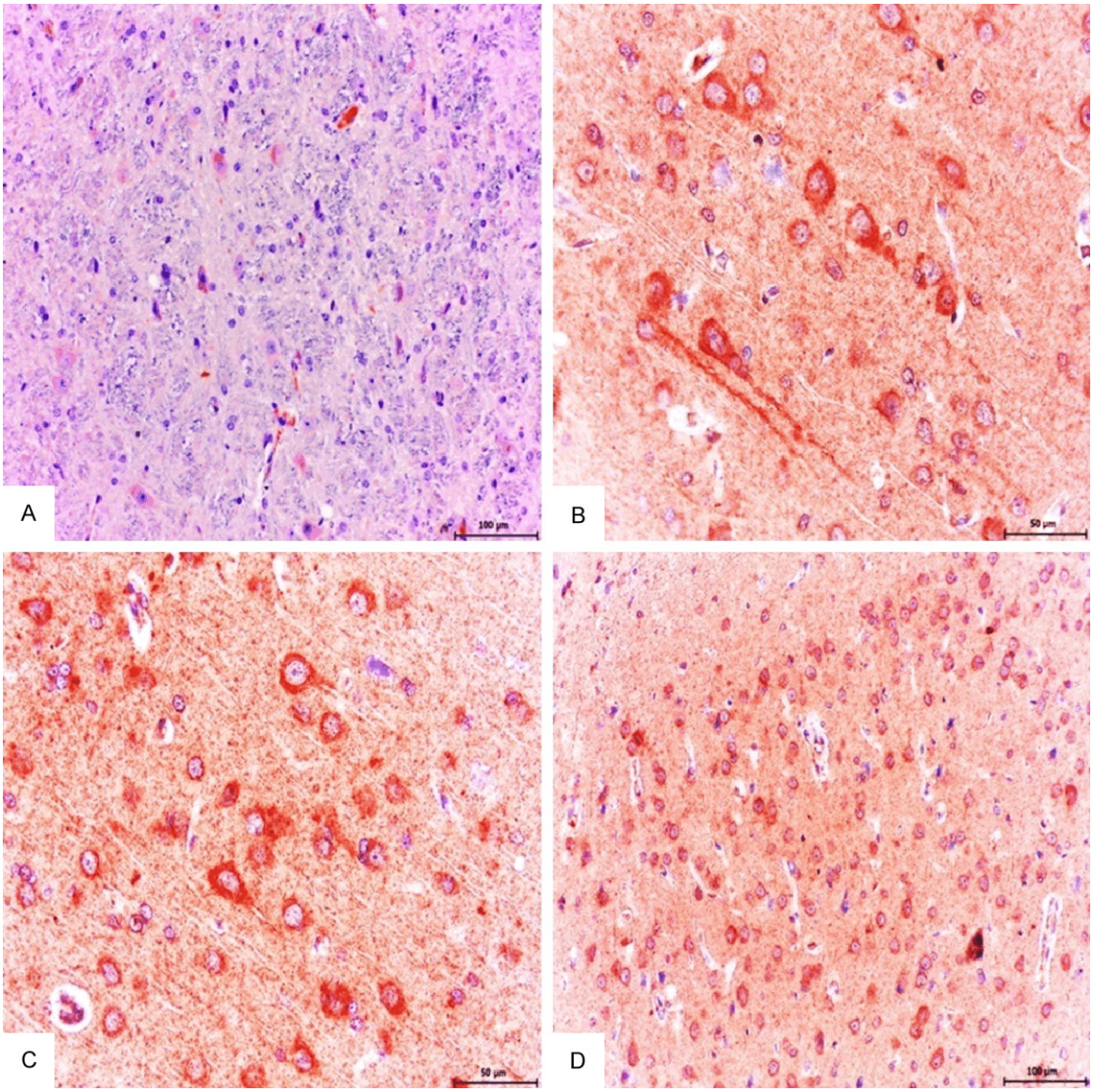

Figure 2. Control group; mild expression of ADAMTS-13 in neuronal and glial cells were detected. ABC technique (anti-ADAMTS-13), Mayer's hematoxylin counterstain, Bar, 100 µm (A) Diabetic group (B-D); Strong expression of ADAMTS-13 in neuronal and glial cells detected. ABC technique (anti-ADAMTS-13), Mayer's hematoxylin counterstain, Bar, 50 µm (B) Strong expression of ADAMTS-13 in neuronal and glial cells detected. ABC technique (anti-ADAMTS-13), Mayer's hematoxylin counterstain, Bar, 100 µm. (C) Strong expression of ADAMTS-13 in neuronal and glial cells detected. ABC technique (anti-ADAMTS-13), Mayer's hematoxylin counterstain, Bar, 50 µm (D).

Ltd.) and used according to the procedure described by Dincel and Kul, 2015 [38]. The pictures of five random fields selected and consecutive 20x objective microscopic fields were captured by the Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions) at a setting identical to the image system. For examining the staining for each antibody, we used

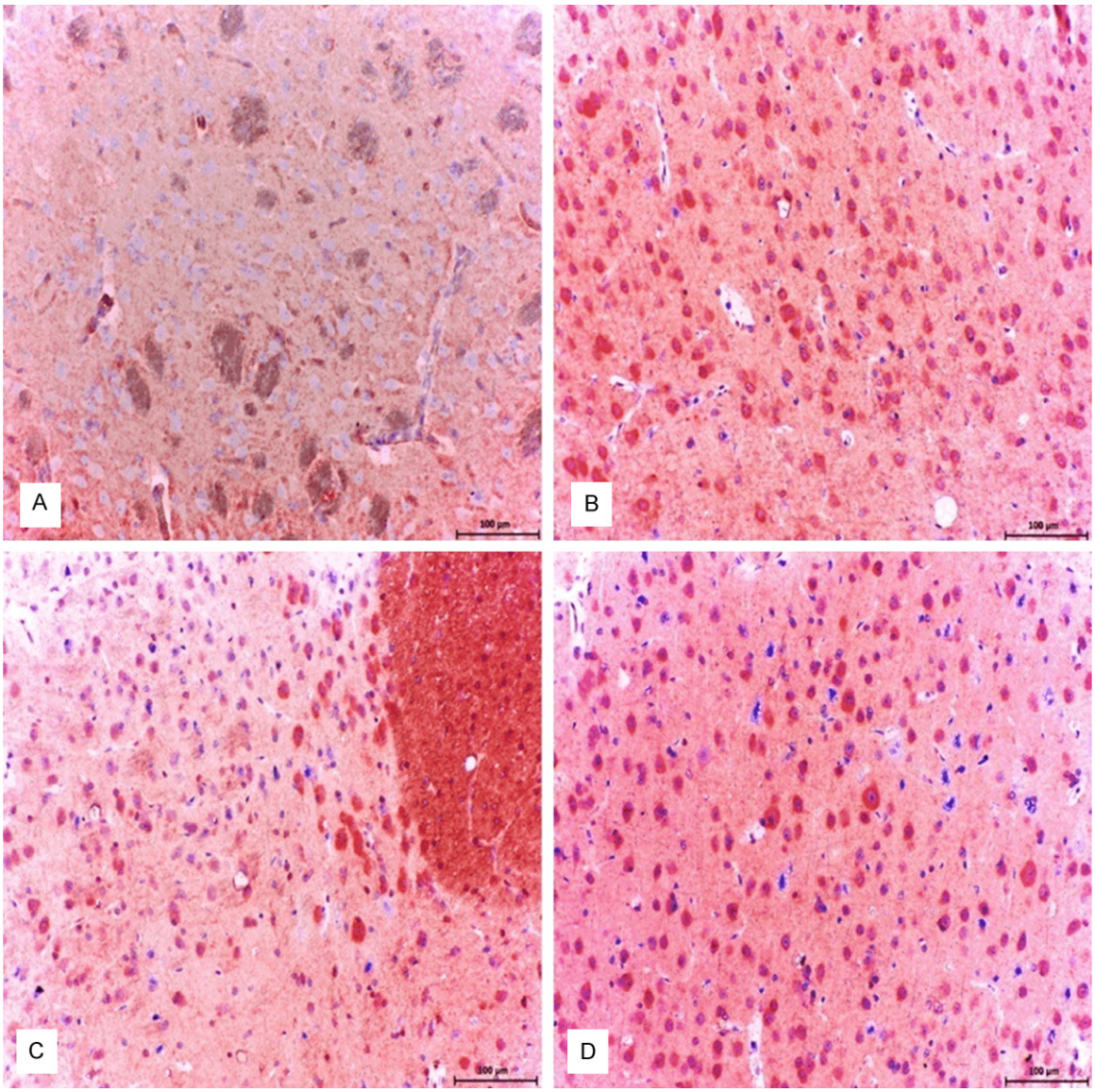

Figure 3. Control group; mild expression of nNOS in neuronal cells was noted. ABC technique (anti-nNOS), Mayer's hematoxylin counterstain, Bar, 100 µm (A). Diabetic group (B-D); (A) Great number of nNOS expressed neurons were determined. ABC technique (anti-nNOS), Mayer's hematoxylin counterstain, Bar, 100 µm. (B) Strong expression of nNOS in neuronal and glial cells were determined. ABC technique (anti-nNOS), Mayer's hematoxylin counterstain, Bar, 100 µm. (C) Strong expression of nNOS in neuronal and glial cells were determined. ABC technique (anti-nNOS), Mayer's hematoxylin counterstain, Bar, 100 µm (D)

the same setting for all slides. Integrated optical density of all the positive staining of ADAMTS-13, nNOS, NSE, NF and GFAP in each photograph was measured. For the quantification, mean was quantified as the ADAMTS-13, nNOS, NSE, and GFAP-positive area/total area were measured and calculated by Leica Qwin Plus on the pictures. All images were collected under the same lighting conditions. To avoid observer bias, all sections were quantified by a blinded investigator. Data were statistically described in terms of mean and standard deviation (mean ± SD) for area %. After calculating the proportion (% pixels) of stained area to the whole field, the mean (in % pixels) staining area for each slide was determined. For evaluating the non-parametric data, Mann-Whitney U-test was performed to compare ADAMTS-13, nNOS, NSE, NF and GFAP immunoreactive cells and immunopositively stained areas in the diabetic animals versus the healthy controls. A p value of < 0.05 and < 0.005 was considered sig-

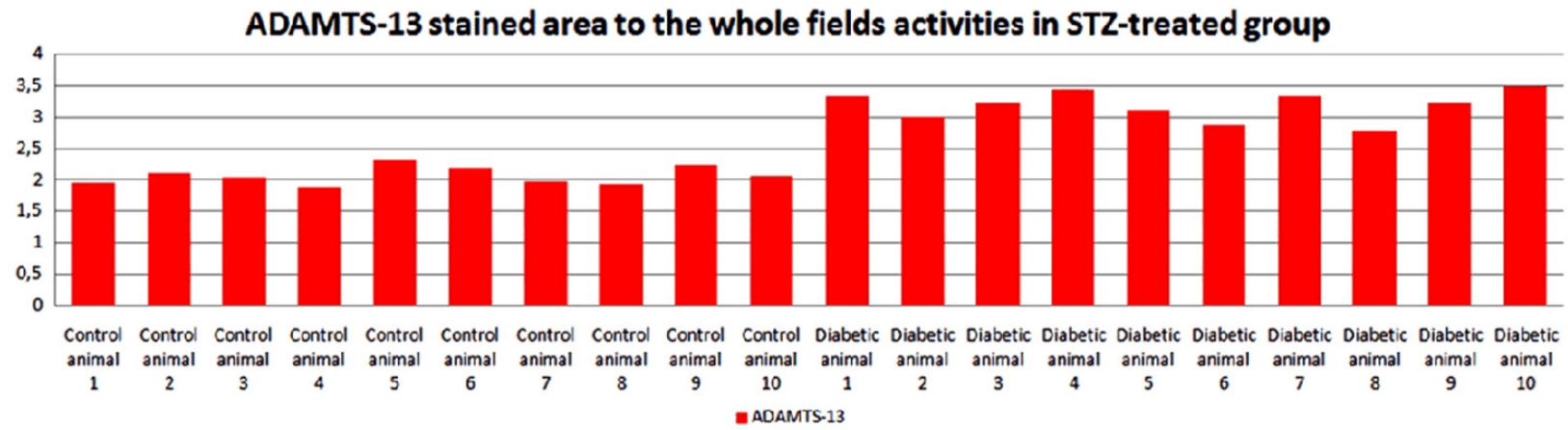

Figure 4. Calculating the proportion (% pixels) of ADAMTS-13 stained area to the whole field activities in I STZ-induced diabetic animals.

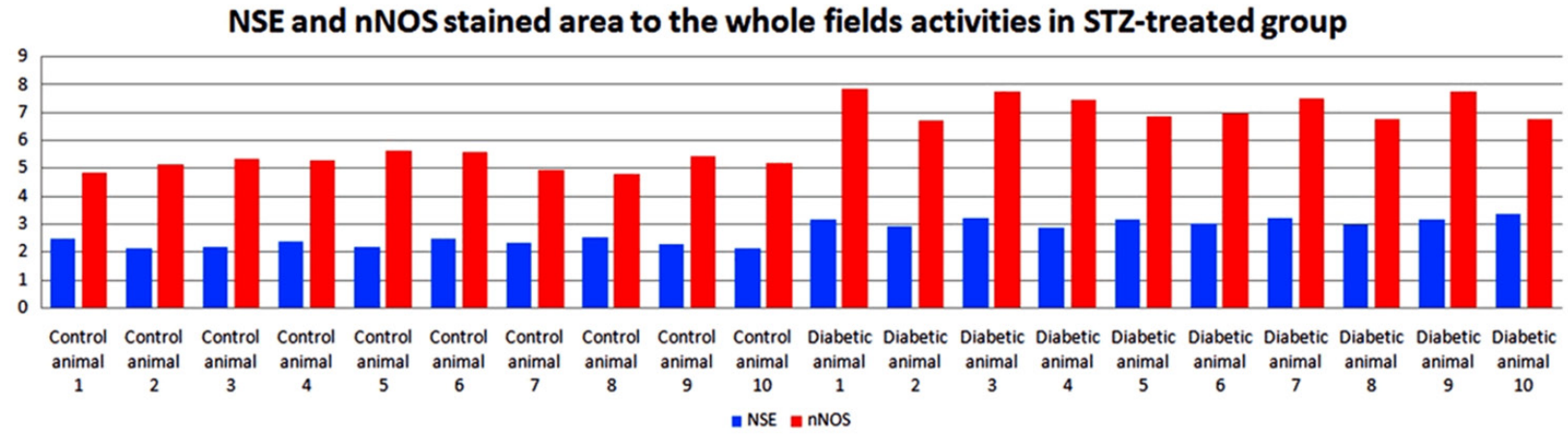

Figure 5. Calculating the proportion (% pixels) of NSE and nNOS stained area to the whole field activities in I STZ-induced diabetic animals.

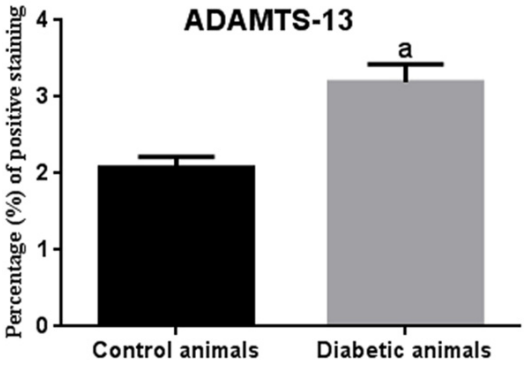

Figure 6. Statistical difference is indicated as letters. "a" represent values statistically higher than control group. Statistical analysis was performed according to Mann-Whitney U-test. The values represent means ± S.D. $P < 0.05$ was considered statistically significant.

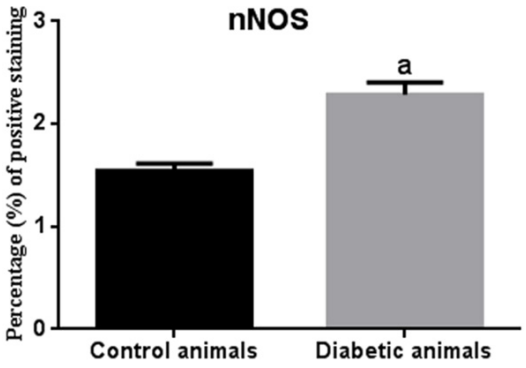

Figure 7. Statistical difference is indicated as letters. "a" represent values statistically higher than control group. Statistical analysis was performed according to Mann-Whitney U-test. The values represent means ± S.D. $P < 0.05$ was considered statistically significant.

nificant. For the statistical analyses, MS-Excel 2003 and SPSS were used for Win. Ver.15.0 (SPSS Inc., Chicago IL, USA) programs. The data were presented as means ± SD. All statistical analyses and graphs were prepared using GraphPad Prism version 6.0 (Graph Pad Software, La Jolla California, USA). $P < 0.05$ was considered statistically significant.

Results

Histopathologic findings

Microscopic lesions were observed in the brains of all STZ-induced diabetic animals. The normal laminar arrangement of neurons and glial cells were not observed. In addition, hematoxylin and eosin (H&E)-stained brain sections from healthy control animals exhibited normal architecture. Neurohistopathologic changes were characterized by neuronal necrosis and central chromatolysis (**Figure 1A**, **1B**). Some degenerated neurons had cytoplasmic vacuolization.

Immunoperoxidase findings

We analyzed protein expression levels of ADAMTS-13, nNOS, NSE, NF and GFAP in the brain tissues from STZ-induced diabetic animals and healthy control animals. Immunohistochemical analysis showed significant up-regulation of ADAMTS-13 ($P < 0,001$), nNOS ($P < 0,001$), and NSE ($P < 0,001$) expression and significant down-regulation of GFAP ($P < 0,001$) expressions in the STZ-induced diabetic animals in comparison to the control animals. Statistical analysis of the data on ADAMTS-13, nNOS, NSE, NF and GFAP expressions in the brain, measured by immunostaining in all the groups, are presented in **Table 1**.

A disintegrin and metalloprotease with thrombospondin type I repeats-13 (ADAMTS-13) and neuronal nitric oxide synthase (nNOS) expression

Fairly weak ADAMTS-13 (**Figure 2A**) and nNOS (**Figure 3A**) expression was observed in neurons and glial cells in healthy control group. We measured protein expression of ADAMTS-13 and nNOS in all parts of the brain. ADAMTS-13 (**Figure 2C**, **2D**) and nNOS (**Figure 3B**, **3D**) expression increased significantly within and in the periphery of the lesion in comparison to the healthy control groups. This assessment has also been interpreted quantitatively and difference was statistically significant [ADAMTS-13 (**Figure 4**) and nNOS, (**Figure 5**)].

ADAMTS-13 was expressed by some glial cells in the cerebral cortex but was predominantly expressed by the neurons (**Figure 2B-D**). The most conspicuous finding of the present study was that ADAMTS-13 and nNOS (**Figure 3B-D**) expression were markedly increased in the neurons (**Figure 6**). The number of nNOS-expressing neurons increased in all parts of the brain (**Figure 7**).

The results suggested that the increased expression of ADAMTS-13 and NO may play an

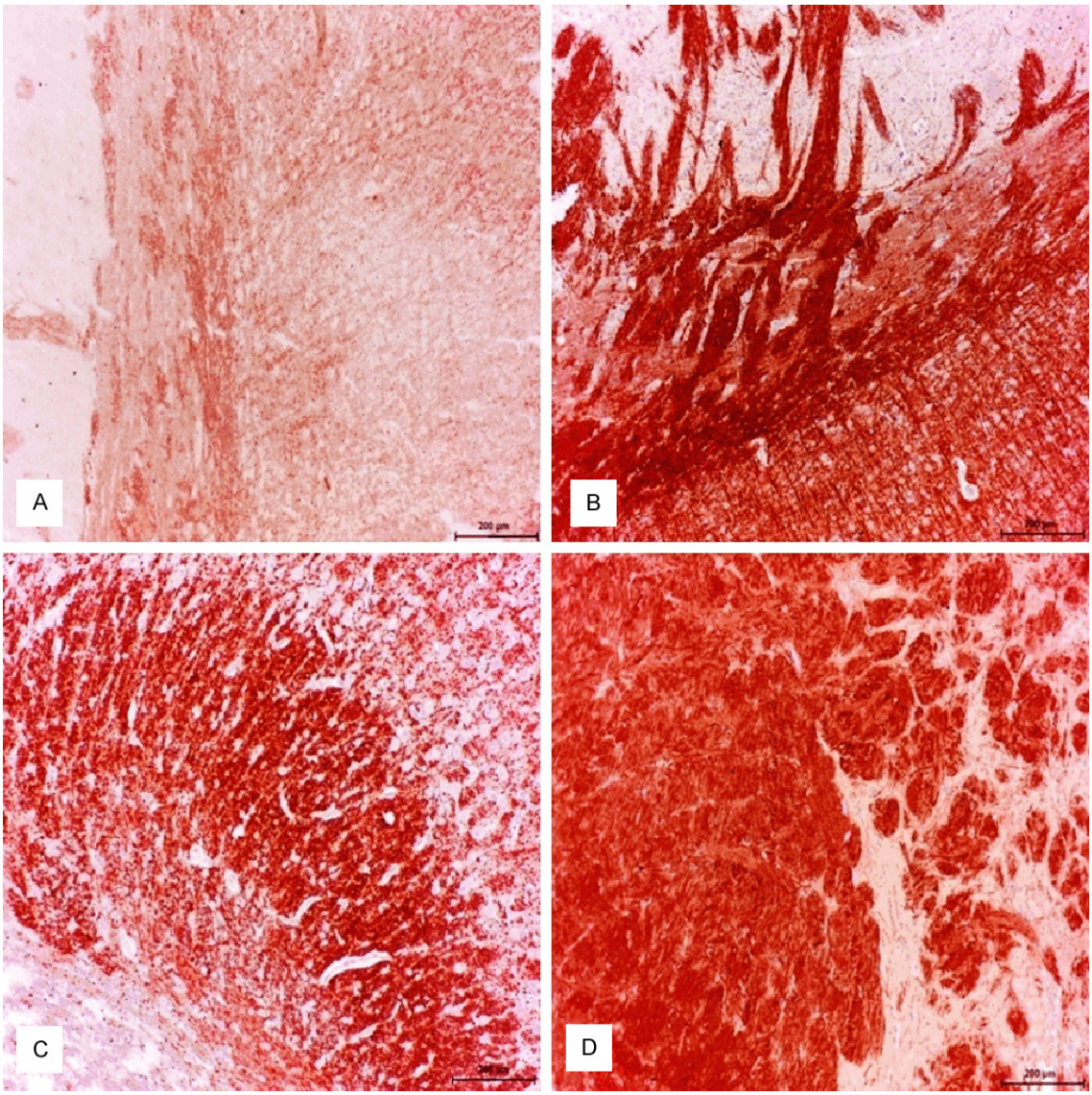

Figure 8. Control group; normal accumulation of NF was noted. ABC technique (anti-NF), Mayer's hematoxylin counterstain, Bar, 200 µm (A). Diabetic group (B-D); Abnormal massive accumulation of NF was detected in the STZ-induced diabetic animal brains. ABC technique (anti-NF), Mayer's hematoxylin counterstain, Bar, 200 µm. (B) Abnormal massive accumulation of NF was detected in the STZ-induced diabetic animal brains. ABC technique (anti-NF), Mayer's hematoxylin counterstain, Bar, 200 µm. (C) Abnormal massive accumulation of NF was detected in the STZ-induced diabetic animal brains. ABC technique (anti-NF), Mayer's hematoxylin counterstain, Bar, 200 µm (D).

important role in the regulation and protection of the CNS microenvironment in STZ-induced diabetic animals. Moreover, increased levels of NO may contribute to neuropathology related with STZ-induced diabetes.

STZ-induced diabetic animal brains showed enhanced levels of nNOS and ADAMTS-13, and prolonged release of NO, which may contribute to neurotoxicity and parenchyma degeneration. This may be responsible for the severity of the disease and the permeability of the blood-brain barrier.

Neurofilament (NF), neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) expression

Fairly weak NF (**Figure 8A**) and NSE (**Figure 9D**) expressions were observed in neurons and glial cells in healthy control group. Strong/moderate GFAP expression was observed in glial cells in

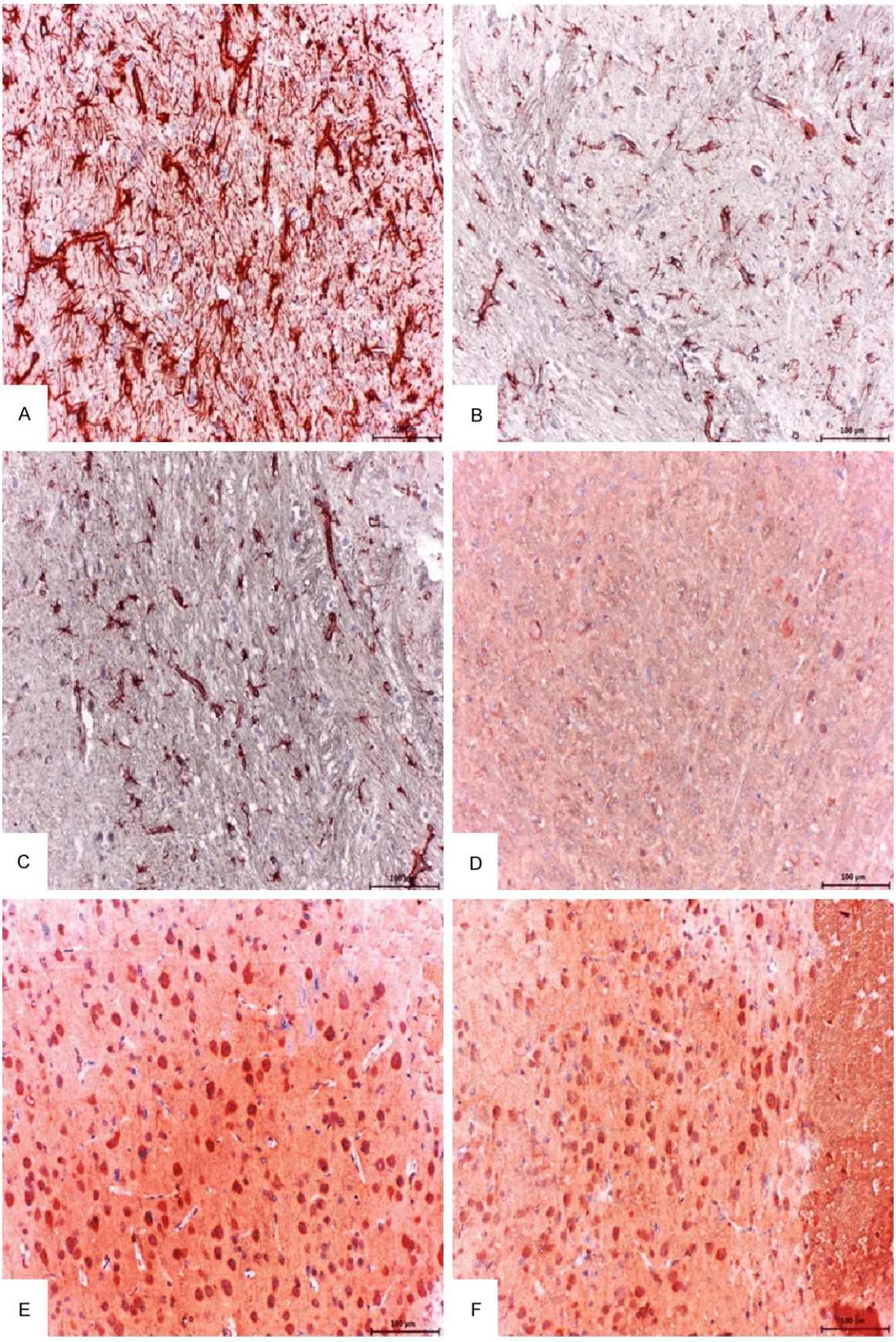
A
B
C
D
E
F

Figure 9. Control group; moderate expression of GFAP in astrocytes were noted. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain, Bar, 100 μm. (A) Diabetic group (B and C); Mild/no expression of GFAP in astrocytes were noted. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain, Bar, 100 μm. (B) Mild/no expression of GFAP in astrocytes were noted. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain, Bar, 100 μm (C) Control group; mild expression of NSE in neuronal cells were determined. ABC technique (anti- NSE), Mayer's hematoxylin counterstain, Bar, 100 μm. (D) Diabetic group (E and F); Strong expression of NSE in neuronal cells were determined. ABC technique (anti- NSE), Mayer's hematoxylin counterstain, Bar, 100 μm. (E) Strong expression of NSE in neuronal and glial cells were determined. ABC technique (anti- NSE), Mayer's hematoxylin counterstain, Bar, 100 μm (F).

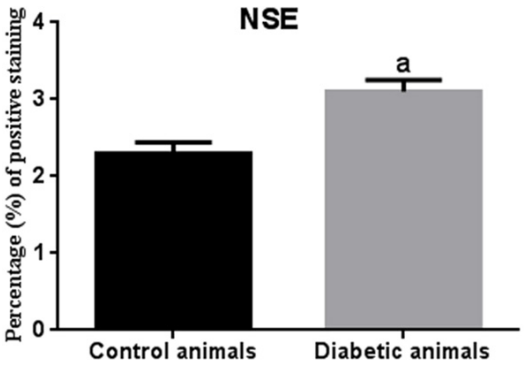

Figure 10. Statistical difference is indicated as letters. "a" represent values statistically higher than control group. Statistical analysis was performed according to Mann-Whitney U-test. The values represent means ± S.D. $P < 0.05$ was considered statistically significant.

healthy control group (**Figure 9A**). Intense immunoreactivity for NSE expression was observed in cerebral sections (**Figure 9E**, **9F**). NSE expression in the brain was higher in STZ-induced diabetic animals than in the control animals (**Figure 5**). The number of NSE-expressing neurons increased in all parts of the brain (**Figure 10**). Importantly, a significant decrease in the expression of GFAP was observed in the brain parenchyma in STZ-induced diabetic animals (**Figures 9B**, **9C**, **11**, **12**).

Sites of enhanced NF immunoreactivity localized to the necrotic lesions and this phenomenon was significantly more pronounced in STZ-induced diabetic animals than in the healthy control animals (**Figures 8B-D**, **13**, **14**).

Increased expression of NSE, NF and decreased expression of GFAP might give an idea of progress of the disease and be critical for diagnosis of the disease.

Discussion

In the recent years, there have been studies on the role of ADAMTS-13 expression in the CNS, but the function of this protein has not been fully revealed [11, 14, 15, 47, 48]. For instance, expression of ADAMTS-13 in mice brain tissue in experimental diabetes and its possible roles in the pathogenesis of the disease have not been studied before. In this study, we suggest that ADAMTS-13 may take an important role in the inflammatory processes and the BBB permeability. Moreover, we indicated that NO that is derived from nNOS causes degeneration of the CNS. nNOS is severely expressed in neurons and glial cells. In this study, the severity of the degeneration was indicated with NSE and NF expressions.

Blood-brain barrier (BBB), which is the physical and metabolic barrier between the peripheral circulation and CNS, is involved the regulation and protection of the microenvironment in the CNS [49]. Significant alteration in the level of systemic glucose, as well as structural and functional disorders that occur in the BBB plays important roles in the pathogenesis of neurological disorders that happen in DM [50-52]. *In vivo* and *in vitro* studies show that diabetes deteriorates the integrity of the BBB and increases the permeability of it [27, 28, 30, 31]. ADAMTS-13 closely associates with vWF and in mice inhibition of vWF induces very significant increase in the BBB permeability [53]. Dincel and Kul, 2015, showed that ADAMTS-13 is highly expressed in the brains of small ruminants, which is infected with border disease virus and is suggested to decrease the degeneration by reducing permeability of the BBB [11]. In a similar study with Toxoplasmic Encephalitis model, severe ADAMTS-13 expression in the brain of mice was suggested to protect the permeability of BBB and alleviate the severity of infection [15]. In this research, we suggest that permeability of the BBB may be decreased by severe expression of ADAMTS-13 in order to suppress the negative effects of hyperglycemia on the BBB and protect its integrity. This situation indicates the tight connection between ADAMTS-13 and the BBB perme-

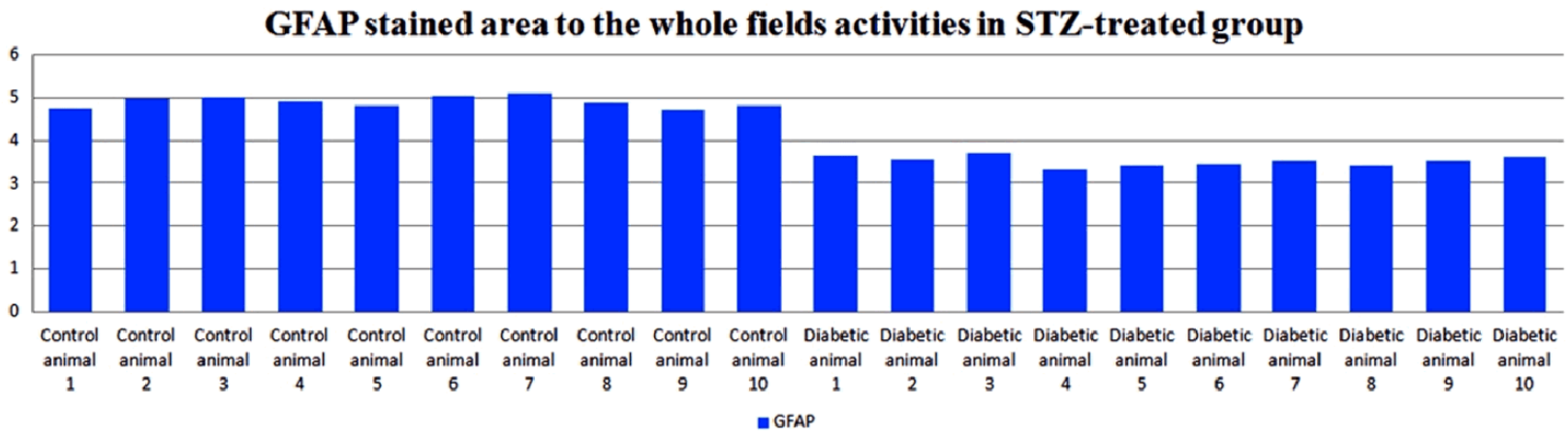

Figure 11. Calculating the proportion (% pixels) of GFAP stained area to the whole field activities in I STZ-induced diabetic animals.

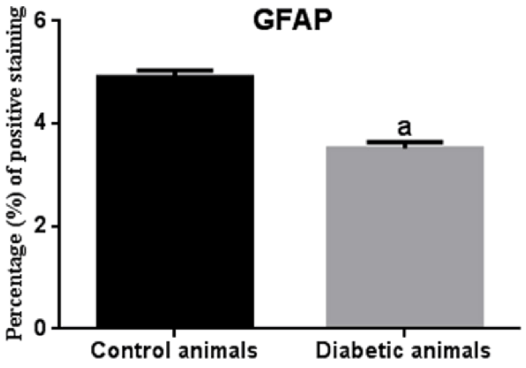

Figure 12. Statistical difference is indicated as letters. "a" represent values statistically higher than control group. Statistical analysis was performed according to Mann-Whitney U-test. The values represent means ± S.D. $P < 0.05$ was considered statistically significant.

ability. Therefore, discovering the effects of ADAMTS-13 on the BBB permeability may contribute to the understanding of the pathogenesis of diabetic neuropathy and the control of the disease.

ADAMTS-13 contributes to the healing and remodelling process of the injured tissue [54]. In mice, inhibition of ADAMTS-13 induces a severe inflammatory response and causes a rise in the infarct area in the heart [55-57] and brain [58, 59] depending on the ischemic/perfusion injury. When the recombinant human ADAMTS-13 protein was injected to these mice, a decrease in the infarct area was detected [58, 59]. These findings indicate that ADAMTS-13 acts as a systemic protection against myocardial and cerebral infarction. In this study, we suggest that significantly high level of ADAMTS-13 expression in brain tissue of diabetic animals may help to alleviate the existing tissue degeneration and induce the healing process. Therefore, it is considered that understanding the link between biosynthesis, expression and inhibition of ADAMTS-13 with diabetic neuropathy and its complications will be crucial for revealing the pathogenesis of the disease and for the development of new treatment protocols.

Vascular complications and endothelial dysfunctions related to Diabetes Mellitus are pathology that are widely studied and well defined [60-62]. Injured or damaged endothelial cells lose some of the essential molecules that play role in homeostasis and blood flow. Thus, in damaged endothelial cells there is an increase in the expression of adhesive molecules, procoagulant activity and predisposition to the development of thrombosis [63, 64]. Von Willebrand Factor (VWF) plays a key role in blood clotting and thrombus formation in severe vascular injury and damage [19, 20]. UL-VWF multimers that has high thrombotic activity is cleaved into less active VWF fragments by ADAMTS-13 [16, 65]. There are studies showing that diabetes increases the tendency of the thrombosis and the risk of diabetes related stroke. However, in diabetes related stroke, complications that occur do not play an essential role. Dincel and Atmaca, 2015a found that ADAMTS-13 has a function in prevention of thrombus formation and may help for the inhibition of a possible stroke [15]. The most striking finding of this study was the absence of any thrombus related pathology even though the veins that were damaged by hyperglycemia were a potential reason for microthrombus. On the other hand, in this study we suggest that increased ADAMTS-13 expression in endothelial cells has a role in prevention of a potential microthrombus or pathology that are related to microthrombus. It is possible that the reason for why microthrombus and pathology related to microthrombus do not occur may be this severely expressed, protective protein, ADAMTS-13.

Nitric oxide (NO) when produced at high levels, like other free radicals, induces neurotoxic effects [66]. There are studies showing that pathology occurring in the CNS may be based on severe level of NO [38, 67, 68]. In this study, it was suggested that NO produced in neurons may be responsible for degeneration and necrosis in the CNS seen in DM model. We think that NO expressed at pathological levels greatly contributes to degeneration and neuronal necrosis. It was described that NO at physiological levels inhibits apoptosis by blocking cytochrome c release [69]. Thus, it prevents the intrinsic apoptosis pathway. On the other hand, NO when produced above physiological limit, is known to induce apoptosis in CNS [37, 38, 40, 42]. It was shown that severe iNOS expression causes neurotoxicity in the hypothalamus and triggers neuronal apoptosis [70]. Along with this, severe iNOS expression was also shown to induce apoptosis [38, 71, 72]. Moreover, nNOS derived NO was shown to

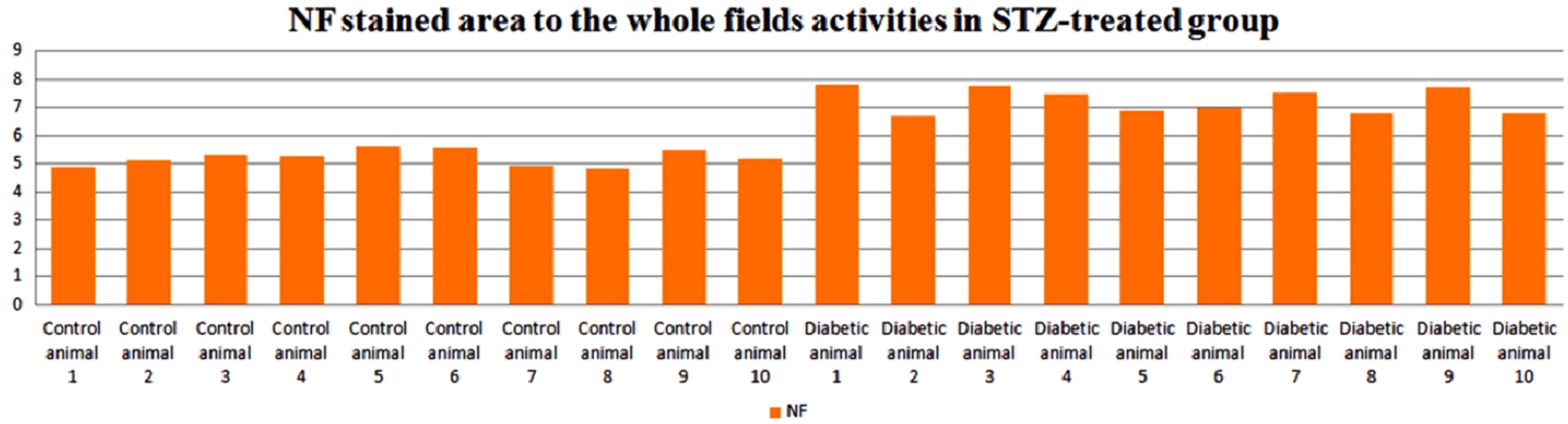

Figure 13. Calculating the proportion (% pixels) of NF stained area to the whole field activities in l STZ-induced diabetic animals.

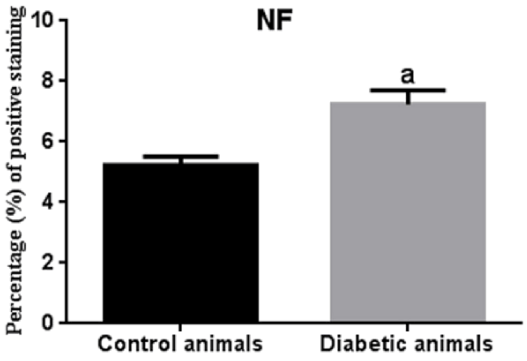

Figure 14. Statistical difference is indicated as letters. "a" represent values statistically higher than control group. Statistical analysis was performed according to Mann-Whitney U-test. The values represent means ± S.D. $P < 0.05$ was considered statistically significant.

cause degeneration by inducing apoptosis of neuronal and glial cells [11, 15]. In this study, nNOS derived NO expressed above the physiological limits in the CNS cells in diabetic animals is considered as the reason of apoptosis that was previously observed in diabetes [1, 2, 43, 44].

In cerebral ischemia, NSE can be used as a marker to identify the severity of the neuronal damage [73, 74]. A statistically significant increase in the expression of NSE indicates severe degeneration of CNS. Therefore; in this study, NSE expression may help us to make comment on the severity of the neuronal degeneration that occurs in the patients with diabetes. The reason for the severe reduction in the expression of GFAP in DM model is thought to be due to the apoptosis/degeneration of astrocytes. With the finding that there is a negative correlation between NSE and GFAP, this study helps us to comment about the severity of the pathology that occur in CNS.

Neurofilaments are the most important components of the neuronal cytoskeleton and they are necessary for the maintenance of normal neuronal function [75, 76]. Abnormal deposition of NF is diagnostic indicator of parenchymal destruction in neurodegenerative and cerebral diseases [11, 46, 77, 78]. Thus, by looking at the expression of NF in the cerebrospinal fluid, we may have an idea about the severity of the degeneration [78]. In the present study, significantly high-level of NF expression in the parenchymal tissue of diabetic animals was observed. A key finding of the present study was that the NF might contribute to the diagnosis of the disease and provide insight about the severity of diabetes-related neuropathology.

Diabetes mellitus was defined to be associated with demyelination in the brain and axonal injury [25]. There are myelin sheath disorders and oligodendrocyte abnormalities in the STZ-induced diabetic animals. It was reported that the quality of myelin decreases [79] and the number of oligodendrocytes decreases in these animals [80]. It is announced that ADAMTS-13 may closely related to myelin production in the border disease that is characterized by hypomyelination [11]. Since we know that there is severe ADAMTS-13 expression and also major complications in myelin production in DM, we think that knowing the role of ADAMTS-13 in the hypomyelination and demyelination would support the understanding of the pathogenesis of diseases such as DM and other diseases associated with myelin damage.

It is thought that ADAMTS-13 acts a protective role in cerebral pathology, in prevention of the increase in BBB permeability or damage in endothelial cells that are induced by parasites, viruses or metabolic diseases. Considering this and other similar studies that we have done previously, we believe that ADAMTS-13 protects CNS microenvironment and damaged endothelial cells and prevents neurons, endothelial and glial cells from an ischemic damage. It is clear that the knowing the correlation between diabetes and biosynthesis and expression of ADAMTS-13 will help to understand possible neuropathology and will help in the follow-up of the disease. In this study we show that there is severe level of neuronal degeneration and a decrease in the astrocytic activity that is far below the physiological level. This situation shows that astrocytes decrease in number when they are exposed to severe NO levels that results in apoptosis/degeneration or the disruption in their functions. Subsequent to these findings, it is believed that NF may be important in identifying the severity and the disease progression during the follow-up, and NF levels may also be used clinically. The findings of this research show the degeneration that occurs in the CNS may not only originate from hypergly-

cemia, at the same time it may originate from nNOS derived NO expressed above on the physiological limits from neuronal and glial cells. The research also suggests that ADAMT-13 that is expressed severely in the brain tissue of diabetic animals may be associated with myelin formation in addition to being closely associated with BBB protection. ADAMTS-13 contributed to the alleviation of degeneration in the brain and the healing process of the tissue is also supported by the findings.

Acknowledgements

This work was funded and supported by the Scientific Research Projects Commission of the Gümüşhane University, Gümüşhane, Turkey (Project Code: 15.B0421.02.2). This study was presented as an oral presentation in the 32nd World Veterinary Congress, 13-17 September 2015, Istanbul.

Disclosure of conflict of interest

None.

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