International Immunopharmacology 74 (2019) 105672

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Preventive role of metformin on peripheral neuropathy induced by diabetes

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ARTICLE INFO

Keywords:
Diabetic neuropathy
Schwann cells
Hyperglycemia
Myelinated nerve fibers
Peripheral nerve disease

ABSTRACT

Metformin is the first line drug in the treatment of type 2 diabetes, however, little is known about its therapeutic potential to prevent or delay damage to the peripheral nerve. Thus, the aim of this study was to investigate whether metformin is able to attenuate the neuroinflammatory response in sciatic nerve of insulin-dependent diabetic mice. Swiss Webster mice were divided into four groups: Control, Diabetic (STZ), Diabetic +100 mg/kg/day of metformin (STZ + M100) and Diabetic +200 mg/kg/day of metformin. Diabetes was induced by streptozotocin (90 mg/kg, i.p.). Only animals with glycemia ≥270 mg/dl were considered diabetics. Metformin prevented atrophy of myelinated axons, and reduced expression of inflammatory mediators (interleukin-1β, inducible nitric oxide synthase and nitric oxide). However, treatment with 200 mg of metformin was more effective in increasing neurotrophic (myelin basic protein and neural growth factor), angiogenic (vascular endothelial growth factor) and anti-inflammatory (inhibitor kappa B-alpha and interleukin 10) factors. Thus, metformin treatment, especially at the dose of 200 mg, protected the nerve from damages related to chronic hyperglycemia.

1. Introduction

Diabetic distal polyneuropathy is a common comorbidity in diabetic patients [1]. Hyperglycemia compromises peripheral nerve homeostasis, which results in various sensory disorders ranging from a painful symptomatology to cutaneous anesthesia in limbs extremities, affecting the quality of life of patients and increasing ulcerative wounds and limb amputations risk [2,3].

The pathogenesis of diabetic distal polyneuropathy is multifactorial because hyperglycemia generates accumulation of toxic metabolites in all cells of peripheral nervous tissue. The effect of hyperglycemia on endothelial cells compromises the *vasa nervorum* vasodilatation mechanism, which results in reduction of blood flow in nerve tissue [4]. In addition, the uptake of excess glucose by the insulin-independent glucose transporter (GLUT-1) mainly by Schwann cells causes metabolic disturbances in these glial cells, which in turn transfer the excess

Abbreviations: STZ, streptozotocin; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; MBP, myelin basic protein; NGF, neural growth factor; VEGF, vascular endothelial growth factor; IkBα, inhibitor kappa B-alpha; IL-10, interleukin 10; TNFα, tumor necrosis factor alpha; NF-κB, nuclear factor kappa B; NADPH, nicotinamide adenine dinucleotide phosphate; AMPK, adenosine monophosphate protein kinase

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https://doi.org/10.1016/j.intimp.2019.05.057
Received 28 March 2019; Received in revised form 21 May 2019; Accepted 28 May 2019
Available online 10 June 2019
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glucose and even products of metabolic stress to the axon [5,6].

High glucose levels inside the nervous fiber saturate the main glycolytic pathway, activating secondarily the polyol pathway, which generates accumulation of advanced glycation end products, sorbitol and fructose and, therefore, causes structural and functional impairment to Schwann cell and nerve. The activation of polyol pathway reduces adenine dinucleotide phosphate (NADPH) availability, an important intracellular antioxidant [7], and induces the expression of several inflammatory factors, such as TNF-α and IL-1, contributing to activation of the NF-kB pathway [8]. These cytokines are closely related to the mechanism of neuropathic pain in injured peripheral nerves and M1 macrophages recruitment to the lesion site, increasing tissue inflammation [9].

Insulin resistance characterizes type 2 diabetes, whereas type 1 diabetes is associated with systemic insulin deficiency. Metformin is an agonist of AMPK, which promotes glucose uptake through the phosphorylation of Akt and other enzymes necessary to the translocation of glucose transporter type 4 (GLUT4), increasing insulin sensitivity in type 2 diabetes [42].

Recent studies indicate that metformin, first line drug in the treatment of type 2 diabetes, has a neuroprotective potential in chemotherapy-induced peripheral neuropathy model [10], decreasing neuropathic allodynia in model of peripheral nerve injured [11] and attenuating diabetes-induced hyperalgesia in a model of painful diabetic neuropathy induced by streptozotocin [12].

Hitherto, only one study evaluated the preventive potential of metformin in the onset of diabetic peripheral neuropathy. However, according to this study metformin-treated diabetic group had statistically lower glycemic levels than the diabetic group, even on the 4th day after diabetes induction, which may have contributed to avoiding the development of neuropathy due to lower exposure to hyperglycemia [13].

Thus, the aim of this study was to investigate whether metformin is able to attenuate the neuroinflammatory response in hyperglycemia-induced damage to sciatic nerve of type 1 diabetic mice.

2. Experimental procedures

2.1. Induction of diabetes and treatment with metformin

Forty Swiss Webster mice aged 12 weeks and weighing of 40 ± 4 g were used, maintained at a temperature of 23 ± 2 °C and with access to food and water ad libitum. This study was approved by the Ethics Committee on the use of animals of Aggeu Magalhães Institute/Oswaldo Cruz Foundation (81/2015 - CEUA/FIOCRUZ).

The animals were divided into four experimental groups: Control group (Control), consisting of healthy animals; Diabetic group (STZ), diabetes-induced animals that did not receive treatment with metformin; Diabetic group treated with 100 mg/kg/day of metformin (STZ + M100); Diabetic group treated with 200 mg/kg/day of metformin (STZ + M200).

For induction of type 1 diabetes we performed the protocol described by Oliveira et al. [14], consisting of intraperitoneal injection of 90 mg/kg streptozotocin [15] diluted in citrate buffer (pH 4.5), for two consecutive days. The animals were fasted for 12 h before diabetes induction. The control group animals received intraperitoneal injection containing only citrate buffer.

Four days after induction of diabetes, blood samples were collected from the caudal vein of each animal to measure glucose levels using a blood glucose meter (Lifescan, One Touch UltraSmart). Animals with glycemia ≥ 270 mg/dl were included in the study [16]. Body weight and glycemia were measured weekly and diabetic animals did not received insulin to control hyperglycemia. The animals with blood glucose lower than 270 mg/dl for two consecutive weeks after induction were excluded from the experiment, discarding possible spontaneous recovery of endogenous beta cell as previously suggested [17].

The treatment started on the same day of diabetes confirmation and consisted on administration orally by gavage of two daily metformin doses diluted in saline (0.9%), 100 mg/kg/day and 200 mg/kg/day [14], for 9 weeks. STZ + M100 group received the first dose and STZ + M200 group the last dose, respectively. Control and STZ groups received saline (0.9%) orally by gavage.

2.2. Processing and histomorphometric analysis

The animals were submitted to intracardiac perfusion with physiological saline followed by Karnowisky solution (2.5% glutaldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer, pH = 7.4). Sciatic nerve fragments were removed and maintained in fixative solution of Karnowisky for 24 h and post fixed in 1% osmium tetroxide in sodium cacodylate buffer 0.1 M (pH 7.4) for 2 h, immersed in 5% uranyl for 24 h, dehydrated in increasing concentration of aqueous solutions of acetone and embedded in Epoxy resin.

Semi-thin transverse cross-sections (5μm thick) were stained with toluidine blue solution (1%) and analyzed in a microscope (Zeiss MicroImaging) connected to a camera (AxioCam MRm Zeiss) at the magnification of 1000×.

Mesurim Pro 0.8 software was used to quantify the absolute number of myelin fibers in each nerve section and ImageJ 1.50i software was used to measure axonal and fiber diameters of at least 200 myelinated fibers of four animals per group [18] and to calculate the thickness of myelin sheath (fiber diameter - axonal diameter/2) [19,20].

2.3. Processing for immunohistochemistry

The sciatic nerve fragments were overnight fixed in 4% paraformaldehyde diluted in 0.1 M phosphate, pH 7.4. The samples were dehydrated, cleared and embedded in paraffin. Transversal sections of 4 μm thickness were used and adhered to 3-amino-propyl-triethoxysilane-treated slides (Sigma-Aldrich, St. Louis, MO, USA).

The sections were rehydrated and treated with 20 mM citrate buffer (pH 6.0) at 100 °C for 30 min and endogenous peroxidase was blocked with 3% H_2O_2 diluted in methyl alcohol. Subsequently, nonspecific sites were blocked in solution containing 1% bovine serum albumin, 0.2% Tween 20 and PBS.

The sections were incubated for 12 h at 4 °C with anti-NGF (SC-548; Santa Cruz Biotechnology, 1:100), anti-IκBα (sc-371; Santa Cruz Biotechnology, 1:100), anti-IL-1β (sc-7884; Santa Cruz Biotechnology, 1:100), anti-TNFα (500-P64; Prepotech, 1:50) and anti-iNOS (ab3523; Abcam, 1:50).

The slides were incubated with the biotin-conjugated secondary IgG antibody, amplified using the DakoCytomation kit (Biotinylated Link Universal HRP; K0690; USA) and visualized by incubation with the 3′-3-diaminobenzidine (DAB) chromogen at room temperature.

The slides were mounted in Entellan (1079610100; Merck, USA) to obtain five photomicrographs of sciatic nerve of two different animals per group at 1000× magnification. Images were quantified using GIMP 2.6.11 software (GNU Image Manipulation Program software, Australia).

2.4. Immunofluorescence assay

Similar to the initial processing for immunohistochemistry, the slides were overnight incubated with primary MBP antibody (ab7349; Abcam, 1:200). Then the sections were incubated with the secondary antibody conjugated to Alexa 488 goat anti-mouse fluorophore (A11008, Invitrogen, 1:500), followed by incubation with DAPI (Vector laboratories, USA, 1: 500) for nuclear labeling. The slides were mounted and the images were obtained from a fluorescence microscope (DM2500, Leica Microsystems, Germany) coupled with digital camera (Leica DFC345 FX).

2.5. Western blotting

Sciatic nerves of four animals per group were collected and immediately frozen in liquid nitrogen. The fragments were homogenized in a tube containing extraction cocktail (10 mM/l EDTA, 2 mmol/l phenylmethane sulfonyl fluoride, 100 mmol/l sodium fluoride, sodium 10 mmol/l, 10 mmol/l sodium orthovanadate, 10 μm aprotinin and 100 mmol/l Tris-aminomethane - ph 7.4) using a manual macerator for tissue breakdown and protein release. Thereafter, the homogenate was centrifuged and the supernatant extracted, obtaining a pool of samples of each group.

In an electrophoresis gel, 20 μg of total protein per group was applied. The proteins were transferred to nitrocellulose membranes (BioRad 162-0115), blocked for 1 h with skim milk (5%) diluted in PBS and overnight incubated at 4 °C with primary antibody anti-IL-10 (ab33471; Abcam, 1:1000), anti-VEGF (ab1316, Abcam, 1:1000), anti-MBP (ab62631, Abcam, 1:1000), anti-NGF (ab6199, Abcam, 1:1000) and β-actin (A2228, Sigma-Aldrich, 1:3000). The technique was performed in duplicate for confirmation of results and β-actin marker was used as a control by ratio of protein/β-actin in each group.

The bands were detected by a chemiluminescent reagent (Super Signal, 34080) and visualized in C-DiGit Blot Scanner (LI-COR Biosciences, USA) and Image Studio Digits software (LI-COR Biosciences version 5.0, USA). ImageJ software version 1.50i was used to quantify the pixels density of bands.

2.6. Measurement of nitric oxide

After anesthesia, intracardiac blood puncture was performed for serum collection. Sciatic nerve fragments were removed in the absence of fixative and immediately frozen in liquid nitrogen. Afterwards, the animals were submitted to euthanasia by cervical dislocation.

Nitric oxide was measured using the Griess reaction (1% sulfanilamide and *N*-1-naphthyl ethylenediamine diluted in 2.5% H_3PO_4). In duplicate, samples were added to an ELISA plate in equal proportion of sample per Griess reagent. A standard curve was also added to the plate and consisted of sodium nitrite solution at the initial concentration of 100 μM and serially diluted in PBS. The results were obtained through the absorbance values of the samples compared to the standard curve using a spectrophotometer (490 nm).

2.7. Statistical analysis

The data were analyzed in the Prisma 6.0 program. Initially, the Shapiro-Wilk normality test was used. One-way analysis was used for the data that presented a normal distribution, followed by Tukey's post hoc test. The variables without normal distribution were analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. The values of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Treatment with metformin does not reduce hyperglycemia

Blood glucose monitoring performed weekly in all experimental groups confirmed the validity of the model used in this study. All animals presented glycemic values lower than 150 mg/dl before induction and, after diabetes induction, the animals of STZ, STZ + M100 and STZ + M200 groups presented hyperglycemia, which was maintained throughout the experiment and differed from glycemic level of control group (Fig. 1A).

Body weight monitoring showed that only STZ group had significant reduction in body weight by intragroup comparison at the end of the experiment compared to that before induction of diabetes, whereas no significant difference was observed in the body weight of control and diabetic groups treated with metformin (Fig. 1B).

3.2. Treatment with metformin prevents axonal atrophy and fiber degeneration

Absolute quantification of myelin fibers revealed that STZ group presented a decreased number of myelin fibers when compared to control group, whereas treatment with metformin prevented axonal loss related to diabetes. The sciatic nerve of STZ group presented axonal atrophy in comparison with control group, which was prevented by treatment with 100 mg of metformin. By its turn, the 200 mg dose of metformin not only was effective in preventing thickness reduction of myelin sheath, but also resulted in myelin fibers with greater diameters than those found in STZ group (Fig. 2).

3.3. Metformin induces expression of neurotrophic and angiogenic factors

Protein levels of NGF expressed by Schwann cells and axons were evaluated in this study (Fig. 3). Treatment with 200 mg of metformin potentiated NGF expression, above physiological levels, promoting better conditions for maintenance of axonal trophism, despite tissue exposure to excess glucose (Figs. 3B and 4J).

Myelin basic protein (MBP), one of the components of the myelin sheath, showed higher expression in treated groups in comparison to STZ group, indicating activation of repairing mechanisms of myelin sheath (Figs. 3C and 4K–N).

To verify the expression of the protein responsible for stimulation of angiogenesis and maintenance of vascular integrity, the western blot technique for vascular endothelial growth factor (VEGF) was performed. Nerve tissue of diabetic animals showed increased expression of VEGF levels when compared to control group (Fig. 3B), whereas animals treated with 200 mg of metformin showed higher VEGF levels when compared to STZ group (Fig. 3B).

3.4. Metformin attenuates inflammation of nerve tissue exposed to chronic hyperglycemia

The neuroinflammation caused by diabetes was confirmed by overexpression of TNF-α and IL-1β in sciatic nerve of STZ group. Despite the treatment with metformin did not reduce TNF-α release (Fig. 5E), both doses of metformin in this study were able to inhibit IL-1β expression (Fig. 5J).

Furthermore, levels of serum and tissue nitric oxide, increased due chronic hyperglycemia, were reduced by metformin in STZ + M100 and STZ + M200 groups (Fig. 6). In addition, by analyzing the expression of iNOS isoform it was possible to confirm that the higher production of tissue nitric oxide was related to iNOS overexpression in STZ group and that metformin treatment reduced this pro-inflammatory stimulus, mainly at 200 mg dose (Fig. 5O).

3.5. Metformin increases the expression of anti-inflammatory markers

The IκBα expression in axons and cytoplasm of Schwann cells was analyzed. STZ group had no significant difference when compared to control group. However, STZ + M100 group had an increased expression of this protein in comparison with STZ group, whereas STZ + M200 group presented significantly IκBα overexpression when compared to all other groups (Fig. 4E).

Finally, Western blot technique was performed for the anti-inflammatory cytokine IL-10 and a significantly increase in IL-10 expression in metformin-treated groups (STZ + M100 and STZ + M200) was detected, both in comparison with control and STZ groups (Fig. 3D).

4. Discussion

The present study assessed metformin effects at a dose of 100 mg/kg and 200 mg/kg on histomorphometry as well as expression of

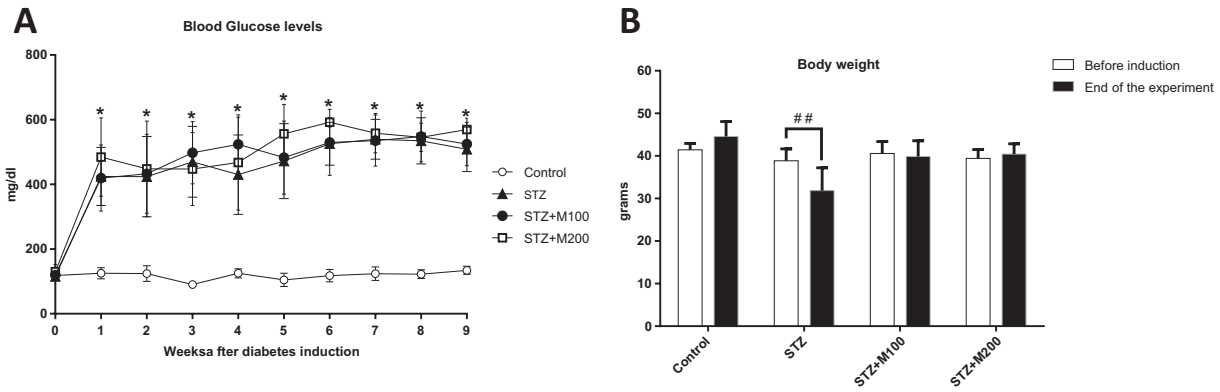

Fig. 1. Weekly monitoring of blood glucose levels (A) and body weight (B) in the experimental groups. Data were expressed as mean ± standard deviation, n = 7 animals per group. *p < 0.05 STZ, STZ + M100 and STZ + M200 groups vs control group. ##p < 0.01 body weight in the end of the experiment vs before induction.

inflammatory, anti-inflammatory, neurotrophic and angiogenic factors of sciatic nerve of mice induced to type 1 diabetes.

The maintenance of hyperglycemia in diabetic groups, even in those who received daily metformin treatment, reinforces the inability of this drug to normalize glycemic levels in type 1 diabetes without supplemental insulin administration [21]. In addition, the maintenance of hyperglycemia was essential for this study, because we aimed to evaluate anti-inflammatory and neuroprotector potential of metformin in peripheral nerve exposed to chronic hyperglycemia. Results obtained by other authors utilizing rats instead of mice reported a reduction in glycemic levels in metformin-treated animals 4 days after induction to diabetes with 65 mg/kg of streptozotocin [13]. However, some pancreatic beta cells could resist to this streptozotocin dose or could be spontaneously regenerated [17]. In the present work, we excluded the animals that did not developed hyperglycemia in order to evaluate metformin effect on hyperglycemia-induced damage in neurons. Interestingly, no animals had normalized glycemic levels 3 weeks after metformin treatment.

As expected, diabetic animals presented alteration of murinometric parameter, such as weight loss [12,22]. In type 1 diabetes, deficient insulin production causes depletion of energy reserve and increase in protein catabolism, generating reduction in body weight [23], as was verified in STZ group. On the other hand, metformin prevented weight reduction in diabetic animals (STZ + M100 and STZ + M200 groups), possibly due to the recently showed adipogenic effect of metformin mediated by a signaling pathway not mediated by AMPK [24].

Structural alterations found in sciatic nerve of diabetic animals (STZ) evidenced that hyperglycemia during nine weeks promoted histomorphometric alterations, which is in agreement with earlier studies [15,18]. In contrast, the present study demonstrated that metformin was able to prevent axonal atrophy and fiber degeneration, as well as to promote increased NGF expression, a known neurotrophic factor responsible for neuronal survival, neuroprotection and regeneration of peripheral nervous system [25].

Hyperglycemia promotes an increase in influx of glucose to neurons, generating intracellular accumulation of glycolysis products, causing oxidative stress due non-enzymatic reaction with nucleotides, lipids and proteins, changing the structure and function of proteins from different regions of peripheral nerve [6]. Ma et al. [12] showed that metformin at 200 mg dose was able to reduce the levels of malondialdehyde and advanced glycation end-products, two important markers of oxidative stress in sciatic nerve [26]. Another nervous structure that must be considered as suffering directly with the chronic hyperglycemia is the myelin sheath. In the pathogenesis of diabetic neuropathy, advanced glycation end-products bind irreversibly to components of the myelin sheath, becoming target of phagocytic macrophages to be replaced by new proteins [27]. Depending on the speed and replacement capacity of the modified proteins, the integrity of myelin layer may be impaired, reducing conduction velocity of nerve fibers [28].

Data from the present study revealed that 9 weeks after induction to diabetes there is reduced expression of MBP, resulting in reduction of myelin sheath thickness. This reduction is expected in the progression of neuropathy pathogenesis, regardless of the myelin fiber size [29], even in newborn mice induced to diabetes [15]. On the other hand, metformin treatment at dose of 200 mg increased expression of MBP by Schwann cells, suggesting that overexpression of MBP in this group contributed to remyelination process verified by the increase of myelin sheath thickness in STZ + M200 group in comparison to STZ group. Similarly, in a model of perinatal hypoxia-ischemia, rats treated with metformin presented regeneration of myelin sheath associated with increased MBP expression in brain of rats [30].

VEGF promotes angiogenesis through stimulation of endothelial cell proliferation and previous studies showed that AMPK activators can indirectly modulate VEGF expression as a mechanism of response to tissue hypoxia [31–33]. Considering the microvascular changes in the course of diabetic neuropathy [33], it was shown here that STZ group had increased expression of this angiogenic factor, but the treatment with 200 mg of metformin induced overexpression of VEGF, suggesting an increase of the vascular network due to metabolic demand in response to stress caused by hyperglycemia.

The increased levels of the inflammatory mediators IL-1β and TNFα found in STZ group in this study are in agreement with previous study, in which chronic hyperglycemia causes an increase in expression of these markers [34]. Although metformin did not reduced the expression of TNF-α, both diabetic and metformin-treated groups presented a reduction of IL-1β, whose production, along with TNF-α in Schwann cells, recruits macrophages to initiate the process of Wallerian degeneration [35]. These findings are in accordance with the results of an in vitro assay in which metformin inhibited lipopolysaccharide-induced inflammation in macrophages through inhibition of mitochondrial I complex [36].

In addition, metformin has been shown to have a beneficial effect on nervous tissue by increasing the expression of IL-10, an anti-inflammatory cytokine, described as an important factor in the regeneration of damaged nerve [37].

Inevitably, the maintenance of high glycemic levels generates activation of the polyol pathway in peripheral nerve, promoting conversion of excess glucose to sorbitol by the action of aldose reductase, which leads to degradation of NADPH cofactor in this reaction [38]. Depletion of intracellular NADPH compromises the physiological production of NO and glutathione restoration, an important intracellular antioxidant [38,39].

Nitric oxide is a signaling molecule involved in various physiological processes and during inflammation it is produced by the inducible

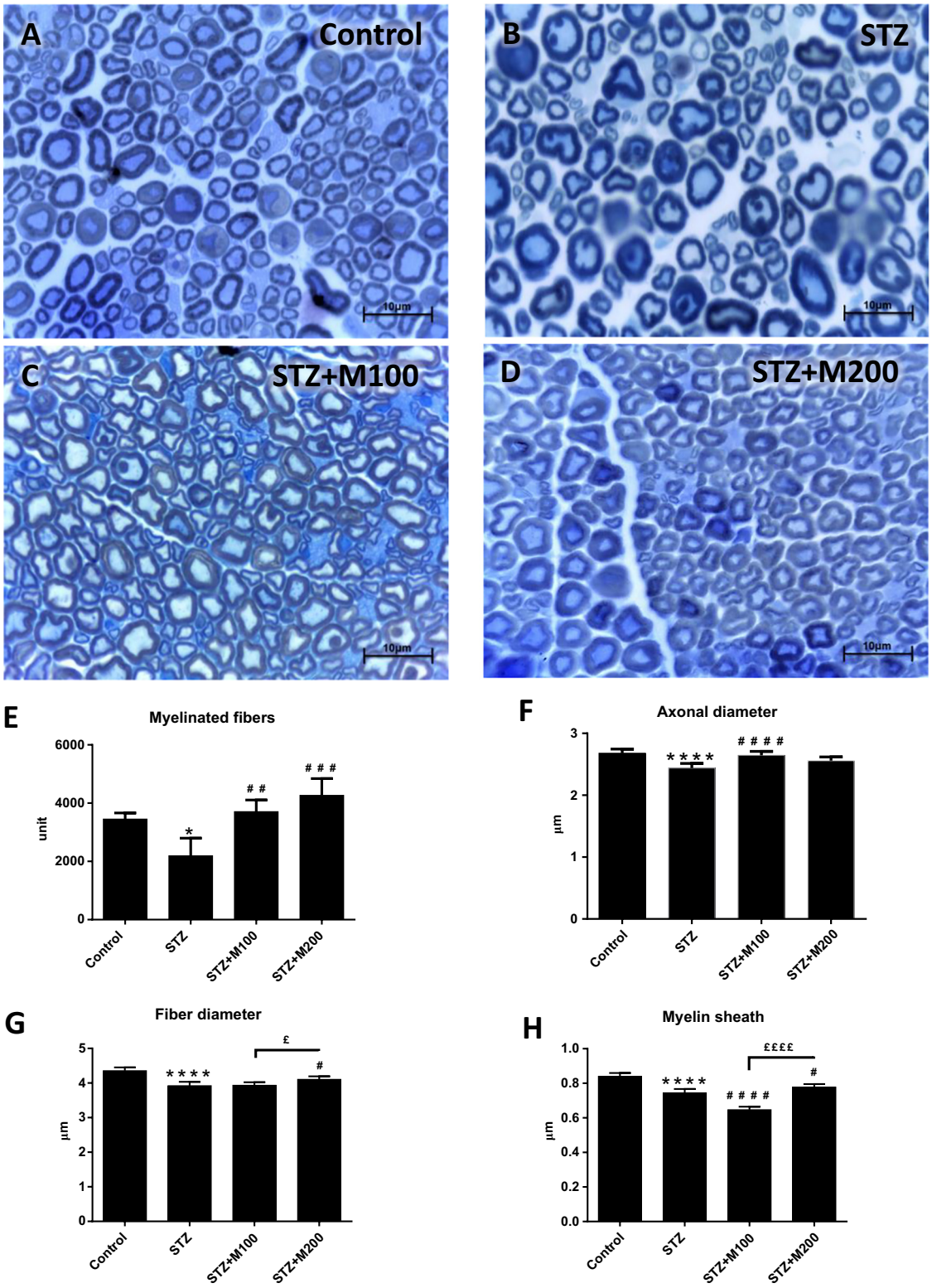

Fig. 2. Effects of metformin on morphology of peripheral nerve. Images A-D show semi-thin toluidine blue-stained transversal section of sciatic nerve at magnification of 1000× in Control group (A), STZ group (B), STZ + M100 group (C) and STZ + M200 group (D). Quantification of myelinated fibers (E) was expressed as mean ± SD. Measurement of axonal diameter (F), fiber myelinated diameter (G) and myelin thickness (H) was expressed as mean with 95% CI. *$p < 0.05$ and ****$p < 0.0001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ vs STZ group; $^{£}p < 0.05$ and $^{££££}p < 0.0001$ vs STZ + M100 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

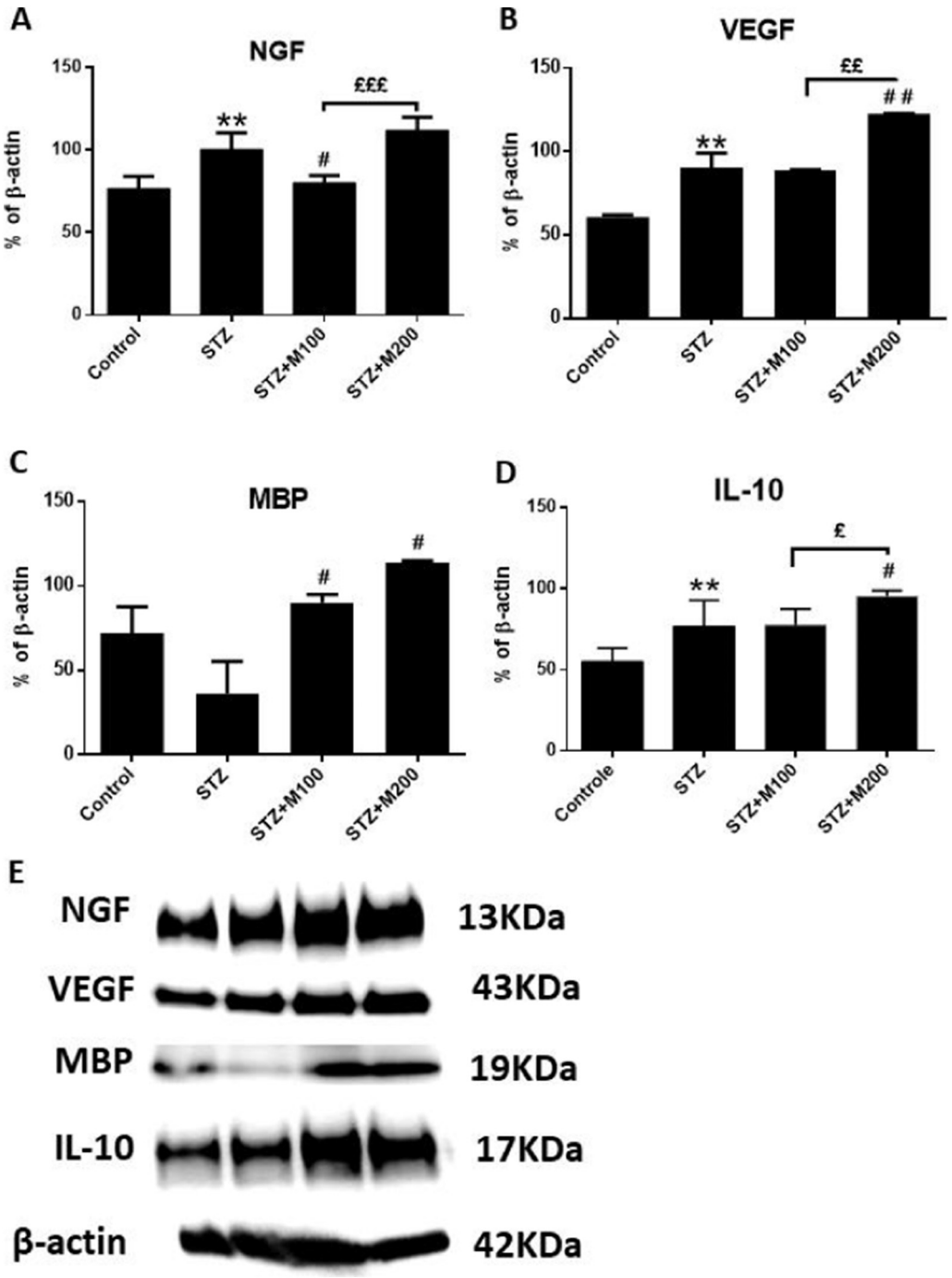

Fig. 3. Effects of metformin on expression of NGF (A), VEGF (B), MBP (C), and IL-10 (D) in the sciatic nerve of diabetic mice (n = 4 animals per group). The results were expressed as mean ± SD. ** $p < 0.01$ vs control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs STZ group; $^{£}p < 0.05$, $^{££}p < 0.01$ and $^{£££}p < 0.001$ vs STZ + M100 group.

isoform of NO synthase (iNOS) in macrophages and several other cell types. Of note, when NO is produced in excess it can be cytotoxic and lead to nitrosative stress [40]. The levels of tissue and serum NO in our study were increased in STZ group in comparison with control group, confirming the inflammatory feature of diabetes mellitus, which is mediated by inflammatory cytokines such as TNF-α and IL-1β [34,40]. Treatment with metformin reduced iNOS levels in sciatic nerve, especially at dose of 200 mg, resulting in lower levels of NO.

In the diabetic neuropathy, TNF-α, IL-1β and iNOS expression are increased due to activation of the NF-κB pathway, which increases transcription of these and other inflammatory cytokines when translocated to the nucleus [41]. Previous study from our laboratory demonstrated that 200 mg/kg of metformin increased ratio of p-AMPK/AMPK [14], promoting intracellular increase of IkBα [14,42]. In this study, the expression of IkBα was increased in treated groups, inhibiting inflammation mediated by NF-κB pathway in peripheral nerve [43–45].

5. Conclusions

The findings of this study show that nine-week treatment with metformin initiated immediately after confirmation of type 1 diabetes protects peripheral nerve from chronic hyperglycemia-mediated damage by inhibiting the expression of inflammatory cytokines. In

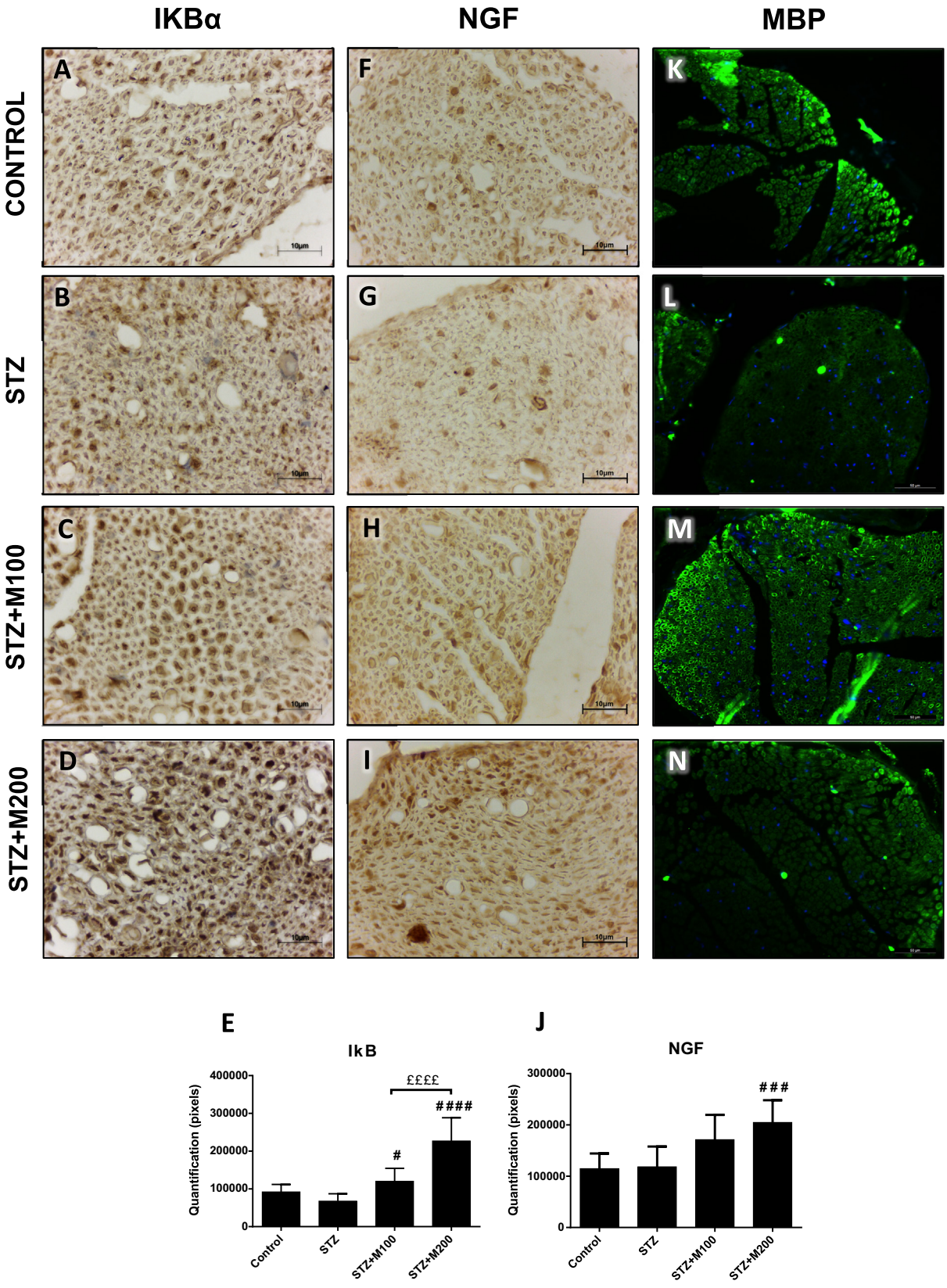

Fig. 4. Effects of metformin on the expression of IkBα, NGF and MBP in the sciatic nerve. Quantification of IkBα and NGF (five areas, n = 2 animals per group) was performed at 1000× magnification. MBP was performed at 500× magnification. The results were expressed as mean ± standard deviation. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ vs STZ group. ANOVA one-way followed by Tukey's post hoc test.

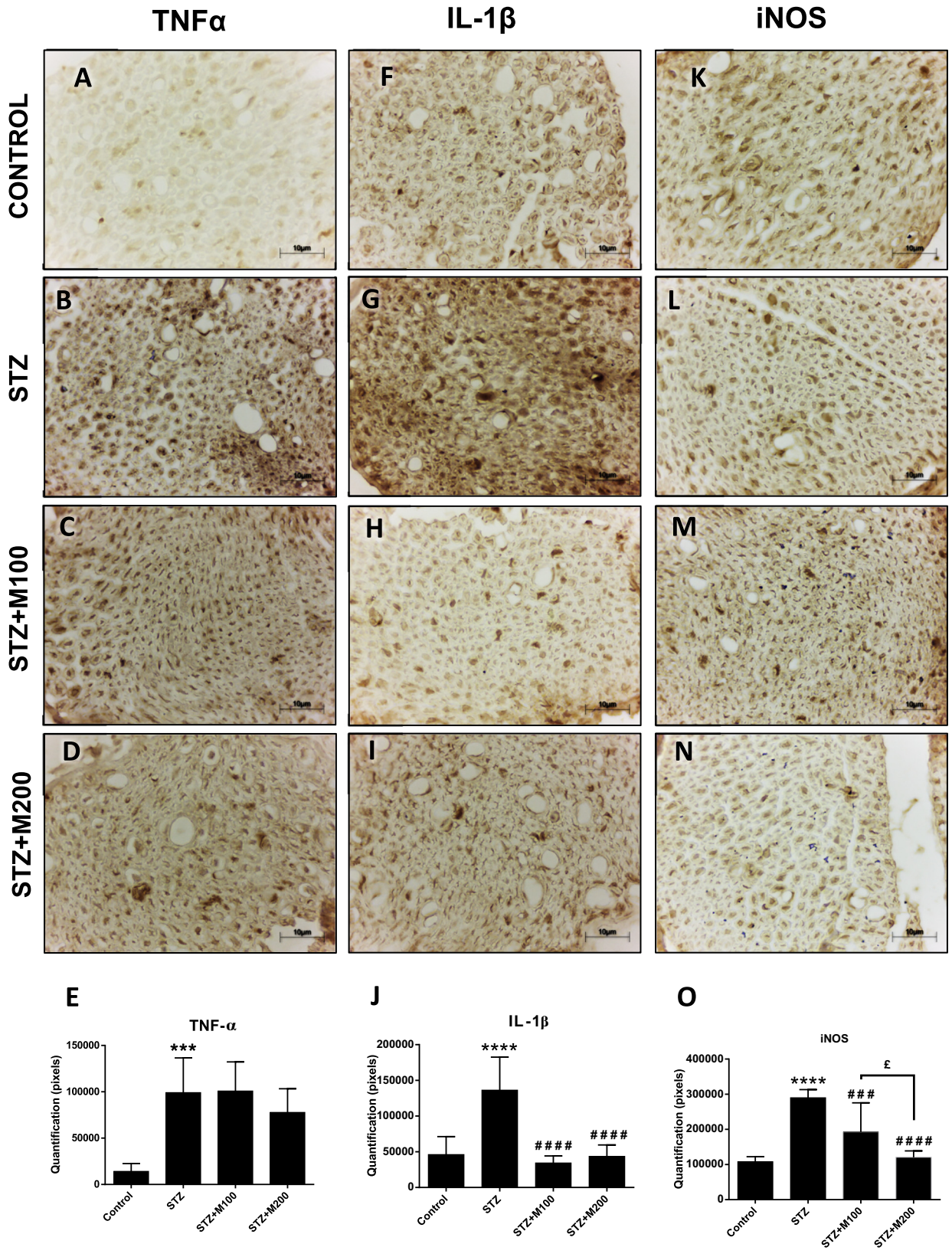

Fig. 5. Effects of metformin on the expression of TNFα, IL-1β and iNOS in the sciatic nerve. Quantification of immunohistochemistry (five areas, n = 2 animals per group) was performed at 1000× magnification. The results were expressed as mean ± standard deviation. $^{***}p < 0.001$ and $^{****}p < 0.0001$ vs control group; $^{###}p < 0.001$ and $^{####}p < 0.0001$ vs STZ group; $^{£}p < 0.05$ vs group STZ + M100 group. ANOVA one-way followed by Tukey's post hoc test.

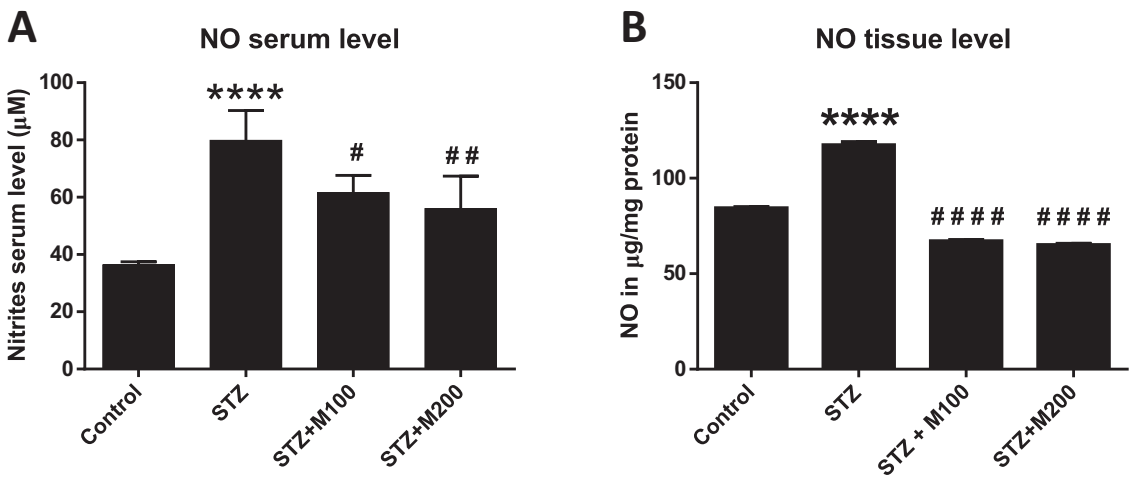

Fig. 6. Effects of metformin on the serum and tissue levels of nitric oxide in the sciatic nerve of diabetic mice. The results were expressed as mean ± SD. $^{****}p < 0.0001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#\#}p < 0.0001$ vs STZ group. ANOVA *one-way* followed by Tukey's post hoc test.

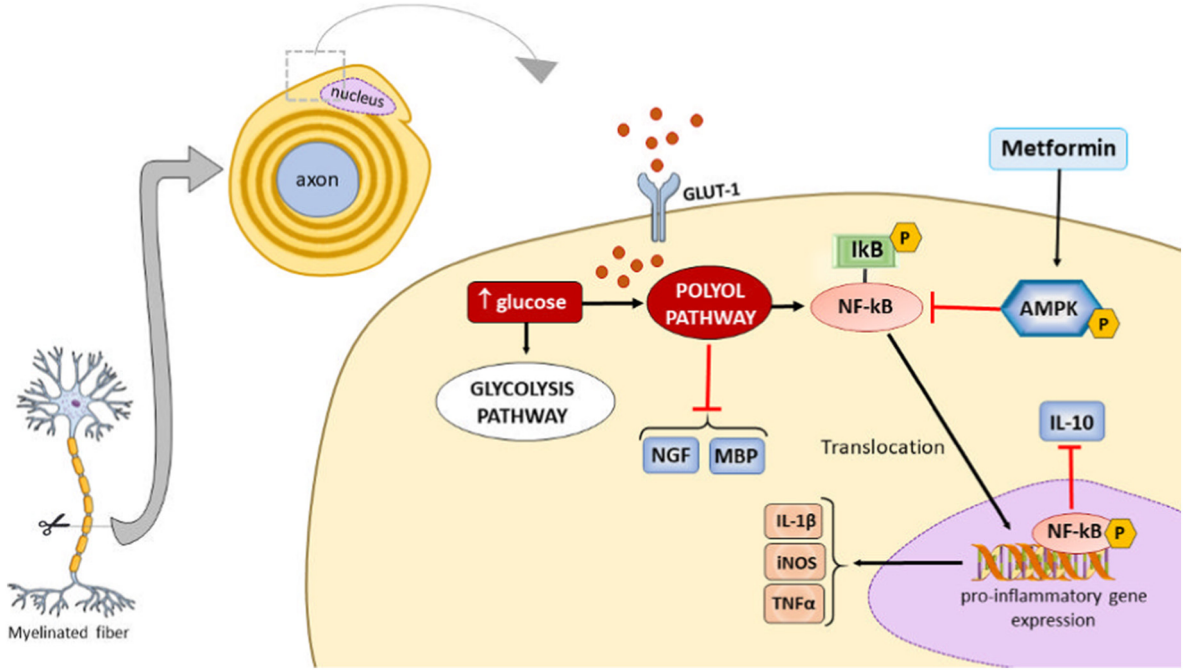

Fig. 7. Schematic summarizing the effects of glucose excess in the Schwann cell and anti-inflammatory action of metformin. Black arrows indicate activation and red bar-headed line indicates inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition, metformin treatment increases the expression of anti-inflammatory mediators, as well as angiogenic and neurotrophic factors in axons and Schwann cells of mice sciatic nerve. Thus, metformin has a therapeutic potential to prevent or delay the hyperglycemia-induced peripheral nerve damage (Fig. 7).

Funding

The authors would like to express their gratitude to Oswaldo Cruz Foundation of Pernambuco (FIOCRUZ-PE), the Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM; #465489/2014-1) and National Council for Scientific and Technological Development (CNPq; #301777/2012-8) for research support. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Declaration of Competing Interest

None.

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