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Exploring Neuroprotective Effects of Gabapentin in Experimental Diabetic Retinopathy

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ABSTRACT

Introduction: *Diabetic retinopathy (DR) is a main reason for vision loss worldwide. It is manifested by neurovascular dysfunction rather than pure vascular lesion.*

Aim of the work: *This research aimed to examine the impact of gabapentin on diabetic retinal neurodegeneration induced by streptozotocin (STZ), identifying the crucial role of neurodegeneration in DR.*

Material and methods: *Forty-two adult male albino rats were randomly classified into three groups. Control group I: subdivided into subgroup Ia; plain control, subgroup Ib; vehicle control and subgroup Ic; positive control. Group II (Diabetic or STZ-treated group); administered STZ one time intraperitoneally at a dosage of fifty milligrams per kilogram, that was dissolved freshly in a 0.1 mole per liter citrate-buffered solution (pH 4.5). Animals detected with high levels of blood glucose of 250 milligrams per deciliter or greater forty-eight hours after STZ administration were classified as diabetic and included in the research. Group III (Gabapentin-treated diabetic group); The diabetic rats administered gabapentin orally by intragastric tube at a dosage of 300 milligrams per kilogram dissolved in distilled water twice daily for four weeks, following the administration of STZ at the same previous dosage. After four weeks, preparation of specimens of retinae was done for histological & immunohistochemical study using anti-caspase-3, anti-GFAP and anti-VEGF antibodies. Results were morphometrically & statistically studied.*

Results: *Diabetic group II revealed a significant decrease in the retinal sections thickness, reduced cell number of inner & outer nuclear layers and also a significant decrease in ganglion cells number. Significant upregulation of caspase-3, GFAP and VEGF immune reaction was observed. Gabapentin-treated diabetic group III showed preserved histological structure with retinal thickness restoration and down regulation of caspase-3, GFAP & VEGF immune reactions.*

Conclusion: *Gabapentin effectiveness in the prevention of neurodegeneration in rat models of diabetes was proved. Therefore, gabapentin might be considered as a valuable drug for future neuroprotective therapy.*

Keywords: *Diabetic retinopathy, Gabapentin, Neurodegeneration, Rat*

INTRODUCTION

Diabetes mellitus (DM) is considered as one of the most common metabolic disorders. Diabetes is manifested by hyperglycemia due to deficient insulin production and/or insulin resistance. Exposure to chronic high blood glucose levels leads to multiple complications [1]. Diabetic complications involve diabetic retinopathy (DR), cardiomyopathy, neuropathy, nephropathy, liver diseases and impaired healing [2,3].

Diabetic retinopathy (DR), a significant complication of DM, is an important reason of visual affection and irreversible vision loss globally. With the continued increase in the prevalence of DM, the burden of DR is significantly increasing. Beyond the well-recognized vasculopathy as a hallmark of DR; recent studies suggest that neurodegeneration is a crucial part in occurrence of DR [4].

Streptozotocin (STZ) caused diabetes is a widely utilized animal model for investigating the retinal complications of DM. Streptozotocin causes many histological and biochemical changes. Glucose transporter two (GLUT2) facilitates the transport of streptozotocin into pancreatic beta cells, where it functions as a toxic glucose analogue. Glucose and methyl nitrosourea are the products of STZ's cleavage. The pancreatic beta cells are damaged as an outcome of the multiple DNA strand breaks and fragmentations that are induced by the potent alkylating agent, methyl nitrosourea [1,5,6].

Gabapentin is a widely used medication for management of seizures and neuropathic pain, particularly in diabetic patients. Gabapentin, a structural analogue of gamma-aminobutyric acid (GABA), has been approved as medication in the nineties. It acts through inhibition of the alpha 2 delta accessory subunits of voltage-dependent calcium channels [7]. Gabapentin binding at the pre- & post-synaptic membranes leads to suppression of excitatory neurotransmitters secretion [8,9]. Gabapentin is documented to display anti-oxidant and anti-apoptotic actions in rats with DR [7].

This research has been designed to elucidate the retinal neurodegenerative and microvascular alterations in adult male albino rats following induction of diabetes by STZ & to assess possible neuroprotective effect of gabapentin as a promising therapy against diabetic retinal neurodegeneration.

MATERIAL AND METHODS:

Chemicals:

Vials containing 1 gm streptozotocin (STZ) (Sigma Aldrich, catalogue number 327506, USA) were used. The vials should be stored at -20° C.

Gabapentin (300 mg capsule) is a product of Pfizer, United Kingdom, marketed under the trade name Neurontin.

Experimental protocol and animals:

The present experimental research was done at Medical Experimental Research Center (MERC), which is a part of Faculty of Medicine at Mansoura University. The experiment was conducted in accordance with the international guidelines for the use and care of laboratory animals. Faculty of Medicine, Mansoura University Institution Research Board approval was obtained (code number: MD/17. 06.37). Forty-two adult male albino rats three months old weighing (180-200) grams were utilized. Rats were obtained from MERC. Acclimatization of rats for a week before the experiment was done. They were housed in plastic cages in controlled environment with adequate temperature and ventilation. A standard laboratory diet and water were provided without restriction, and the animals were exposed to a twelve-hour light-dark cycle.

Induction of Type 1 Diabetes Mellitus:

The rats were fasted overnight prior to i.p administration of a single dosage of fifty milligram per kilogram of STZ dissolved freshly in 0.1 moles per liter citrate-buffered solution (pH 4.5). Within 48 hours of STZ injection, animals were provided with 10% sucrose water after STZ treatment to prevent fatal hypoglycemia resulting from extensive pancreatic beta cell necrosis and abrupt insulin release after STZ injection [10]. Tail vein blood samples were collected to determine the fasting serum glucose level after 48 hours of STZ injection utilizing a One Touch Ultra Glucose meter (Roche, USA). Animals detected with high levels of blood glucose ≥ 250 mg/dL were classified as diabetic, while those with levels ≤ 250 mg/dL were excluded from the experiment [11].

Animal Grouping:

The rats were classified into 3 groups:

Group I: Control Group (n=18)

The animals were further subclassified into 3 subgroups, 6 rats each;

Subgroup Ia: Rats in the non-diabetic negative control subgroup were fed a standard diet only all over the experimental period.

Subgroup Ib: Non-diabetic vehicle control group. The animals administrated 0.1 moles per liter citrate-buffered solution (pH 4.5) intraperitoneally once (solvent of STZ).

Subgroup Ic: Non-diabetic gabapentin-treated group. The rats received gabapentin (Neurontin) orally by gastric intubation, at a dosage of 300 milligrams per kilogram dissolved in distilled water, twice daily all over the experimental period (12).

- After 4 weeks of the experiment. Rats were sacrificed

Group II: Diabetic (STZ-treated group) (n=12)

Diabetes type I was induced as previously described and the diabetic rats were sacrificed 4 weeks after diabetes confirmation test.

Group III: Gabapentin -treated diabetic Group (n=12).

The diabetic rats received oral gabapentin, by intragastric tube, at a dosage of 300 milligrams per kilogram dissolved in distilled water, twice daily [12]. The rats were sacrificed 4 weeks after diabetes confirmation test.

Just prior to sacrifice, the animals were anaesthetized with sodium pentobarbital (forty milligrams per kilogram) intraperitoneally [13]. Samples of blood were gathered from the vein of the tail for

biochemical study to measure fasting serum glucose level. Initially, the rats were perfused with 500 milliliters of 0.1 moles per liter phosphate buffer (pH 7.4), then by 500 milliliters of phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde, administered through the left ventricle.

METHODS:

1. Biochemical Study:

The samples of blood were obtained from the tail vein 4 weeks after diabetes confirmation test for biochemical study to determine the fasting serum glucose level, utilizing a One Touch Ultra Glucose meter (Roche, USA) [11].

2. Histological Study:

Light Microscopic Study:

Right eyeball was enucleated from each animal then the lens was extracted, and the rest of the eyeball was preserved in 10% buffered formaldehyde for twenty-four hours, subsequently dehydration was done in alcohol (ascending grades), followed by clearing within xylene. This is followed by impregnation and sagittally embedding the specimen in paraffin. Transverse consecutive paraffin sections (4-5um) thickness were cut. Lastly, staining of the specimens was done using hematoxylin and eosin (H&E) stain [14] & immunohistochemical stain [15] using anti- Caspase-3 antibody as apoptotic marker [16], anti- Glial fibrillary acidic protein (GFAP) antibody as a marker for astrocytes and Müller cells (gliosis) [17], and anti-Vascular endothelial growth factor (VEGF) antibody as an angiogenesis marker [18].

Immunohistochemical staining technique:

The detection kit Ultravision Antipolyvalent, horseradish peroxidase /Diaminobenzidine, catalogue number 092806E was used. Thermo Fisher Scientific Company, UK (ready to use). The reagents used in the detection kits constitute a labelled streptavidin-biotin- (LSAB) immunoenzymatic antigen detection system (K 0673, DakoCytomation, Denmark). First, the specimens were deparaffinized in xylol for thirty minutes, rehydrated in alcohol (descending grades) (2 changes, five minutes each), then distilled water for five minutes. Next the specimens were incubated in hydrogen peroxide for fifteen minutes at 37° for inhibiting endogenous peroxidase activity, thin were rinsed in phosphate buffered saline (2 times, five minutes each). Antigen unmasking was done by specimens heating at 95°C in citrate buffer pH 6.0 for ten to twenty minutes, then cooling for twenty minutes at standard room temperature, followed by washing in phosphate buffer saline 4 times. Ultraviolet block was applied to the specimen to block nonspecific background staining. Incubation of specimens was done at standard room temperature for 5 minutes. Anti-caspase-3 antibody (rabbit polyclonal antibody, class IgG1, catalogue number: RP096, Diagnostic Biosystem, Pleasanton, California, United States) at dilutions of 1:100 (according to Manufacture Company), anti-GFAP antibody (mouse monoclonal antibody, (2A5), class IgG1 catalogue number: ab4648, abcam, Cambridge, United Kingdom) at dilutions of 1/500 (according to Manufacture Company) and anti-VEGF antibody (mouse monoclonal antibody, catalogue number: IHC682, GenomeMe, Richmond, Canada) at dilutions of 1:200 (according to Manufacture Company) were added to the specimens. Humidity chamber was used for incubation of specimens, for sixteen minutes at standard room temperature. Then sections were rinsed in phosphate buffer saline (3 times, 5 minutes each). Biotinylated goat antipolyvalent antibody (from the LSAB 2 Kit) was added to the specimens then incubated for ten minutes in humidity chamber at standard room temperature. Then rinsing of the specimens was done in phosphate buffer saline (three times, five minutes each). Following this, the sections were treated with solution of avidin–biotin–peroxidase complex (also from the LSAB 2 Kit) and incubated with 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma Chemicals Co., St. Louis, MO, USA) in a 10 millimoles solution of Tris-buffered saline to visualize the sites of binding of the antibody to the tissue. Lastly, Mayer's hematoxylin, a counterstain, was utilized for 1 minute. Next the Tap water was used for washing specimens, then dehydration in alcohol (ascending grades) two changes, five minutes each was done, after that xylol was used for clearing of the specimens, finally mounting of the specimens in Canada balsam was done to be cover-slipped. Negative controls were included in the specimens, where instead of the primary antibodies, the specimens were incubated in Tris-buffered saline [19]. Human palatine tonsil [20], rat cerebellum [21], and human breast carcinoma (according to Manufacture Company) were used as positive control sections, to

demonstrate anti-caspase-3, anti-GFAP and anti-VEGF immunoreactivity respectively.

3. Morphometric Study:

Six non-overlapping microscopic fields, covering entire retina of every rat in all groups, images were captured by an Olympus digital camera (E420, China) mounted on an Olympus microscope with a 0.5X photo adapter and an objective lens X 40 or X 100 (TX 31Philippines). Image analysis was performed using VideoTest Morphology software (VideoTesT, Russia, Saint-Petersburg) on an Intel Core I3 computer. The following measurements were obtained:

1. The total thickness of the retina and the thickness of the outer & inner nuclear layers (µm) in H & E-stained slides (X 400).
2. The mean number of ganglion cells in the ganglion cell layer in H & E-stained slides (X 400).
3. The percentage area of Caspase-3 positive reaction within the ganglion cell layer, inner plexiform layer, inner & outer nuclear layers in Caspase-3 immunohistochemical stained slides (X 400).
4. The percentage area of GFAP positive immune reaction in GFAP immunohistochemical stained sections (X 400).
5.The mean number of blood vessels in the inner nuclear layer, inner plexiform layer, ganglion cell layer and nerve fiber layer in VEGF immunohistochemical stained sections (X 1000).

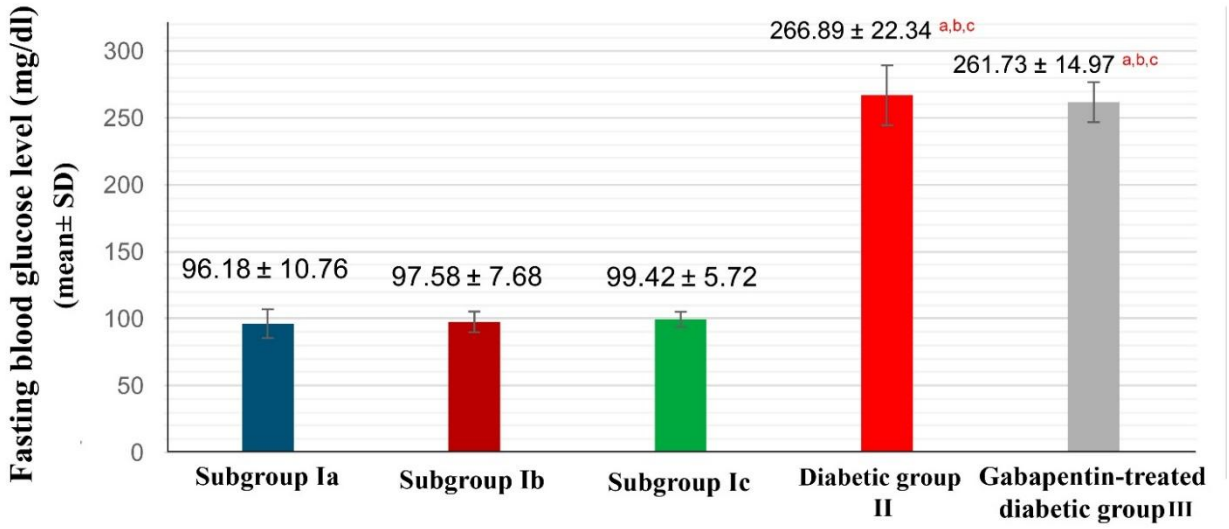
4. Statistical Study:

Data from biochemical and morphometric analyses were analyzed using statistical package for social sciences (SPSS) software version 15.0 (SPSS, Inc., Chicago, IL, USA). Parametric data were represented as (mean ± SD) and ANOVA (one-way analysis of variance) then post-hoc Tukey test were used to compare the different groups of the study. Non parametric data were represented as range and median and Kruskal-Wallis test was used. P-value < 0.05 was statistically significant [22].

RESULTS:

A. Biochemical results:

Diabetic group II and gabapentin-treated diabetic group III rats demonestrated a significant elevation ($P < 0.05$) in fasting blood glucose level compared with control group I. However, there was insignificant difference ($P > 0.05$) in the fasting blood glucose level between diabetic group II and gabapentin-treated diabetic group III rats (Histogram 1).



Histogram 1. The mean value (± SD) of the fasting blood glucose level in the different groups of the study. Significance: $P < 0.05$. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic.

B. Light microscopic results:

1. H and E stain:

The sections from the retinas of all subgroups of control rats (Ia, Ib & Ic) showed similar histological structure. The retina revealed well-organized layers from the outside inward; the pigmented epithelial

layer (RPE), the photoreceptor cell layer (PRL) with its deeply stained inner segments & pale-stained outer segments, the outer limiting membrane (OLM). Outer nuclear layer (ONL) is present inner to OLM containing closely packed deeply stained cones and rods nuclei. Outer plexiform layer (OPL) was observed as a pale eosinophilic narrow area. Inner nuclear layer (INL) was observed less thick than ONL with fewer cells which vary in density, size and shape. Inner plexiform layer was observed as acidophilic fibers forming a loose network. Ganglion cell layer (GCL) showed cells which are arranged in a single row with lightly stained open face large nuclei. The nerve fiber layer (NFL) appeared thin and made up of axons of ganglion cells. The inner limiting membrane (ILM) was considered the innermost layer (Fig. 1A).

Diabetic group II revealed no apparent structural alternations in the pigmented epithelium layer. Spaces among the inner & outer segments of the photoreceptor processes were noticed. Nuclei of ONL & INL appeared darkly stained and less packed with empty spaces in between. Total thickness of retinal layers and ONL& INL thickness were significantly reduced ($P < 0.05$) compared to the control group I (Fig. 1B and histograms 2-4). The ganglion cells seemed shrunken with condensed dark nuclei and wide spacing areas around. The mean ganglion cells number revealed a significant reduction ($P < 0.05$) compared to control group I. Furthermore, ILM appeared interrupted (Fig. 1B and histogram 5)

Gabapentin-treated diabetic group III showed retinal histological architecture more or less similar to group I control rats. The photoreceptor layer was intact with their dark stained inner segments and pale-stained outer segments. The total thickness of the retinal layers and thickness of ONL & INL were significantly elevated ($P < 0.05$) compared to diabetic group II and non-significantly ($P > 0.05$) reduced compared to control group I (Fig. 1C and histograms 2-4). The ganglion cells had large vesicular nuclei. Mean number of ganglion cells was significantly increased ($P < 0.05$) compared to diabetic group II and non-significantly ($P > 0.05$) reduced compared to control group I. Inner limiting membrane appeared intact (Fig. 1C and histogram 5).

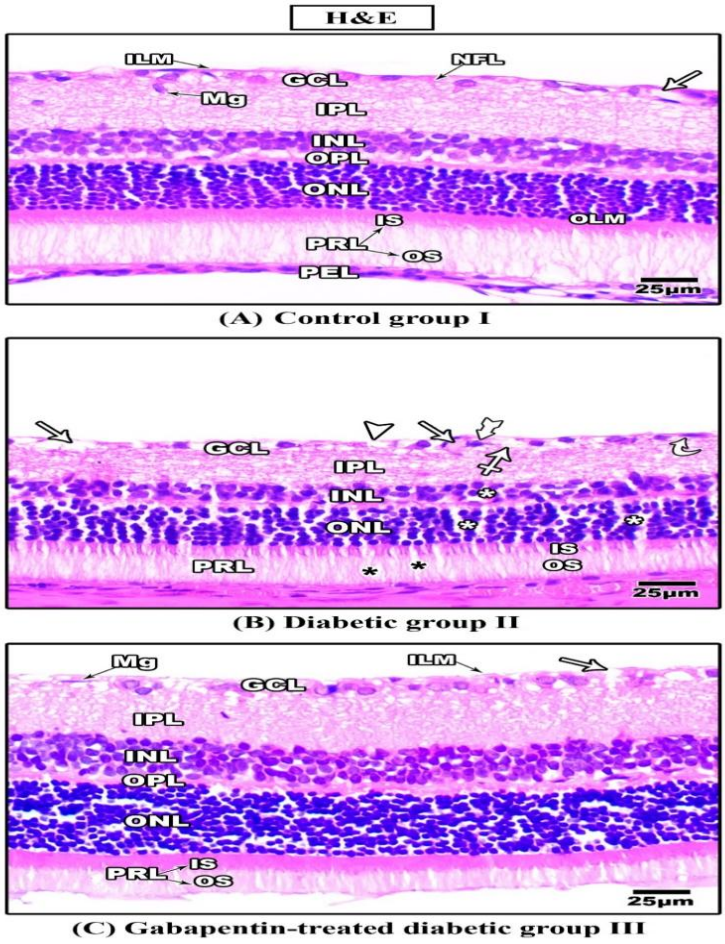
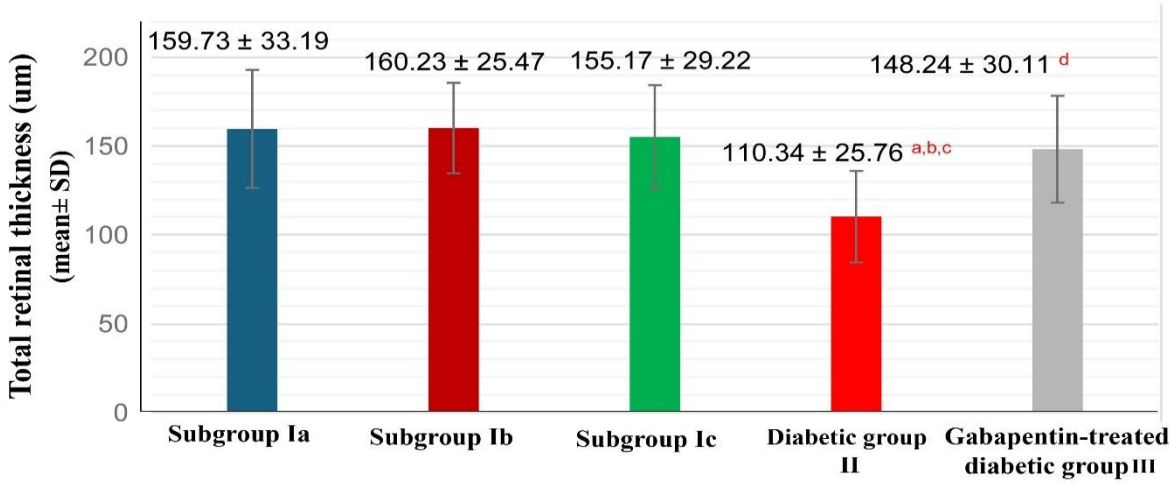
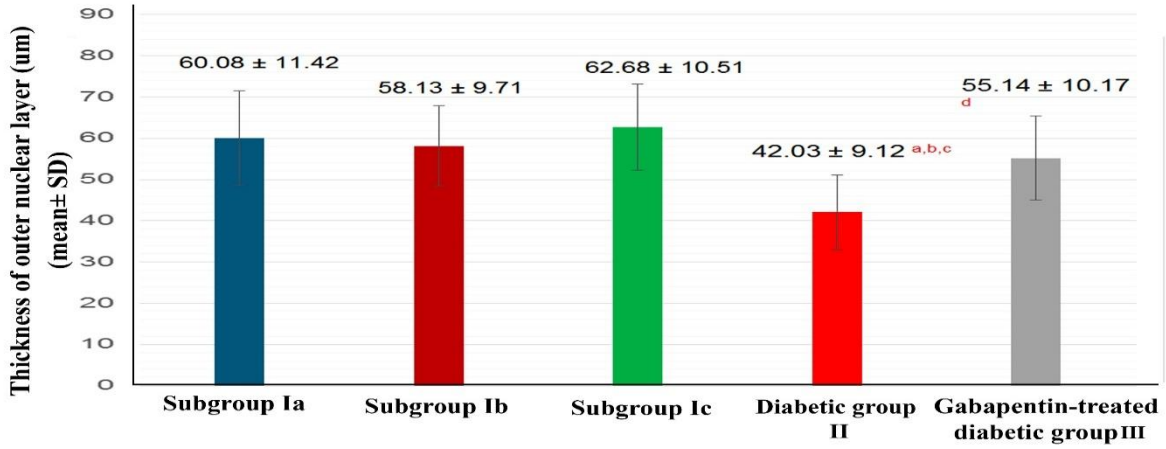


Fig. 1. Retinal sections stained with H & E (A, Control group I; B, Diabetic group II; C, Gabapentin-treated diabetic group III). A: showing the different layers of the retina from the outermost to innermost; the retinal pigmented epithelium (RPE), outer (OS) and inner (IS) segments of

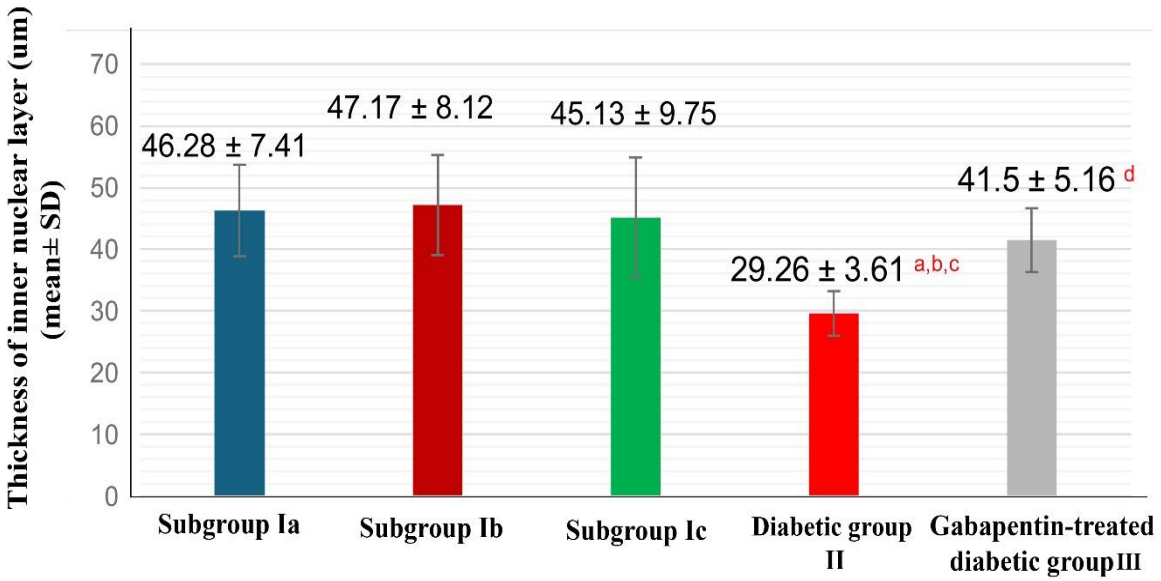
photoreceptor layer (PRL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) containing ganglion cells with vesicular nuclei, nerve fiber layer (NFL) and inner limiting membrane (ILM). Microglia (Mg) in the IPL is seen. A small blood vessel (arrow) lined with endothelial cells in the GCL is observed. B: mild spacing (black asterisks) between the photoreceptor processes of the photoreceptor layer (PRL) is seen. The nuclei of the outer nuclear layer (ONL) and the inner nuclear layer (INL) are darkly stained and less packed with empty spaces (white asterisks) in between. Apparent decrease in the thickness of the retina and the thickness of the ONL and the INL are noticed. Shrunken ganglion cells with condensed dark nuclei (tailed arrow) and wide spacing around them (crossed arrow) are seen. Apparent reduction in the ganglion cells number is also noticed. Microglia (curved arrows) in the IPL& GCL and many blood vessels (arrows) in the GCL are seen. Notice interrupted inner limiting membrane (arrowhead). C: Photoreceptor layer (PRL) with pale stained outer segments (OS) and dark stained inner segments (IS) is seen. The retinal thickness and the thickness of the outer nuclear layer (ONL) & inner nuclear layer (INL) are relatively similar to the control group I. The ONL& the INL show packed nuclei. The ganglion cells in the GCL have large vesicular nuclei. The inner limiting membrane (ILM) appears intact and continuous. Notice the microglial (Mg) cell nucleus and the blood vessel (arrow) in the GCL. (H&E stain X 400).



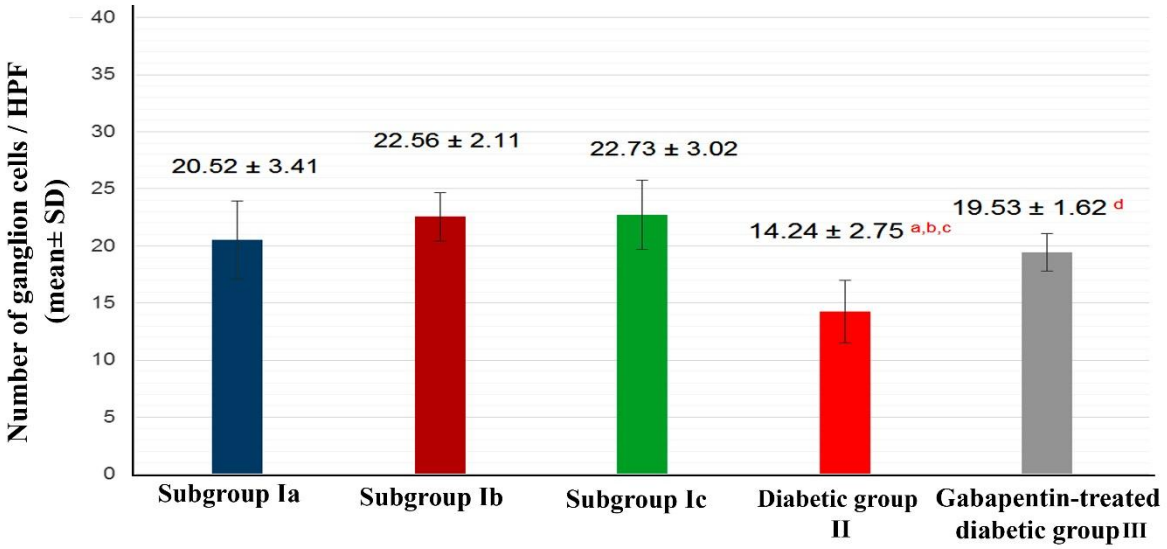
Histogram 2. The mean value (± SD) of the total retinal thickness (µm) in the different groups of the study. Significance: P < 0.05. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.



Histogram 3. The mean value (± SD) of the thickness of the outer nuclear layer (µm) in the different groups of the study. Significance: P < 0.05. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.



Histogram 4. The mean value (± SD) of the thickness of the inner nuclear layer (µm) in the different groups of the study. Significance: P < 0.05. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.



Histogram 5. The mean number (± SD) of ganglion cells/ HPF (X400) in the different groups of the study. Significance: P < 0.05. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.

2. Immunohistochemical staining for caspase-3:

The control group I showed nearly a negative cytoplasmic and nuclear immune reaction for caspase-3 in all layers of retina (Fig. 2A). However, diabetic group II revealed a positive caspase-3 immune reaction that was mainly cytoplasmic in the IPL and to less extent in the OPL. Ganglion cells showed positive nuclear reactions. A positive reaction in the cytoplasm around nuclei of some neurons of the INL & ONL was observed (Fig. 2B). The Gabapentin-treated diabetic group showed positive cytoplasmic caspase-3 immune reaction in few cells in GCL (Fig. 2C). Percent area of caspase-3 immune reaction was significantly elevated (P < 0.05) in diabetic group II compared to the control group I. Moreover, it was significantly reduced (P < 0.05) in gabapentin-treated group III compared to diabetic group II and significantly elevated (P < 0.05) compared to the control group I (Histogram 6).

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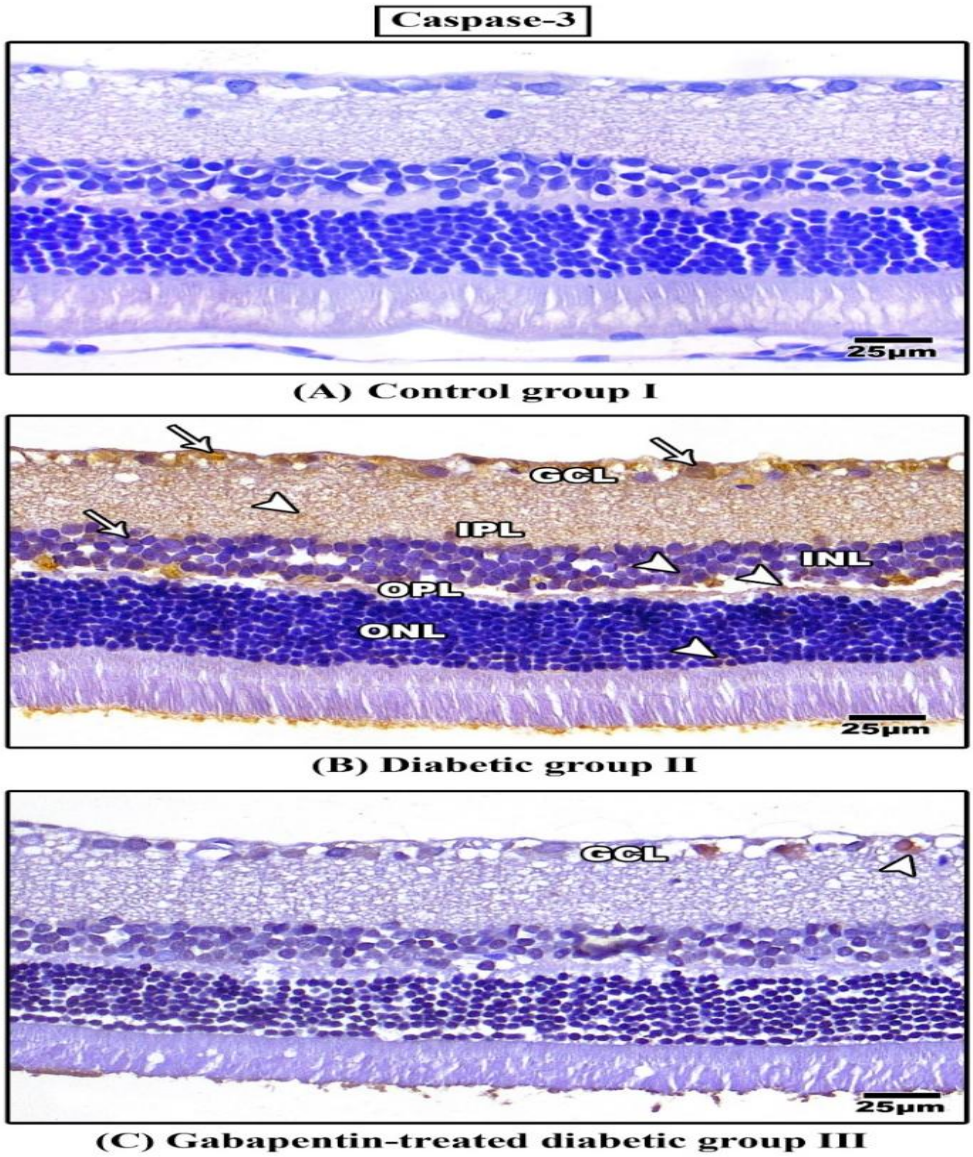
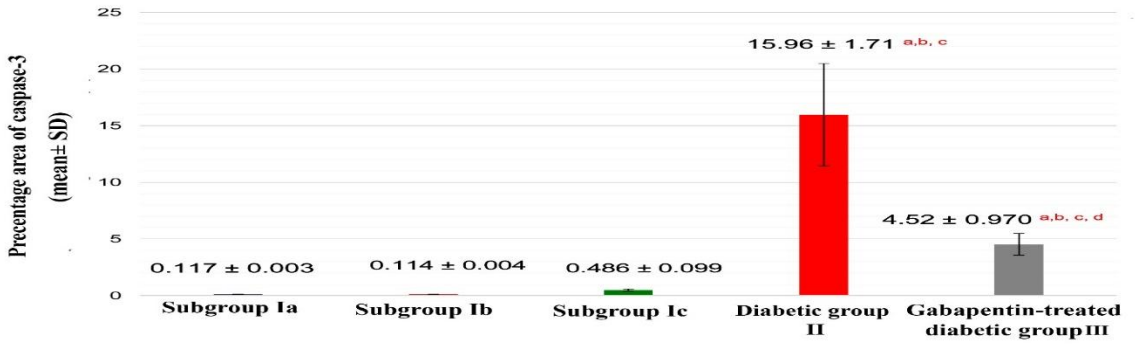


Fig. 2. Caspase-3 expression in immunohistochemically stained retinal sections (A, Control group I; B, Diabetic group II; C, Gabapentin-treated diabetic group III). A: showing nearly a negative caspase-3 immune reaction in all layers of the retina. B: showing the positive caspase-3 immune reaction is mainly cytoplasmic (arrowheads) found in the inner plexiform layer (IPL) and to less extent in the outer plexiform layer (OPL). Ganglion cells in the ganglion cell layer (GCL) show positive nuclear reactions (arrows). The inner nuclear layer (INL) and the outer nuclear layer (ONL) show positive reactions (arrowheads) in the cytoplasm around nuclei of some neurons. C: showing positive cytoplasmic (arrow head) caspase-3 immune reaction in few cells in the ganglion cell layer (GCL). (IHC of caspase-3 X 400).



Histogram 6. The mean value (± SD) of the percentage area (%) of caspase-3 positive reaction the different groups of the study. Significance: $P < 0.05$. a: Significant difference in relation to subgroup

Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.

3. Immunohistochemical staining for GFAP:

The control group I administered a positive cytoplasmic GFAP immune reaction in the few astrocytes of NFL & GCL (Fig. 3A). Diabetic group II showed positive cytoplasmic GFAP immune reaction in many astrocytes of NFL & GCL. A positive reaction also found in processes of Müller cells extending between the inner & outer limiting membranes (Fig. 3B). Gabapentin-treated diabetic group III showed positive cytoplasmic GFAP immune reaction in few astrocytes of GCL & NFL and in few number of Müller cells processes (Fig. 3C). The percent area of GFAP immune reaction was significantly elevated ($P < 0.05$) in diabetic group II compared to control group I. Moreover, it was significantly reduced ($P < 0.05$) in gabapentin-treated group III compared to diabetic group II and non-significantly elevated ($P > 0.05$) compared to the control group I (Histogram 7).

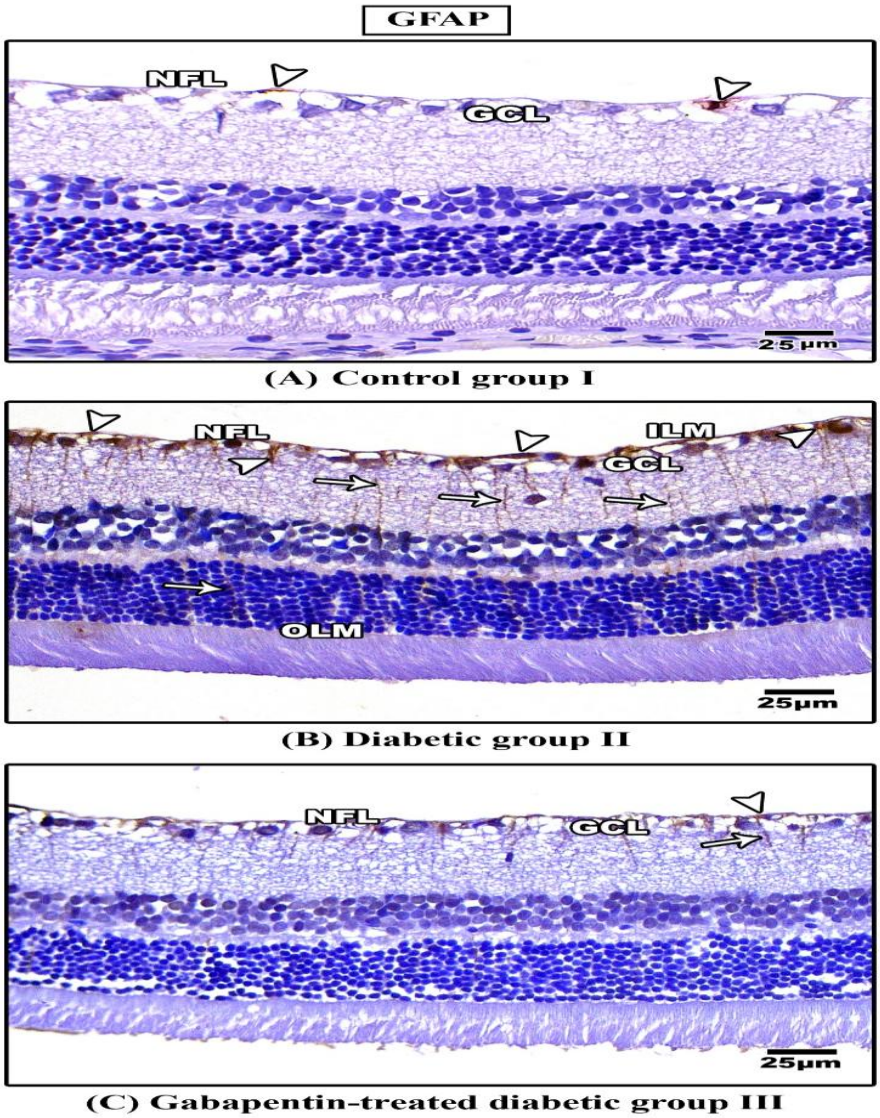


Fig. 3. GFAP immunostaining in the retinal sections (A, Control group I; B, Diabetic group II; C, Gabapentin-treated diabetic group III). A: showing a positive cytoplasmic GFAP immune reaction in the astrocytes (arrowheads) of ganglion cell layer (GCL) & nerve fiber layer (NFL). B: showing a positive cytoplasmic GFAP immune reaction in the astrocytes (arrowheads) of the ganglion cell layer (GCL) & nerve fiber layer (NFL). Müller cell processes (arrows) that extend from the outer limiting membrane (OLM) to the inner limiting membrane (ILM) also showed a positive cytoplasmic GFAP immune reaction. C: showing a positive cytoplasmic GFAP immune reaction in few astrocytes (arrowhead) in the ganglion cell layer (GCL) & nerve fiber layer (NFL) and in few number of Müller cell processes (arrow). (IHC of GFAP X 400).

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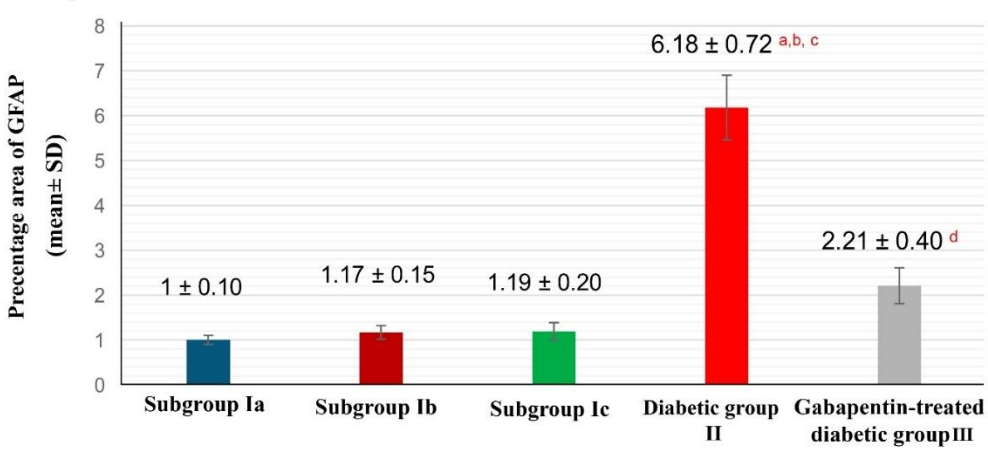


Histogram 7. The mean value (± SD) of the percentage area of GFAP positive cells in the different groups of the study. Significance: P < 0.05. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.

4. Immunohistochemical staining for VEGF:

The control group I demonestrated a positive cytoplasmic VEGF immune reaction in the blood vessels endothelial cells of GCL (Fig. 4A). Diabetic group II revealed positive cytoplasmic VEGF immune reaction in the endothelial cells of many blood vessels of INL & GCL (Fig. 4B). The Gabapentin-treated diabetic group III showed a positive cytoplasmic VEGF immune reaction in the endothelial cells of occasional blood vessels of GCL (Fig. 4C). The number of blood vessels positively stained with VEGF was significantly elevated (P < 0.05) in diabetic group II compared to control group I. Moreover, it was significantly reduced (P < 0.05) in gabapentin-treated group III compared to diabetic group I and insignificant (P > 0.05) to control group I (Histogram 8).

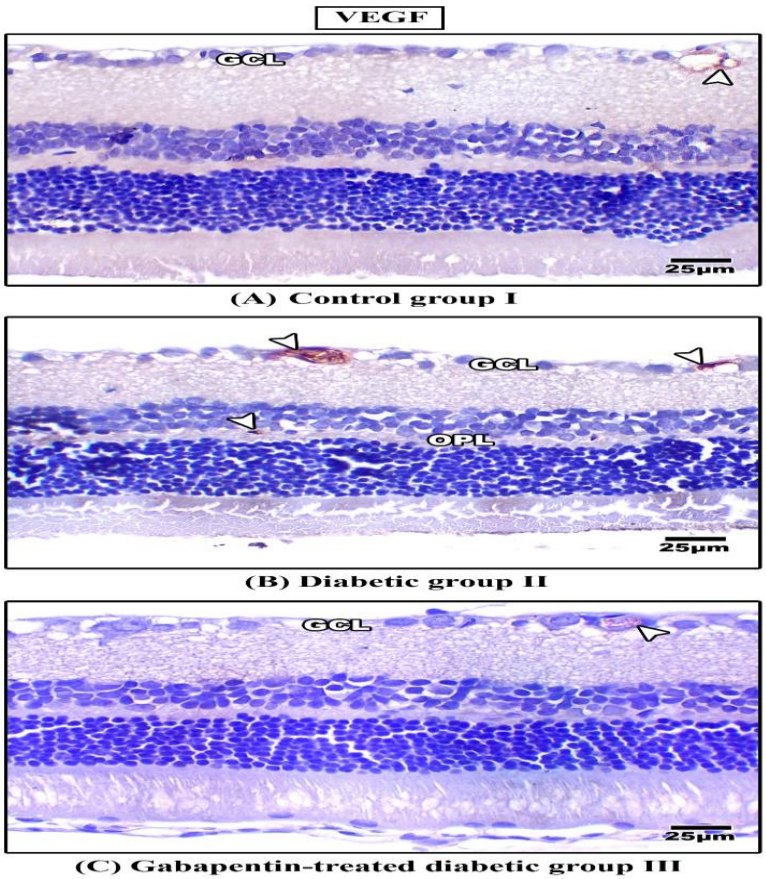
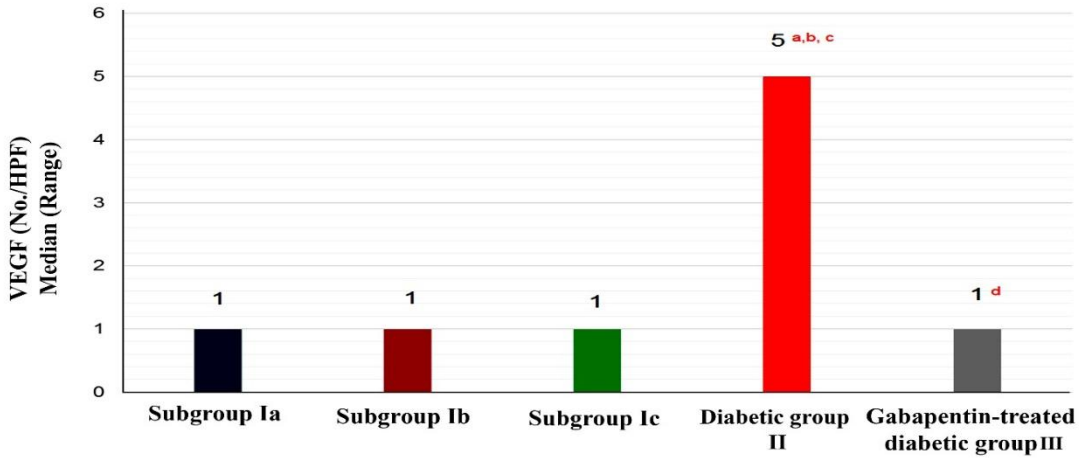


Fig. 4. VEGF immunohistochemical staining in the retinal sections (A, Control group I; B, Diabetic group II; C, Gabapentin-treated diabetic group III). A: showing a positive cytoplasmic VEGF

immune reaction (arrowhead) in the endothelial cells of a blood vessel of the ganglion cell layer (GCL). B: showing a positive cytoplasmic VEGF immune reaction (arrowheads) in the endothelial cells of the blood vessels of the inner nuclear layer (INL) near the outer plexiform layer (OPL) and also of the ganglion cell layer (GCL). C: showing a positive cytoplasmic VEGF immune reaction (arrowhead) in the endothelial cells of a blood vessel of the ganglion cell layer (GCL). (IHC of VEGF X 400).



Histogram 8. The number of blood vessels in VEGF immunohistochemical stained sections in the different groups of the study. Significance: $P < 0.05$. a: Significant difference in relation to subgroup Ia. b: Significant difference in relation to subgroup Ib. c: Significant difference in relation to subgroup Ic. d: Significant difference in relation to diabetic group II.

DISCUSSION:

To our knowledge, the histological and immunohistochemical (GFAP& VEGF) effects of gabapentin on diabetic retina haven't been assessed before. The current study demonstrated degenerative changes in diabetic group II retinal tissue. These results were consistent with the morphometric alternations in the total thickness of the retina and thickness of ONL & INL in addition to the number of ganglion cell nuclei. Similar degenerative changes were reported by Gawish et al. [11] in the retina of STZ treated rats. A decrease in thickness of ONL & INL was also observed and can be explained by apoptosis of the neurons which are incapable to regenerate or proliferate, leading to cell loss and neurodegeneration. The observed empty spaces in group II may be explained by apoptotic neurons phagocytosis by glial cells [23,24,25].

In the current immunohistochemical study, anti-caspase-3 antibody was utilized because caspase-3 activation is common in DR [26]. The Control group I showed nearly negative caspase-3 expression in all layers of the retina. A similar finding was observed by Mohamed and Mubarak [27] and Gawish et al. [11].

A positive caspase-3 immune reaction in the GCL, IPL, INL, OPL & ONL was detected within the retina of diabetic group II. This result was observed by Gawish et al. [11] within retinae of rats administered STZ. These findings were statistically confirmed by a highly significant elevation in the percentage area of a positive caspase-3 immune reaction in the diabetic group II in comparison with the control group I.

Apoptosis and anti-caspase-3 immune reaction in DR can be explained by persistent hyperglycemia which contributes to the overproduction of reactive oxygen species (ROS) & high oxidative stress. This disrupts the mitochondrial membrane with subsequent imbalance of the apoptotic molecules. Pro-apoptotic molecules are elevated while, anti-apoptotic Bcl-2 is reduced [25, 26, 28].

Moreover, hyperglycemia causes glutamate excitotoxicity that leads to the apoptosis of the retinal tissues [29, 30, 31]. The neurotransmitter glutamate, a primary excitatory neurotransmitter, has a vital role in transmission of sensory signals within the retina. N-Methyl-D-aspartate receptor (NMDA) is a 1ry glutamate receptor responsible for calcium influx into the neurons. Glutamate excitotoxicity is a major mechanism implicated in ocular neurodegenerative illnesses, for example optic nerve injury, DR, retinal ischemia and glaucoma. Over activation of NMDA receptors can induce neuronal

cell death through multiple pathways, such as p38 mitogen-activated protein kinase, nitric oxide, cytochrome c and other pathways [32].

GFAP is a non-specific marker of injury or stress of the retinal tissue during DM [33]. GFAP expression in the retina is confined to the astrocytes but not related to Müller cells in normal conditions [17]. Diabetic group II revealed a positive cytoplasmic GFAP immune reaction in the astrocytes & Müller cell processes which extend from ILM to the OLM. This finding comes in agreement with that stated by Jung et al. and Gu et al. [33, 34]. Statistical analysis confirmed this by a significant elevation in the percentage area of a positive GFAP immune reaction in the diabetic group II compared to the control group I.

During hyperglycemia the activated Müller cells secrete neurotoxic factors and proinflammatory mediators, with subsequent reactive gliosis, decline in the function of the neurons and cell death [35, 36, 37, 38].

VEGFs function as signaling proteins for angiogenesis. VEGF is multiple isomers that segregate into VEGF-B, VEGF-A, VEGF-D, VEGF-C and placental growth factor. VEGFA is the primary regulator of both pathological and physiological conditions of angiogenesis [39,40].

Diabetic group II demonstrated a positive VEGF immune reaction in more endothelial cells of the retinal blood vessels compared to control group I. This finding is similar to that observed by Gu et al. [34] who used immunofluorescent stain to reveal VEGF immune reaction in retina of rats administered STZ. Statistical analysis confirmed this result by a significant elevation in the number of endothelial cells of the retinal blood vessels positively immunostained by VEFG in the diabetic group II compared to the control group I .Kusari et al. [41] stated that levels of VEGF protein were significantly increased in vitreous fluid and retinas of male brown Norway diabetic rats 5 weeks from STZ administration at dose of 65mg/kg. However, Foureaux et al. [42] reported no significant change was found in the VEGF immune reaction in retinas among the control and hyperglycemic groups. VEGF immune reaction results in the present study were statistically confirmed by a significant elevation in number of blood vessels in diabetic group II compared to control group I. Elevated levels of VEGF in DR may be explained by the several alterations in the retinal capillaries as a result of hyperglycemia, including reduction of levels of nitric oxide. Consequently, flow of blood in these vessels decreases & neovascularization starts to occur, in order to supply the ischemic retina [42].

Regarding gabapentin-treated diabetic group III, a marked morphological improvement of the retina was observed in spite of the non-significant effect on hyper glycemia occurring in this group. The morphometric data revealed an elevation in number of ganglion cells & in total thickness of retina. These results come in agreement with Ali et al. [30] who used pregabalin as a neuroprotective drug for the diabetic retina. Pregabalin and gabapentin have the same mechanism of action. They bind to the $\alpha 2\text{-}\delta$ accessory subunits of voltage-gated Ca+2 channels. This binding leads to the modulation of Ca+2 influx, inhibition of glutamate release with subsequent inhibition of excitotoxicity [8,30]. Downregulation of caspase-3, GFAP and VEGF immune reaction was demonstrated.

Downregulation of caspase-3 immune reaction in the retina of gabapentin-treated diabetic group III rats comes in agreement with results reported by Ola et al. [43]. This can be explained by reduced expression of the Bax gene & elevated expression of Bcl-2 gene by gabapentin treatment. The downregulation of GFAP immune reaction in the diabetic retina following gabapentin treatment aligns with results reported by Rossi et al. [44] who demonstrated that administering gabapentin effectively reduced reactive gliosis and decreased loss of neurons in the hippocampal CA-1 region during the latency period after status epilepticus. This decrease was indicated by a decrease in GFAP immunostaied area. This can be explained by the ability of gabapentin to reduce glutamate neurotoxicity, that causes production of free radical in brain with overexpression of GFAP [45]. Downregulation of VEGF under the effect of gabapentin treatment of the present study aligns with that observed by Kusari et al. [41] who stated that memantine treatment decreased elevated levels of VEGF protein in the retinas and vitreous fluid of diabetic rats. Both memantine and gabapentin interfere with N-Methyl-D-aspartate receptors activation [46, 47].

CONCLUSION:

Streptozotocin induced diabetic structural degenerative changes in the retinal tissue, and gabapentin could protect against STZ induced diabetic retinal neurodegeneration.

RECOMMENDATION

Gabapentin should be taken as an adjuvant therapy with insulin in diabetes type I to protect the retina from the neurodegeneration. Additional studies are recommended to confirm this assumption or to find other protective substances which may be more effective.

CONFLICT OF INTEREST:

None.

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