

Research Article

Does Metformin Protect Against Diabetic Retinopathy in Albino Rats? An Immunohistochemical Study

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Abstract

Background: Metformin (MT) is a widely used oral anti-hyperglycemic agent for Type II diabetes. However, its role in protection against diabetic retinopathy is not clear.

Aim of the work: The purpose of this study was to investigate the possible protective effect of MT on the diabetic retinopathy in rat model with special consideration on glial cell activation, neuronal apoptosis and neovascularization.

Materials and Methods: Twenty-one male Sprague Dawly rats were divided into control group (n=7) and experimental group (n=14) that developed Type II diabetes by feeding on high fat diet for 8 weeks followed by repeated small doses streptozotocin injections. The experimental group were farther subdivided into Diabetic (DB) group (n=7) were left for another 8 weeks without treatment and MT group (n=7) were left 4 weeks then received MT for another 4 weeks. At the end of the experiment, blood and retinal samples were collected for biochemical, histological and immunohistochemical studies.

Results: Metformin administration significantly decreased diabetic induced hyperglycemia and decreased the serum level of oxidative stress markers to the control level. Also, it significantly suppressed the diabetic induced increase of Glial Fibrillary Acidic Protein (GFAP) and Caspase3 expression. On the contrary, it enhanced the diabetic induced increase of vascular endothelial growth factor (VEGF) expression in the retina.

Conclusion: This study indicated that MT may have an adjuvant role in prevention of diabetic retinopathy, and future studies are recommended to declare the regulatory mechanisms for its antiangiogenic or proangiogenic effects.

Keywords: Diabetes; Gliosis; Metformin; Neovascularization; Retinopathy

Abbreviations: ANOVA= Analysis of Variance; BRB= Blood-Retinal Barrier; Cas₃= Caspase3; CDC= Center for Disease Control and Prevention; CN=Control; DAB= Diaminobenzidine; DB=Diabetic; DR= Diabetic Retinopathy; g=gram; GCL= Granular Cell Layer; GFAP=Glial Fibrillary Acidic Protein; GSH= Reduced Glutathione; HFD= High Fat Diet; hrs=hours; HX&E= Hematoxylin and Eosin; ILM= Internal Limiting Membrane; INL= Inner Nuclear Layer; ip= intraperitoneal; IPL= Inner Plexiform Layer; MDA= Malondialdehyde; MERC= Mansoura Experimental Research Center; MREC= Medical Research Ethics Committee; MT= Metformin; n=number; NFL= Nerve Fiber

Layer; ONL= Outer Nuclear Layer; OPL=Outer Plexiform Layer; PRL=Photoreceptor Layer; ROS= Reactive Oxygen Species; RPE=Retinal Pigmented Epithelium; SPSS= Statistical Package for Social Science; STZ= Streptozotocin; UK=United Kingdom; US=United States; VEGF= Vascular Endothelial Growth Factor

Introduction

Diabetes is an epidemic health issue that currently affects millions of individuals worldwide. According to the Center for Disease Control and Prevention (CDC), 10% of US adults have diabetes, with a projected increase to one in three US adults having diabetes by 2050 [1]. Type II diabetes accounts for most of the diabetes cases, it results from excess caloric intake, insulin resistance and inadequate insulin secretion due to beta

cell dysfunction [2]. Diabetic Retinopathy (DR) is the most common complication of diabetes, affecting over 90% of persons with diabetes and progressing to legal blindness in about 5%. Hyperglycemia-mediated oxidative damage is a vital pathological mechanism involved in the process of DR [3]. Long-term and persistent hyperglycemia induces the overproduction of reactive oxygen species (ROS) and lower the activity of antioxidative enzymes both contribute to accumulation of ROS, and enhance oxidative stress in diabetic patients [4,5]. The vascular features of long-term DR are well documented [6]. The Blood-Retinal Barrier (BRB) breakdown occurs at the early stage of DR [7,8]. The consequent retinal vascular hyper permeability results in macular edema and loss of central vision in diabetic patients. BRB disruption attributes to ischemia, inflammation, and overexpression of vascular endothelial growth factor (VEGF) leading to abnormal angiogenesis [1,9]. The VEGF has a major role in the development of proliferative diabetic retinopathy. VEGF acts as potent proangiogenic factor, involved in both vascular proliferation and increased vascular permeability [10,11].

In addition to vascular changes, neurodegeneration in the diabetic retina is indicated by loss of specific cell types and reduced retinal layer thickness [12]. Little attention has been paid to the neural cells (neurons and glia) of the retina. Neural apoptosis occurs early in the retina and remains constant throughout the duration of diabetes [13]. Hyperglycemia can initiate proapoptotic pathways by enhancing caspase-8 and caspase-3 activity. Caspase-3 is the executioner caspase in the apoptotic pathway, and its activation causes specific DNA cleavage pattern seen in apoptotic cell death [14]. Glial cell proliferation is a well-established late change in proliferative diabetic retinopathy and contributes to epiretinal membrane formation [15]. Glial Fibrillary Acidic Protein (GFAP) is a protein expressed in astrocytes and its increase is indicative of astrocyte activation, which is often termed reactive gliosis [16]. Increased retinal GFAP expression has been noted in the rat model of diabetic before overt vascular changes have occurred [17]. Metformin (MT), the most widely used oral anti-hyperglycemic agent, has been an important drug for treatment of Type II diabetes for decades. It is currently recommended as first line therapy for all newly diagnosed Type II diabetic patients. It was proposed to be useful against DR by suppression of retinal oxidative stress, inflammation, and apoptosis [18]. Our scan of literature revealed no information about the effect of MT on diabetic induced retinal gliosis. There are conflicting reports on the effect of MT on endothelial cell proliferation and neovascularization [19]. Although it was reported to decrease retinal neovascularization in diabetic animals [20,21], recent studies documented MT stimulated angiogenesis by augmenting the expression of VEGF and inhibiting apoptosis in acute hyperglycemia-hypoxia *in vitro* model [22,23], and *in vivo* random-pattern skin flaps [24]. The present work was designed to study the possible protective effects of MT against the diabetic induced altered expression of GFAP,

Caspase 3 and VEGF in the retina of Type II diabetic rat model using immunohistochemically stains. Up to our knowledge this is the first study to examine the effect of MT against diabetic induced retinal gliosis.

Materials and Methods

Animals used

Twenty-one adult male Sprague Dawley rats, weighting 200-250 gm and aging 9-12 weeks were purchased from Mansoura Experimental Research Center (MERC), Egypt. They were housed under controlled conditions of temperature (23 °C±3), and humidity (50±10) with *ad libitum* access to food and water and fixed 12:12 hours light/dark cycle. All rats were maintained under specific pathogen-free conditions in the animal house. All experiments were carried out according to the rules and regulations of the Medical Research Ethics Committee (MREC) of Mansoura Faculty of Medicine.

Drugs used

Streptozotocin (STZ) was purchased from Sigma Aldrich (S0130), dissolved in 0.1 mM citrate buffer (pH 4.4) [25]. Metformin (MT) was purchased from CID pharmaceutical company, dissolved in distilled water and administrated orally via gastric tube [26].

Experimental design

One week after acclimatization, the rats were randomly divided into control (n=7) and experimental (n=14) groups. The control (CN) group fed on normal chow diet and received citrate buffer intraperitoneal (i.p) for four consecutive days. Type II diabetes was induced in the experimental group, and then the rats were further divided into Diabetic (DB) and Metformin (MT) groups. To induce Type II diabetes, the rats were kept on a High Fat Diet (HFD) containing 60% (kcal) fat (soybean oil and lard that contains 0.95 mg cholesterol/g lard), 20% (kcal) carbohydrates and 20% (kcal) protein for eight weeks. Then, the rats were fasted for 16 hours then injected with a low dose of STZ (10 mg/kg i.p) for four consecutive days [25]. Rats with fasting plasma glucose more than (250 mg/dl) 24h after the STZ injection were considered diabetic [26]. After diabetes induction, the rats were fed on normal chow diet and left for four weeks to establish diabetic complication. The rats were then subdivided into DB group; received no treatment for further 4 weeks and MT group; received MT (50 mg/kg) via gastric tube for 4 weeks. At the end of the experiment (17th week), all the rats were sacrificed.

Assessment of body weight

The body weight was measured at the beginning of the experiment, after eight weeks of HFD intake, and at the end of the experiment.

Specimens collection

At the end of the experiment, blood samples were collected from the tail vein, centrifuged at 3,000g for 10 min, serum and packed cell pellets were frozen and transported to the laboratory for analysis. The rats were then anaesthetized by chloral hydrate injection (350 mg/kg i.p) [27], and the eyes were enucleated and the rats were scarified. To prevent retinal detachment, the eyes were placed in 100 ml of Davidson's fixative solution for 18-36 hours, and then transferred to 50% followed by 70% ethanol [28].

Biochemical study

Monitoring of blood glucose level

The glucose level was evaluated before STZ injection, 24 hours after STZ injection and then weekly till the end of the experiment with the ACCU-CHEK glucose meter (Roche Diagnostic Co., Germany).

Assessment of oxidative stress state

Lipid peroxide (Malondialdehyde (MDA) and reduced Glutathione (GSH) were measured in the serum samples [29], using the appropriate kits (Bio diagnostic kits, Giza, Egypt).

Histopathological and immunohistochemical study

The eye balls were dehydrated using graded alcohol concentrations, then cleared in xylene and embedded in soft then hard paraffin wax. Transverse sections were cut at 5 μ m, stained with hematoxylin and eosin stain, and examined for histopathological changes. For immunohistochemical staining, the retinal sections (5 μ m) were deparaffinized and rehydrated by descending alcohol concentrations. To abolish endogenous peroxidase-like activity, the retinal tissues were exposed to 3% hydrogen peroxide (H_2O_2). These sections were treated for 30 minutes with citrate-buffer (0.01 M, pH 6.0) at 95 °C in a boiling water bath. The sections were incubated over night at room temperature with primary antibodies; Anti-Cas₃ (caspase-3) (CPP32, Diagnostic bio systems, Pleasanton, CA, at 1/100 dilution) [30], Anti-GFAP antibody (ab7260, Abcam chemicals, Kemet, Egypt at 1/1000 dilution) [31], and Anti-VEGF antibody (ab16883, Abcam, Cambridge, UK, at a 1:200 dilution) [32]. After rinsing with phosphate-buffered saline, the streptavidin complex was applied, and the reactions were visualized with Diaminobenzidine (DAB) chromogen, and counter stained with hematoxylin.

Morphometric study

Four fields from central retina of each eye were examined and photographed using Olympus® CX41light microscope (X400) with Olympus® SC100 digital camera installed on it. Morphometric study was done using program NIH Image J program (National Institutes of Health, Bethesda, MD, USA), according to the program instruction.

Analysis of HX & E stained sections

The thicknesses of Inner Nuclear (INL) and Outer Nuclear (ONL) layers were measured using the distance parameters in interactive measurements menu. The number of cells in the Granular Cell Layer (GCL) was quantified with respect to nuclei in this layer (excluding nuclei of blood vessels). The number of blood vessels in the inner retina (Internal Limiting Membrane (ILM), Ganglion Cell Layer (GCL) and Inner Plexiform Layer (IPL)) was counted.

Analysis of immunohistochemical-stained sections

The retina was determined in a rectangular frame in each field. Then, the positive expression of GFAP, Cas₃ and VEGF proteins was considered as the area percentage occupied by brown pixels in the retina. Also, the average optical density was measured for Cas₃ immune-staining [33].

Statistical Analysis

The data were tabulated, coded and analyzed with the computer program SPSS (Statistical Package for Social Science) version 22 using descriptive and analytical statistics. To compare the significance of difference between the different groups, we either used Analysis of Variance (ANOVA) for more than two groups of parametric data followed by post-hoc Tukey test, or Kruskal–Wallis test for more than two groups of non-parametric data followed by Mann-Whitney test for multiple comparisons. $P < 0.05$ was considered statistically significant. All graphic representations of the data were performed with Microsoft® Excel® for windows®. (Microsoft Inc., USA).

Results

Body weight

After eight weeks of HFD administration, there was a high significant increase in body weight of the experimental animals (339.5±23.8) as compared to the CN (245±31.5) group. At the end of the experiment, the DB group showed a significant decrease in body weight (156.2±8.3) as compared to the CN group (291.7±30.6). On the other hand, MT treatment restored the body weight (287.5±11.8) nearly to the control level (Figure 1A).

Biochemical results

Blood glucose

After eight weeks of HFD intake, there was significant increase in blood glucose of the experimental group (184.5±28.8) as compared to CN group (112.4±22.79). At the end of the experiment, although the blood glucose in MT group (288.5±36.1) was significantly lower than DB group (466.8±40.4), the blood glucose of both DB and MT groups was significantly higher than the CN group (Figure 1B).

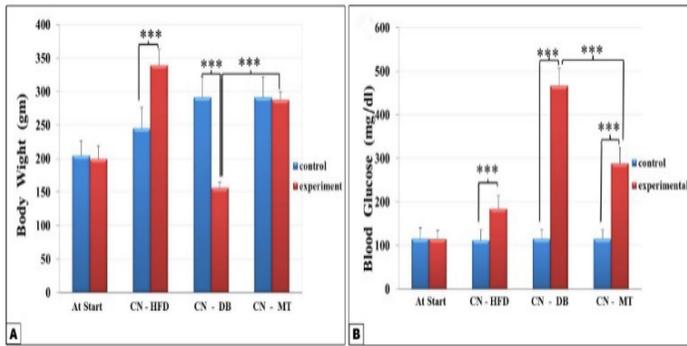


Figure 1: Histograms showing body weight (A) and blood glucose level (B) in different groups. Both body weight and blood glucose show significant increase after eight weeks of high fat diet. However, body weight decreased and blood glucose increased significantly after eight weeks of diabetic state induced by repeated small doses STZ injection in rats after this high fat diet. Treatment of four weeks' diabetic rats with metformin for another four weeks restored the body weight nearly to the control level and significantly reduced the blood glucose, however still higher than the control group. CN=Control; HFD=High Fat Diet; DB=Diabetic group; MT=Metformin treated group *** $p < 0.0005$.

Serum level of MDA and GSH

There was a high significant increase in MDA serum level of DB group (15.9 ± 1.9) as compared to CN (7.3 ± 0.5) group which indicated enhancement of lipid peroxidation. Treatment with MT significantly decreased the MDA level (6.7 ± 0.8) towards the CN level (Figure 2A). Although the serum level of GSH was significantly decreased in both DB (0.7 ± 0.08) and MT (2.77 ± 0.8) groups when compared to CN (3.97 ± 0.5) group, its level was significantly higher in MT group as compared to DB group (Figure 2B).

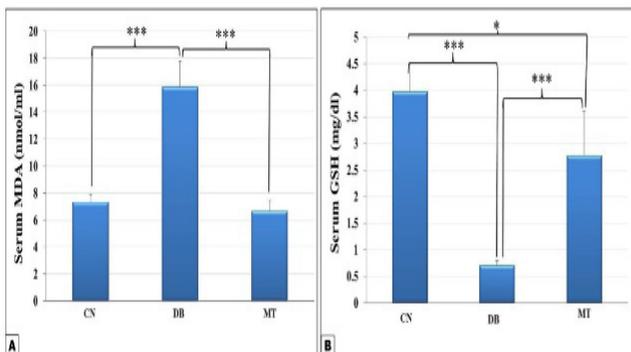


Figure 2: Histograms showing serum level of MDA (A) and GSH (B) in different groups. Eight weeks' diabetes induced oxidative stress indicated by elevated serum level of MDA and decreased serum level of GSH. Metformin treatment significantly reduced the oxidative stress and reversed the serum level of both MDA and GSH. CN=Control; DB=Diabetic group; MT=Metformin treated group. * $p < 0.01$, *** $p < 0.0001$.

Histopathological results

By routine Hx & E stain, the central retina of CN group showed normal histological architecture, demonstrating well organized retinal layers. The cells in the GCL were densely packed with little space intervening. Few small blood vessels were observed in the inner retinal layers indicated normal retinal vasculature (Figure 3A). Obvious retinal changes were observed in the DB group. The cells of the inner and outer nuclear layers (INL, ONL) were less packed with empty spaces in between (Figure 3B, 3C). On the other hand, the changes were less evident in MT group as the cells of ONL, INL were more packed (Figure 3D). On measuring the thickness of INL and ONL, there was significant decrease in DB group (56.2 ± 11 and 68.3 ± 21.1) and MT group (60.5 ± 21.3 and 52.5 ± 18.6) as compared to control group (85.4 ± 17.17 and 117.2 ± 18) with non-significant difference between DB and MT groups ($P > 0.05$) (Figure 4A, 4B). No significant difference in the ganglionic cells number were observed in DB (11.3 ± 3.5) and MT (13 ± 7.2) as compared to CN group (14.9 ± 3.9) ($p > 0.05$) (Figure 4C).

An increased number of slightly large blood vessels was observed in both DB and MT groups (Figure 3B-3D). On counting the number of blood vessels in the inner retina (INL, IPL and GCL) of different groups, there was significant increase in DB and MT groups (10.6 ± 4 and 11.1 ± 3.9 respectively) as compared to CN group (5.1 ± 1.1). Unexpectedly, the number of blood vessels in the inner retina was slightly higher in MT group than DB group but the difference was not significant ($P > 0.05$) (Figure 3D).

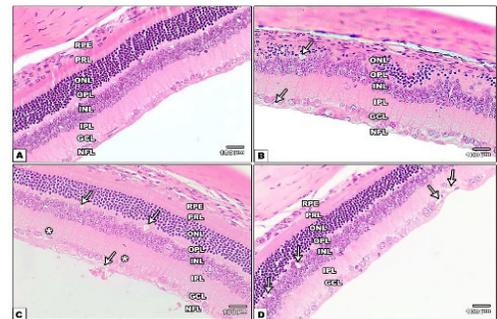


Figure 3: photomicrograph of central retina of different groups. (A): Control group showing normal histological structure of well-organized retinal layers. (B,C): Diabetic group showing evidence of retinopathy in the form of marked disorganization of retinal layers, decreased inner and outer nuclear layer thickness with empty spaces between their nuclei, few disburbed ganglion cells (*), and evidence of neovascularization (arrows). (D) metformin treated group showing improved retinopathy in the form of more packed cells in the inner and outer nuclear layers, and increased number of ganglionic cells, however there is evidence of increased neovascularization (arrows) more than in diabetic group. RPE=Retinal pigmented epithelium; PRL=Photoreceptor Layer; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; INL=Inner nuclear layer; IPL=Inner plexiform layer; GCL=Ganglion cell layer; NFL=Nerve Fiber Layer, (H&E).

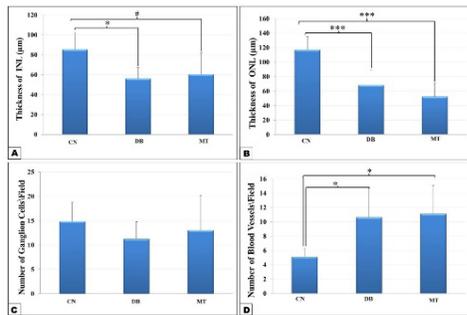


Figure 4: Histograms showing thickness of inner nuclear layer (A), outer nuclear layer (B), number of ganglion cells (C), and number of blood vessels (D) per field in different groups. Both diabetic and metformin treated groups represented significant decrease in thickness of inner and outer nuclear layers and increase in number of blood vessels with mild difference in between. Although the number of ganglion cells decreased in diabetic group as compared to diabetic and control groups, the difference was insignificant between all studied groups. INL=Inner Nuclear Layer; ONL= Outer Nuclear Layer. CN=Control; DB=Diabetic Group; MT=Metformin Treated Group. #p<0.05. *p<0.01. ***p<0.0001.

Immunohistochemistry for GFAP

Little GFAP expression was observed in INL, IPL, and GCL and to less extent in the OPL and ONL in the retinas of CN group (Figure 5A). In DB group, the expression of GFAP was increased significantly (19.7 ± 8.3) as compared to CN group (11.1 ± 3.6) and extended to occupy the whole layers of the retina from ILM to OLM (Figure 5B,5D). It was clear that MT treatment significantly decreased GFAP expression (13.9 ± 5.1) to nearly the same distribution of CN group (Figure 5C,5D).

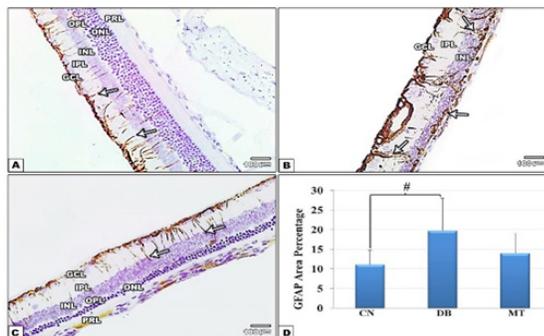


Figure 5: Photomicrograph of central retina of different groups immunostained for GFAP with Hematoxylin counter stain. (A): Control group shows weak reaction (arrows) in the inner retina. (B): Diabetic group shows marked positive immune reaction (arrows) occupying the whole layers of the retina. (C): Metformin treated group shows mild positive immune reaction (arrows) with nearly same distribution of CN group. (D): Histograms of GFAP surface area in different groups. PRL=Photoreceptor Layer; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; INL=Inner Nuclear Layer; IPL=Inner Plexiform Layer; GCL=Ganglion Cell Layer. CN=Control; DB=Diabetic Group; MT=Metformin Treated Group. #p<0.05.

Immunohistochemistry for Cas₃

There was little expression of Cas₃ in the inner retina of CN group (Figure 6A). The expression of Cas₃ in the retinas of DB group extended to occupy the whole layers of the retina (Figure 6B) and represented by a high significant increase in Cas₃ area percentage and optical density (52.7 ± 19.7 and 0.9 ± 0.18) as compared to CN group (3.1 ± 0.54 and 0.01 ± 0.003 respectively) (Figure 6D) (Figure 7). As regard to MT group, there was positive Cas₃ expression in INL, IPL and GCL and to less extend in the OPL, ONL (Figure 6C). Although, the area percentage of Cas₃ expression in MT group (39.2 ± 6.9) was slightly decreased as compared to DB group ($P > 0.05$) (Figure 6D), there was significant decrease in Cas₃ optical density in MT group (0.7 ± 0.02) as compared to DB group ($P < 0.05$) (Figure 7).

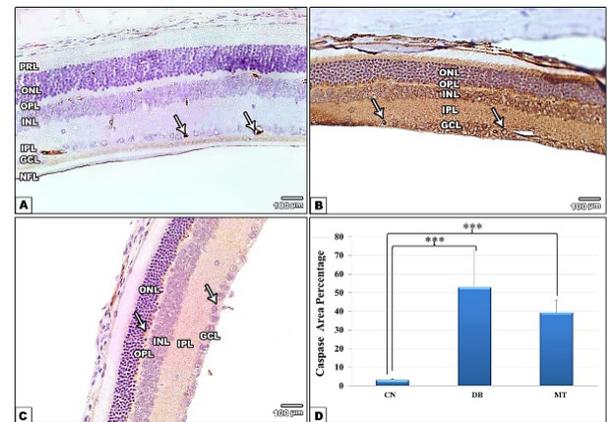


Figure 6: photomicrograph of central retina of different groups immunostained for Cas₃ with Hematoxylin counter stain. (A): Control group shows weak reaction (arrows) in the inner retina indicating few normal apoptotic cells. (B): Diabetic group shows marked positive immune reaction (arrows) occupying the whole layers of the retina indicating marked apoptosis. (C): Metformin treated group shows positive immune reaction (arrows) which is less and lighter than diabetic group. (D): Histograms of Cas₃ surface area in different groups. PRL=Photoreceptor Layer; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; INL=Inner Nuclear Layer; IPL=Inner Plexiform Layer; GCL=Ganglion Cell Layer; NFL=Nerve Fiber Layer. CN=Control; DB=Diabetic Group; MT=Metformin Treated Group. ***p<0.0001.

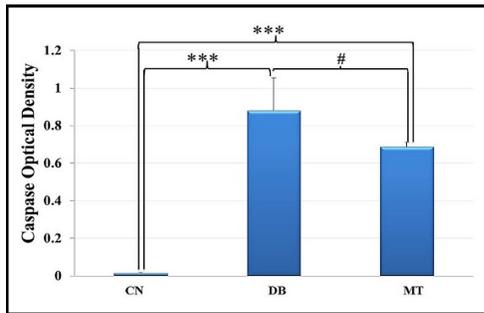


Figure 7: Histogram showing Cas₃ optical density in different groups represents significant increase in diabetic and metformin treated groups. However, the optical density in metformin treated group was significantly lower than diabetic group indicating anti-apoptotic effect. CN=Control; DB=Diabetic Group; MT=Metformin Treated Group. #p<0.05. ***p<0.0001.

Immunohistochemistry for VEGF

There was little expression of VEGF in the inner retinas of CN group (Figure 8A). Increased expression of VEGF was observed in the inner retinas of DB and MT groups (Figure 8B,8C). The area percentage of VEGF stain showed a high significant difference between all studied groups (p<0.0001). There was significant increase in area percentage of VEGF stained sections in DB group (5±5) and highly significant increase in MT treated group (6.6±5.8) as compared to CN group (1.2±0.6). It was notably that VEGF stained area percentage in MT group was slightly higher than DB group (P>0.05) (Figure 8D).

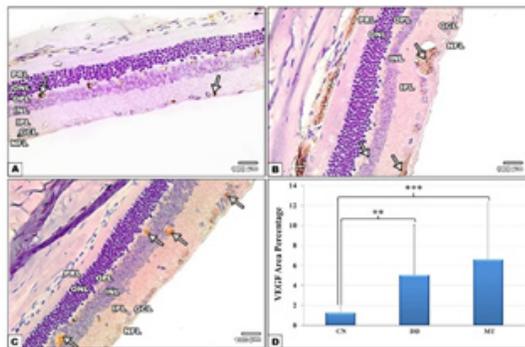


Figure 8: Photomicrograph of central retina of different groups immunostained for VEGF with Hematoxylin counter stain. (A): Control group shows weak reaction (arrows) indicating small number of normally present blood vessels in the inner retina. (B): Diabetic group shows positive immune reaction (arrows) in the inner retina indicating neovascularization. (C): Metformin treated group shows marked positive immune reaction (arrows) indicating increased neovascularization than diabetic group. (D): Histograms of VEGF surface area in different groups. PRL=Photoreceptor layer; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; INL=Inner Nuclear Layer; IPL=Inner Plexiform Layer; GCL=Ganglion Cell Layer; NFL=Nerve Fiber Layer. CN=Control; DB=Diabetic Group; MT=Metformin Treated Group. **p<0.001. ***p<0.0001.

Discussion

The present work was designed to evaluate the possible protective effect of MT on a rat model of DR with special consideration on glial cell activation, neuronal apoptosis and neovascularization using GFAP, caspase 3 and VEGF immune staining.

Nearly all patients with Type I diabetes and more than 60% of patients with Type II diabetes have retinopathy [34]. However, Type II diabetes remains the most common form of diabetes, representing over 90% of diabetic cases [35]. In the present study Type II diabetes was developed in adult male rats using High-Fat Diet/Streptozotocin (HFD/STZ) rat model [25]. Together, these two stressors are designed to mimic the pathology of human Type II diabetes [36]. High fat diet caused excess lipid accumulation in the liver resulted in insulin resistance [37], followed by repeated low doses of STZ that causes partial damage of pancreas islets. The remaining endocrine functions of the pancreas along with HFD induced insulin resistance are in accordance with the definition of Type II diabetes [25]. The present work reported significant increase in the body weight of the animals fed on HFD for eight weeks as compared to same age CN group. However, the body weight of these animals decreased significantly after eight weeks' diabetes. This reduction of body weight may be due to breakdown of tissue proteins in DB rats [38]. Treatment with MT reduced body weight loss in DB group till it became nearly similar to the CN group. This result was in consistent with the result of Kim, et al. (2017a) [39]. This can be explained by reduction of hyperglycemia and protein wasting due to carbohydrate inaccessibility [40]. In agreement with the result of Yang, et al. (2016) [25], we demonstrated a highly significant increase in the blood glucose of the DB group as compared to the CN group. This may be explained by diminished insulin secretion and increased insulin resistant which is concomitant with the hyperglycemia of Type II diabetes [41]. On the other hand, MT treatment significantly reduced the diabetic induced hyperglycemia but its level was still higher than that of CN group. Comparing the level of oxidative stress markers among different groups showed a high significant increase in MDA level and decrease in GSH level of DB group as compared to CN group and this was in agreement with Zuo, et al. (2013) [42].

This can be explained that chronic hyperglycemia which leads to increase free radicals that enhance oxidative stress producing per oxidative damage of the membrane lipids so increase the level of MDA and also produce depletion of antioxidants such as GSH [43]. In consistence with the result of Chukwunonso, et al. (2016) [44], MT increased GSH level and decrease MDA level of the DB animals indicated significant antioxidant effects of MT, so it may be protective against oxidative stress-induced damage during diabetic complications. Histological examination of the central retina of DB group showed structural alternation as compared to that of

CN group. There was significant reduction in thickness of ONL and INL with appearance of empty spaces between their nuclei. In addition, there was mild decrease in the number of ganglion cell and significant increase in the number of blood vessels in the inner retina. The current result was in consistence with the result of Martin, et al. (2004) [45] and Sadek, et al. (2017) [46] who found significant decrease in the thickness of INL and ONL after 12 weeks of diabetic state. This reduction can be explained by progressive apoptosis of retinal neuron. The appearance of empty spaces may be due to phagocytosis of the apoptotic retinal neurons by glial cells [31,47]. Also, the reduction in the number of ganglion cells was in agreement with Foureaux, et al. (2015) [48]. This can be explained by apoptosis of ganglion cells but to a lesser degree than in the other retinal layers [49]. Moreover, there was increase in the number of blood vessels in the inner retina which can be explained by pathological neovascularization that occurs in advanced DR [50]. In the present study, treatment with MT for four weeks caused modest alternation in the thickness of ONL and INL, number of ganglion cells and number of blood vessels as compared to diabetic group. This was in agreement with the study of Kim, et al. (2017b) [51] who found that treatment with MT for four months did not improve retinal neovascularization in high fat diet/hyperglycemic animals. Neuronal stress and cell death lead to glial cells activation, which occurs early in DR. Acute gliosis has useful effect on the stressed retina, since glia and microglia clear debris and cytotoxins, phagocytize apoptotic cells and secrete neurotrophic factors. Though, chronic gliosis in DR is harmful to the neurons and blood vessels of the retina due to inflammatory cytokines, cytotoxic molecules, and vascular growth factors secretion by the activated glial cells and that will produce both microvascular dysfunction and neurodegeneration [52]. One of the most recognized markers of the functional state of gliocytes is glial fibrillary acidic protein (GFAP). In the present work, we studied immunohistochemical expression of GFAP to explore the protective effects of MT against diabetic-induced retinal gliosis. The GFAP expression in retinas of DB group were increased and extended to occupy the whole layers of the retina from ILM to OLM. This result was in agreement with Nedzvetskii, et al. (2016) [31] who stated that increase GFAP expression after 12 weeks of diabetes was indicative of significant activation of astrocytes and Muller cells of the DB group compared with the CN group. For the first time we demonstrated that MT treatment decreased the area percentage of GFAP staining indicated reduction in the reactive gliosis in retinas of MT group as compared to the DB group. Oliveira, et al. (2016) [16] showed similar result in dentate gyrus area of the hippocampus. They found that MT reduced astrocyte and microglia activation, and explained this by the pharmacologic activation of adenosine monophosphate-activated protein kinase (AMPK) by MT that can reduce the reactive gliosis of diabetic animals. The present work evaluated the degree of apoptosis in retinal tissues following eight-week diabetic state by

Cas₃ immunohistochemical stain. In agreement with Huang, et al. (2011) [49], the current work demonstrated significant increase in the caspase3 expression in retina of DB group as compared to CN group and this result might be due to apoptosis of retinal neurons [50]. It is worth mentioning that, MT treatment caused mild decrease in the area percentage and significant decrease in the optical density of Cas₃ stain as compared to DB group. This result was in line with Nasiry, et al. (2017) [18] who found that MT treatment for 2 months decreased the area percentage of Cas₃ expression in retinas of diabetic rats. This can be explained by the ability of AMPK activation induced by MT to prevent retinal cell death [53]. Decrease apoptosis by MT was not marked probably due to the fact that metformin induced hyperhomocysteinemia that caused degeneration of retinal neurons [54]. In addition to the neuronal elements, DR affects the retinal blood vessels. Vascular Endothelial Growth Factor (VEGF) is the major regulator of vascularization, including blood vessel growth and branching, endothelial cell survival and vessel permeability [55,56]. Although VEGF plays a critical role in the development of DR, its regulation is not completely defined [19].

It was reported that, the DB animals represented increased VEGF expression and consequent pathological neovascularization in response to tissue hypoxia [50]. As expected, the present study demonstrated significant increase in the immunohistochemical expression of VEGF in the inner retina of DB group. However, this was contradictory with the result of Foureaux, et al. (2015) [48] who demonstrated a non-significant increase in the VEGF area percentage in the retinas of 30 days' hyperglycemic animals. This discrepancy might be due to longer diabetic duration of the current study. Time-dependent increase in VEGF expression was reported by Yan, et al. (2014) [57] where VEGF immunoreactivity in DR increased with prolonged duration. The present study demonstrated significant increase in VEGF expression in MT group as compared to CN and mild non-significant increase as compared to DB groups. This finding may be explained by MT induced vitamin B12 deficiency and subsequent hyperhomocysteinemia which could contribute to the development of retinal neovascularization through stimulation of VEGF expression by retinal cells [58]. This result was in line with Bakhshab, et al. (2016) [22] who ensured that metformin induced endothelial cells angiogenesis by augmenting the expression of VEGF and reducing angiogenic inhibitors in CD34⁺ cells under hyperglycemia and hypoxia *in vitro*. On the contrary, Zhang, et al. (2017) [19] stated that MT significantly alleviated the severity of DR through attenuating the retinal neovascularization by suppressing VEGF protein. However, Yi, et al. (2016) [20] stated that metformin attenuates the retinal neovascularization by decreasing the phosphorylation of the VEGF receptor 2 (VEGFR2) which inhibited VEGF signaling without affecting total VEGF protein. These conflicting data concerning the angiogenic action of MT indicate its paradoxical

effects on endothelial cells and angiogenesis. It was reported that MT modulates both pro- and anti-angiogenic factors in endothelial cells with no apparent advantage of one over the other [59]. The precise mechanisms, as well as the regulatory components for the contradictory angiogenic properties of MT, still unclear and require further investigations.

Conclusion

In conclusion, the present study demonstrated that MT improved the diabetic induced retinopathy as regard to gliosis and apoptosis, and its mechanism may be due to reduction of hyperglycemia and oxidative stress suppression. However, MT was not able to suppress the diabetic induced neovascularization in the retina, but on the contrary it enhanced the new blood vessels formation. These findings indicated that prevention of DR might require a multi-pronged approach, and MT may have an adjuvant role.

Recommendation

The future studies are recommended to approve or disapprove the protective role of MT in DR particularly in the context of regulatory mechanisms for its antiangiogenic or proangiogenic effects.

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