Life Sciences 232 (2019) 116588

Contents lists available at ScienceDirect

Life Sciences

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

Neuroprotective effect of levetiracetam in mouse diabetic retinopathy: Effect on glucose transporter-1 and GAP43 expression

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ARTICLE INFO

Keywords:
Diabetic retinopathy
Growth-associated protein 43
GFAP
GLUT1
Inflammation
Levetiracetam

ABSTRACT

Aims: Retinopathy is a neurodegenerative complication associating diabetes mellitus. Diabetic retinopathy (DR) is the primary reason of visual loss during early adulthood. DR has a complicated multifactorial pathophysiology initiated by hyperglycaemia-induced ischaemic neurodegenerative retinal changes, followed by vision-threatening consequences. The main therapeutic modalities for DR involve invasive delivery of intravitreal anti-angiogenic agents as well as surgical interventions. The current work aimed to explore the potential anti-inflammatory and retinal neuroprotective effects of levetiracetam.

Main methods: This study was performed on alloxan-induced diabetes in mice (n: 21). After 10 weeks, a group of diabetic animals (n: 7) was treated with levetiracetam (25 mg/kg) for six weeks. Retinal tissues were dissected and paraffin-fixed for examination using (1) morphometric analysis with haematoxylin and eosin (HE), (2) immunohistochemistry (GLUT1, GFAP and GAP43), and (3) RT-PCR-detected expression of retinal inflammatory and apoptotic mediators (TNF-α, IL6, iNOS, NF-κB and Tp53).

Key findings: Diabetic mice developed disorganized and debilitated retinal layers with upregulation of the gliosis marker GFAP and downregulation of the neuronal plasticity marker GAP43. Additionally, diabetic retinae showed increased transcription of NF-κB, TNF-α, IL6, iNOS and Tp53. Levetiracetam-treated mice showed downregulation of retinal GLUT1 with relief and regression of retinal inflammation and improved retinal structural organization.

Significance: Levetiracetam may represent a potential neuroprotective agent in DR. The data presented herein supported an anti-inflammatory role of levetiracetam. However, further clinical studies may be warranted to confirm the effectiveness and safety of levetiracetam in DR patients.

1. Introduction

The World Health Organization announced a prediction of further growth of the prevalence of diabetic retinopathy (DR). Furthermore, the amount of people at risk of vision loss is expected to double by 2030 with the rise of the diabetes mellitus (DM) epidemic [1]. Around 346 million people have DM worldwide; about 10% of diabetic patients suffer from substantial visual impairment, and 2% suffer blindness [2,3]. According to the Diabetes Control and Complications Trial [4] and UK Prospective Diabetes Study [5], hyperglycaemia initiates retinal damage.

Glucose transporter-1 (GLUT1) is the sole transporter of glucose in the retina and is mainly found in vascular cells in the blood-retinal barrier [6]. In DR, neovascular tissue lacks expression of GLUT1 with

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https://doi.org/10.1016/j.lfs.2019.116588
Received 16 February 2019; Received in revised form 14 June 2019; Accepted 17 June 2019
Available online 18 June 2019
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disturbed selective permeability of the retinal capillaries [7]. The retina is an energy-consuming tissue with high metabolic activity supported by neurovascular integrity [8].

Functional retinal signalling requires interactions among three components: vascular, neuronal and glial cells [9]. There are three types of retinal glial cells, including Müller cells, astrocytes and resident microglia. Indeed, astrocytes and Müller cells are responsible for retinal structural and metabolic regulatory roles [10]. However, resident microglia exhibit macrophage-like activity [11–14]. Retinal vascular changes induce glial cell activation, which is characterized by induction of a proinflammatory gene (NF-κB) and upregulation of cytokines such as tumour necrosis factor-α (TNF-α), IL6, and interleukin-1β (IL1β) [15–19]. Additionally, NFκB inhibition reportedly reduces diabetes-induced retinal leucocytosis and expression of vascular endothelial growth factor [20]. Nitric oxide (NO) is also abundant in the retina of diabetic patients [21] as well as DR animal models [22]. Inducible nitric oxide synthetase (iNOS) is responsible for the excessive, pathological production of NO [23].

Growth-associated protein-43 (GAP43) is a neural protein that is expressed in central and peripheral nerve tissues and can be modulated by diabetes and neuronal cell injury [24–26]. Synaptic plasticity comprises alterations in synaptic structure and function [27]. GAP43 expression may be linked to the synapse number [28], synaptic plasticity in the brain [29] and learning and memory abilities [30–32].

Levetiracetam [(*S*)-α ethyl-2-oxo-1-pyrrolidine acetamide] is an FDA-approved third-generation anti-epileptic medication. Levetiracetam has been recognized as effective and safe in preventing different types of epilepsy [31,33–35]. Levetiracetam is a neuromodulator acting through binding to synaptic vesicle glycoprotein (SV2A) and modifies neurotransmitter release [36]. Structurally, SV2A has 12-transmembrane spanning domains. N-terminal six-transmembrane domains are highly homologous to a subfamily of transporters including human GLUT, while C-terminal six-domains show homology to the neurotransmitters transporters in the plasma membrane [37,38]. Levetiracetam reportedly has an anti-inflammatory effect that restores astrocytic electrophysiological properties in vitro [39]. According to a recent study, levetiracetam downregulates the expression of microglia and astrocytes in the spinal cord of mice with diabetic neuropathy induced by streptozotocin [40].

The availability of well-established animal models of DR encourages researchers to further investigate the incompletely elucidated pathogenesis of the disease [41–43]. Based on a previous work from our laboratory, we reported the beneficial use of levetiracetam in cases of diabetic neuropathy and its potential role as a neuroprotector [40]. The aim of the present study was investigate the use of levetiracetam as a modulator of glial activity and synaptic plasticity and studying its protective effect on delicate and vital retinal neuronal structures.

2. Materials and methods

2.1. Experimental animals

Male Swiss albino mice were obtained from Moustafa Rashed Company (weight 20 ± 3 g) and maintained in polyethylene cages in a clean animal room. Experimental conditions were set as temperature equals 24 ± 4 °C, normal light-dark cycle and continuously available food and water. The study protocols were approved by the Institutional Research Ethics Committee at the Faculty of Medicine, Suez Canal University (Ismailia, Egypt).

2.2. Experimental model of mouse type 1 diabetes mellitus

A single dose of alloxan (180 mg/kg, SD Fine Chem Ltd., India) was administered intraperitoneally (i.p.) [44–47]. Mice were left for one week, and fasted animals were tested for diabetes using automated blood glucose measurement (One Touch glucometer, USA) on blood obtained from the mouse tail. Diabetes in animals was confirmed by fasting blood glucose ≥250 mg/dl. Levetiracetam was given orally in 2 ml/kg volume. Pharmacological treatment with levetiracetam (dissolved in distilled water) started from the 10th week and continued till 15th week (end of the experiment).

2.3. Study design

Mice (n: 21) were allocated into three groups (n: 7 mice): (i) saline group: mice injected with saline; (ii) alloxan-diabetic control mice: injected with alloxan and received oral doses of distilled water (drug vehicle, 10 ml/kg) during the last six weeks; and (iii) alloxan-diabetic mice treated with levetiracetam (25 mg/kg) during the last six weeks. At the end of the 15th week, mice were anaesthetized by i.p. ketamine (80 mg/kg) [48,49] and sacrificed for collecting tissue samples for laboratory studies. Ocular tissues were also collected for histopathological studies, immunohistochemistry and RT-PCR measurements.

2.4. Histopathological examination

Retinal tissue obtained from the experimental groups was fixed in buffered formalin, embedded in paraffin wax and used for histopathological assessment. Sections of 5-μm thickness were deparaffinized, hydrated in graded alcohol and stained with HE. Sections were systematically inspected for morphologic changes. Next, morphometric analysis [50,51], including measuring the thickness of total retina, outer nuclear layer (ONL), inner nuclear layer (INL) and counting cells in the ganglion cell layer (GCL), was performed using an Olympus® CX21 microscope with a 400× lens. At least three sections per retina for each animal in the experimental groups were examined.

2.5. Immunohistochemistry staining

Formalin-fixed paraffin-embedded (FFPE) blocks of tissue were sectioned (4-μm thick), mounted on positively charged slides and processed for manual immunohistochemical staining. Retinal sections were incubated with 3% H2O2 for 10 min, washed with PBS for 5 min, and then incubated with primary antibodies (glial fibrillary acidic protein (GFAP), Thermo Scientific Cat. #RB-087-A0, USA, dilution 1:100), rabbit monoclonal anti-GAP43 (Abcam ab75810, UK, 1:500) and GLUT1 (Abcam ab115739, and dilution 1:250) at 4 °C overnight. Next, the sections were PBS-washed and 30 minute-incubated with biotinylated secondary antibodies (at room temperature). The staining was completed using a Power-StainTM 1.0 Poly horseradish peroxidase DAB kit (Genemed Biotechnologies, USA), and the sections were counterstained with haematoxylin.

2.6. Evaluation of immunohistochemical staining

The expression of GFAP and GAP43 in the astrocytic processes and retinal layers was assessed. Regarding GLUT 1 staining intensity, semi-quantitative scoring (0 to 3+) was performed in sections of retinae from the experimental groups based on the relative intensity of staining in the different retinal layers compared to reference structures in the eye with well-recognized GLUT1 expression [7]. At least three sections per retina for each animal in the experimental groups were examined. Sections were captured using a UIS optical system (Universal Infinity System, Olympus®, Japan).

2.7. Quantitative Real-Time Polymerase chain reaction

Briefly, isolation of RNA extracted from FFPE sections was implemented in accordance with the manufacturer's protocol (RNeasy FFPE Kit, Qiagen, Catalogue no. 217504). RNA conversion to complementary DNA was carried out according to a previous method [52]. Reactions were run in duplicate following the steps mentioned in a

previous publication [53] using specific ready-made TaqMan® assays with following manufacturer instruction (Applied Biosystems, assays ID Mm01731290_g1 for Tp53, Mm00440502_m1 for iNOS, Mm00476361_m1 for NF-κB, Mm00446190_m1 for IL-6, and Mm00443258_m1 for TNF-α) and compared to the endogenous control GAPDH (Applied Biosystems, catalogue no 402869). Fold change was calculated using the delta threshold cycle [54].

2.8. Statistical analysis

Statistical tests were performed using the SPSS program. The data showed a normal distribution and are expressed as the means ± SD. Statistical differences in numeric data showing a normal distribution were evaluated using one-way ANOVA followed by Bonferroni's post hoc test for comparing study groups. Data from scoring or quantitative data from PCR results are presented as medians and analysed by a non-parametric ANOVA and a post hoc test. The differences were considered significant when $p < 0.05$. To approve the statistical power of the experimental parameters after exclusion of dead animals, a post hoc power analysis was conducted using G*Power computer program (Version 3.1.9.4 © 1992–2019) [55].

3. Results

Animal survival during the experiment in both saline-treated normal and diabetic animals was 85% (6/7 animals) and 71.5% (5/7 animals) respectively. Dead animals were excluded from statistical analysis. To approve the statistical power of the experimental parameters after exclusion of dead animals, a post hoc power analysis was conducted using G*Power computer program (Version 3.1.9.4 © 1992–2019) [55]. Testing the As demonstrated in Fig. 1A, the retina from the saline-treated group showed normal structure. In comparison with the control group, the diabetic group showed morphologic changes in the retina such as marked disorganization of the retinal layers, angiogenesis and micro-aneurysms, degeneration and reduced numbers of ganglion cells (Fig. 1B). A mild improvement in the morphology was evident in the retinae of the treatment groups. Interestingly, the severity of the pathologic alterations varied between animals in the same experimental group. A difference in the total retinal thickness, INL thickness and ONL thickness was detected between the diabetic and control groups. The total retinal thickness was significantly different in the levetiracetam-treated group compared with that in the diabetic control group. However, INL and ONL thickness showed insignificant difference between the levetiracetam and diabetic control groups (Fig. 1D). Finally, the number of retinal ganglion cells demonstrated a decline in diabetic retinae compared to that in saline-treated retinae. Nonetheless, an increased number of retinal ganglion cells was detected after treatment with levetiracetam (Fig. 1E, p values < 0.05).

Immunohistochemistry images for sections stained for GLUT1 are illustrated in Fig. 2A–C. Retinae from saline-treated mice showed multicellular localization of GLUT1 expression, particularly in the NFL, GCL, photoreceptor cell bodies and RPE. Interestingly, more intense GLUT1 expression was noted in the aforementioned layers of retinae from the diabetic group, whereas the levetiracetam-treated group exhibited GLUT1 expression comparable to that in the normal group in both localization and intensity (p value < 0.05).

Fig. 3A–C shows images for GAP43-stained retinal sections that were apparent in the GCL, INL and IPL. Compared to saline-treated retinae, diabetic retinae showed a significant decline in GAP43 immunostaining in the GCL and IPL. However, compared with the diabetic

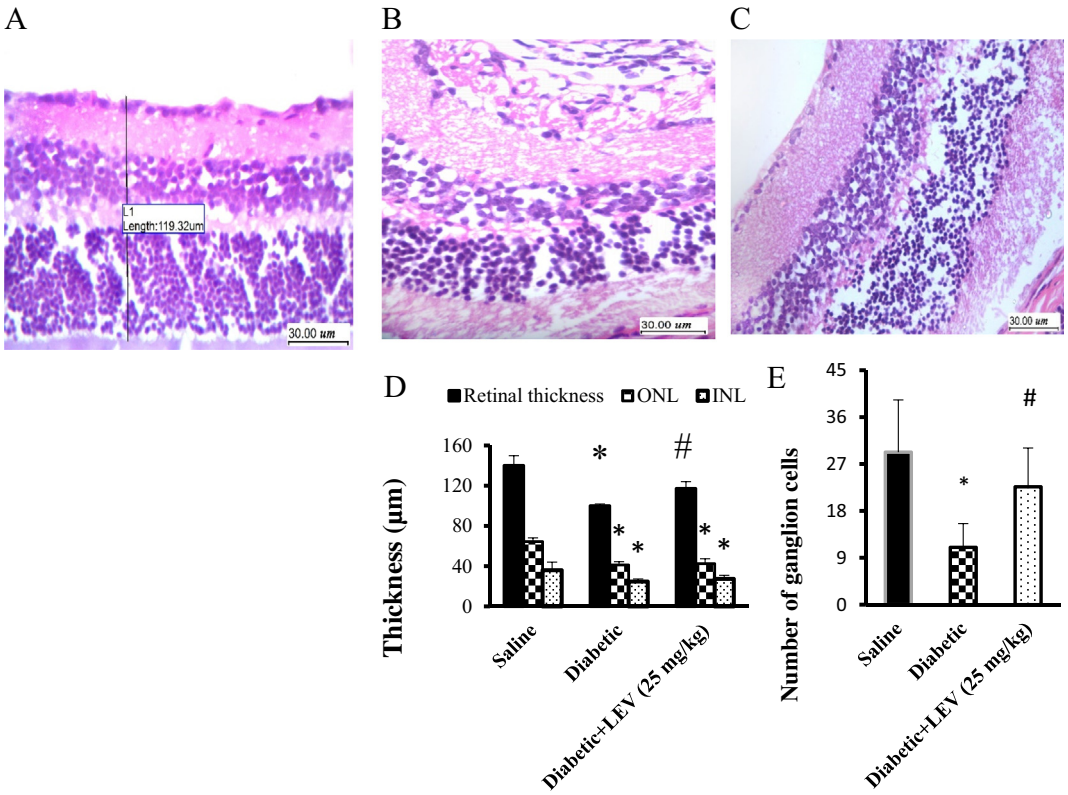

Fig. 1. Histopathological changes in the retina in the study groups. Images for sections from the retinae of experimental groups stained with HE. A: Control group showing a section of normal retina (400×). B: Diabetic group showing disorganization of the retinal layers, angiogenesis and microaneurysms at the ILM, gliosis and thickened NFL, degeneration and reduced numbers of ganglion cells compared to the control group (400×). C: Section from the diabetes + LEV (25 mg/kg) group showing gradual restoration of the retinal layers morphology and the total retinal thickness (400×). ILM: inner limiting membrane, NFL: nerve fiber layer, ONL: outer nuclear layer, INL: inner nuclear layer. D & E: Column charts showing mean ± SD for the measured parameters and analysis was done using one-way ANOVA and a *post-hoc* test. $p < 0.05$ was the accepted level of significance. *Versus saline, #versus diabetic mice.

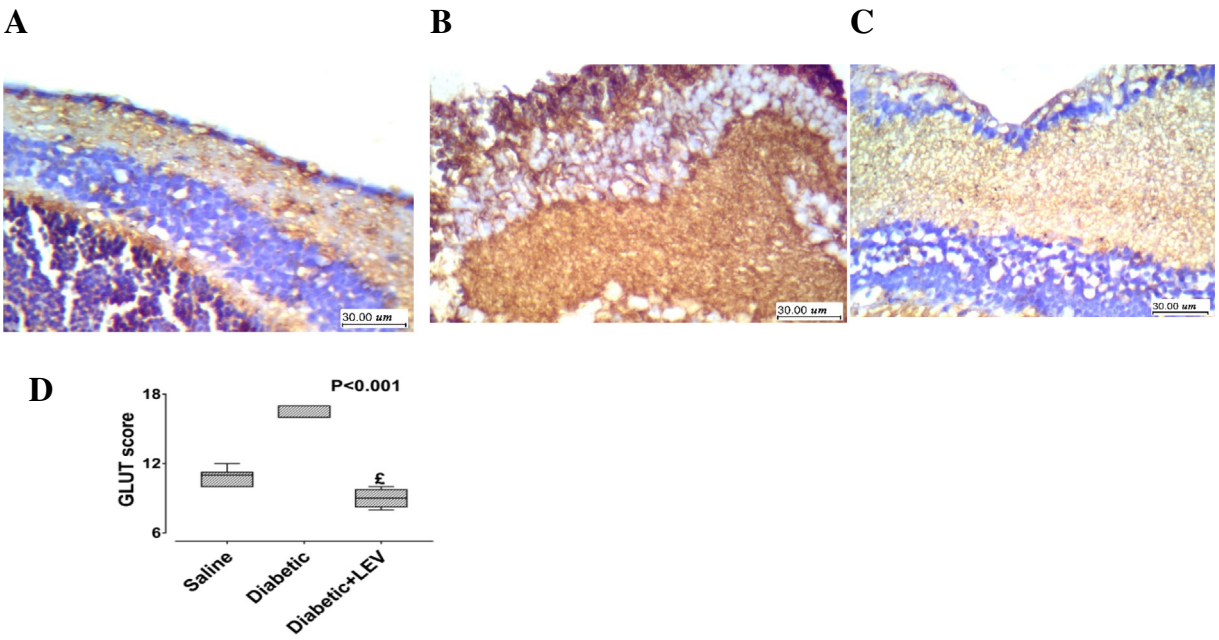

Fig. 2. Immunohistochemical staining for glucose transporter-1 in the retina of mice. A: Image from the saline group showing the staining intensity in the GCL and IPL of normal retina (400×). B: Image from the diabetic control group showing more intense staining in different layers (400×). C: Image from diabetic + LEV (25 mg/kg) group showing moderate staining intensity (400×). D: Showing area of staining in each group. Data are medians in box-plots and analysis was done using Kruskal-Wallis ANOVA and a post-hoc test. $p < 0.05$ was the accepted level of significance. $^{£}$Versus diabetic at $p < 0.05$.

mice, the levetiracetam-treated diabetic mice showed a significant increase GAP43 staining in both the GCL and IPL (Fig. 3D, p value < 0.05).

GFAP immunostaining is illustrated in Fig. 4A–C. GFAP expression was negligible in the NFL/GCL of retinae from the saline-treated group. Retinae of the diabetic group demonstrated a noticeable increase in GFAP expression mainly in the NFL/GCL, IPL and Müller cells spanning the whole thickness of the neural retina. In contrast, the immunostaining area was significantly decreased in mice treated with levetiracetam for 6 weeks (Fig. 4D, p value < 0.05).

In the diabetic animal model, retinal tissues displayed altered expression of inflammatory, oxidative and proliferative markers. Alloxan-diabetic mice exhibited higher mRNA expression of TNF-α, IL6, NF-κB, iNOS, and Tp53 than non-diabetic control mice did. However, in the alloxan-diabetic mice treated with levetiracetam, the relative expression levels of genes were significantly less than those in the alloxan-

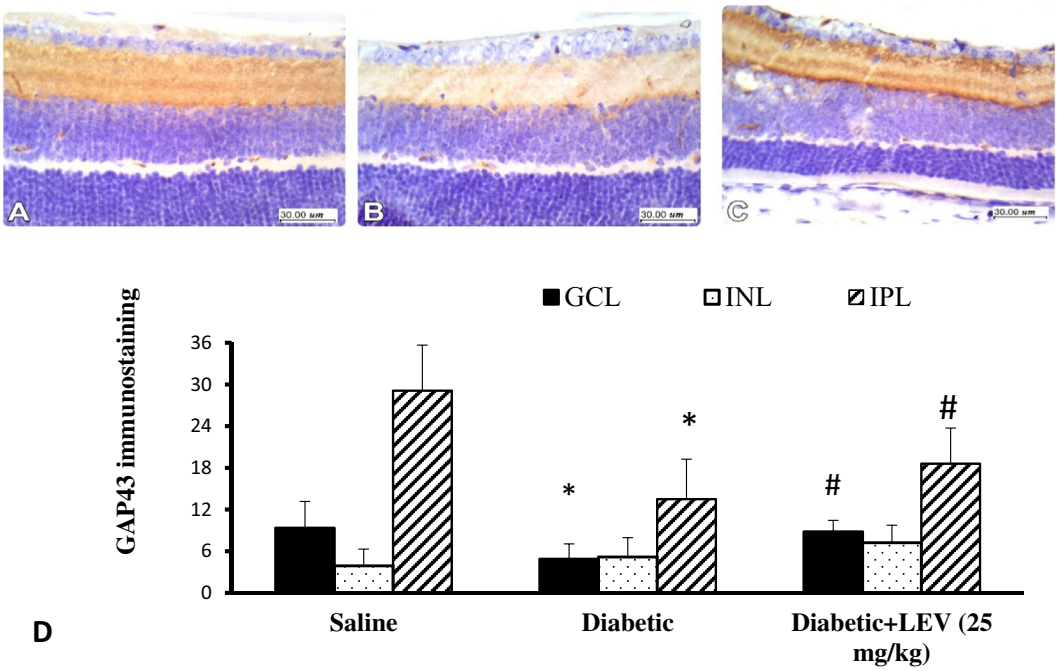

Fig. 3. Immunohistochemical staining for growth associated protein 43 in the retina of mice. A: Image from the saline group showing high staining in the GCL and IPL (400×). B: Image from the diabetic control group showing lower staining in GCL and IPL (400×) C: Image from diabetic + LEV (25 mg/kg) group showing increased staining (400×). D: Column chart showing mean area for staining in the different layers in each group. Data are mean ± SD and analysis was done using one-way ANOVA and a post-hoc test. $p < 0.05$ was the accepted level of significance. *Versus saline, $^{\#}$versus diabetic.

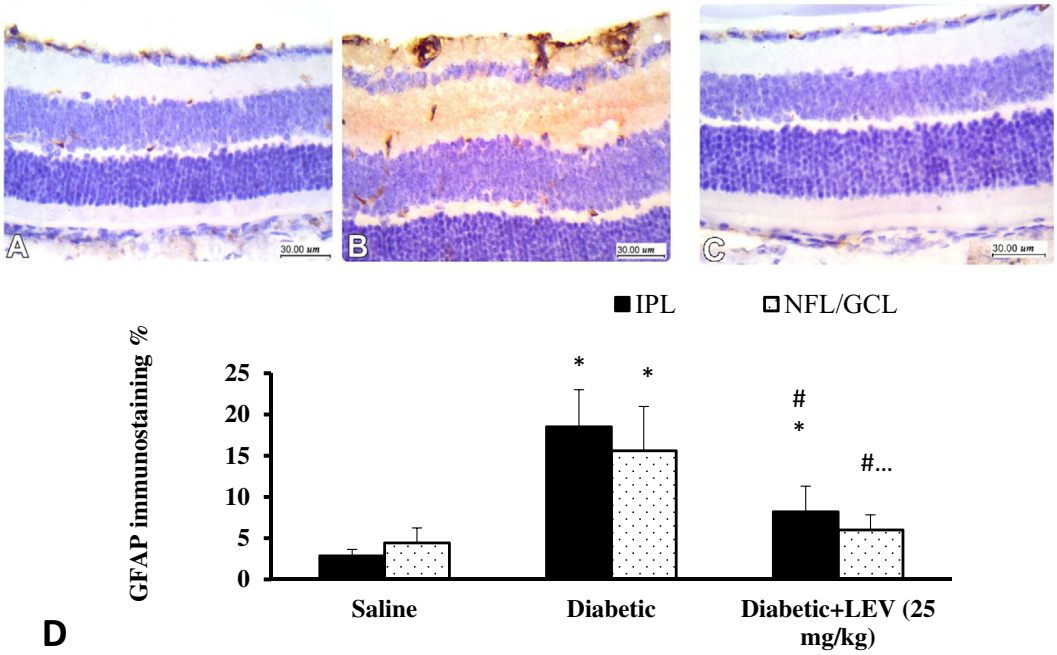

Fig. 4. Immunohistochemical staining for glia fibrillary acidic protein in the retina of mice. A: Image from the saline group showing low staining in the NFL/GCL and IPL (400×). B: Image from the diabetic control group showing greater staining in the NFL/GCL, IPL and Muller Cells (400×). C: Image from diabetic + LEV (25 mg/kg) group showing decreased staining (400×). NFL: nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer. D: Column chart showing mean area for staining in the different layers in each group. Data are mean ± SD and analysis was done using one-way ANOVA and a post-hoc test. $p < 0.05$ was the accepted level of significance. *Versus saline, # versus diabetic.

diabetic control mice. The data reported in the alloxan-diabetic control group showed high variability and distribution which required a non-parametric statistical analysis and presentation using box plots (Fig. 5).

4. Discussion

Visual impairment and blindness are the disabling sequalae of DR, which accounts for 2.6% of cases with blindness [3]. DR is a slowly progressing disease that requires several years for clinical presentation [56]. Maintaining good control of blood glucose levels, serum lipids and systemic blood pressure is useful in reducing the incidence and hindering DR progression [6,56–58]. However, the available therapeutics for DR are limited and involve intravitreal administration of corticosteroids as effective anti-inflammatory agents.

Improving glycaemic control with insulin or oral antidiabetic drugs reportedly inhibits the early stages of DR [59,60], but blood glucose control remains difficult to achieve with many diabetic patients. Therefore, there are substantial efforts to characterize certain pharmacological agents for inhibiting the development of retinopathy. Some therapeutic agents, such as antihypertensive medications [61,62] and fibrates [63], are beneficial in managing DR in some patients with diabetes. Therefore, the existing therapeutics for DR are not equally effective in all diabetes mellitus patients [64].

Hyperglycaemia-induced oxidative stress and low-grade inflammatory changes are the cornerstone initiatives of the pathogenesis of DR [65–67]. The neural and vascular retina maintains the integrity of the eye, including retinal health and functionality. In diabetes, an increase in glucose trafficking into retinal tissues results in oxidative stress through metabolic pathways (accumulation of advanced glycation end products) [65,66]. Consequently, retinal neuronal apoptosis and glial cell activation are earlier pathogenic stages of DR [68–70]. Among pathologic features, glial cell activation with the resultant expression of proinflammatory and inflammatory cytokines [8,71–73]. The present study aimed to investigate the potential role of levetiracetam as a neuroprotective agent in a mouse model of alloxan-induced diabetes.

In the current study, Alloxan-induced diabetes model was used, which is a valid animal model of type-I diabetes mellitus and its microvascular complications [44–47]. The data obtained from the retina of diabetic mice showed extensive retinal morphometric changes, mainly comprising damaged and debilitated retinal layers, thickened NFL, degenerative changes in the GCL, and angiogenesis and microaneurysm formation in the ILM. In support of our data, studies on diabetic rats showed a higher vulnerability of GCL to degenerative changes in DR [50,65]. GCL vulnerability can be attributed to two pathways: (i) ganglion cell mitochondrial stress and enhanced cytochrome-c/caspase apoptotic pathway [65] and (ii) Müller cell-secreted FasL, which acts on FasR expressed on ganglion cells, enhancing apoptosis [71].

Interestingly, in our study, GLUT1, which facilitates retinal hyperglycaemia in favour of DR pathogenesis, was upregulated to non-significant levels in diabetic retinae [7,74,75]. Accordingly, GLUT1 inhibition has been reported as a potential therapeutic strategy in many studies [76,77]. In contrast, other studies have reported down-regulation of GLUT1 expression in animal models of DR [78–80]. This discrepancy may be attributable to differences in the experimental model, species or technique of measurement.

GAP43 is localized to axonal growth cones, involved in operational synaptic plasticity of the nervous system [81] and is downregulated in diabetic rats [26]. There is evidence supporting the activating role of GAP43 in formation and reconstruction of synapse [82]. Neuronal GAP43 is a presynaptic membrane phosphoprotein that guides axonal growth and modulation of the formation of new connections [83] and is reportedly ameliorated in neurodegenerative animal models of epilepsy [84] and the hippocampus of diabetic rats [85]. Another gap junction protein, connexin-43, reportedly plays a role in the maintenance of retinal vascular homeostasis in DR. Indeed, high glucose induced downregulation of connexin-43 expression in human retinal pericytes [86] and diabetic human eyes [87].

The current results revealed greater GFAP staining in diabetic

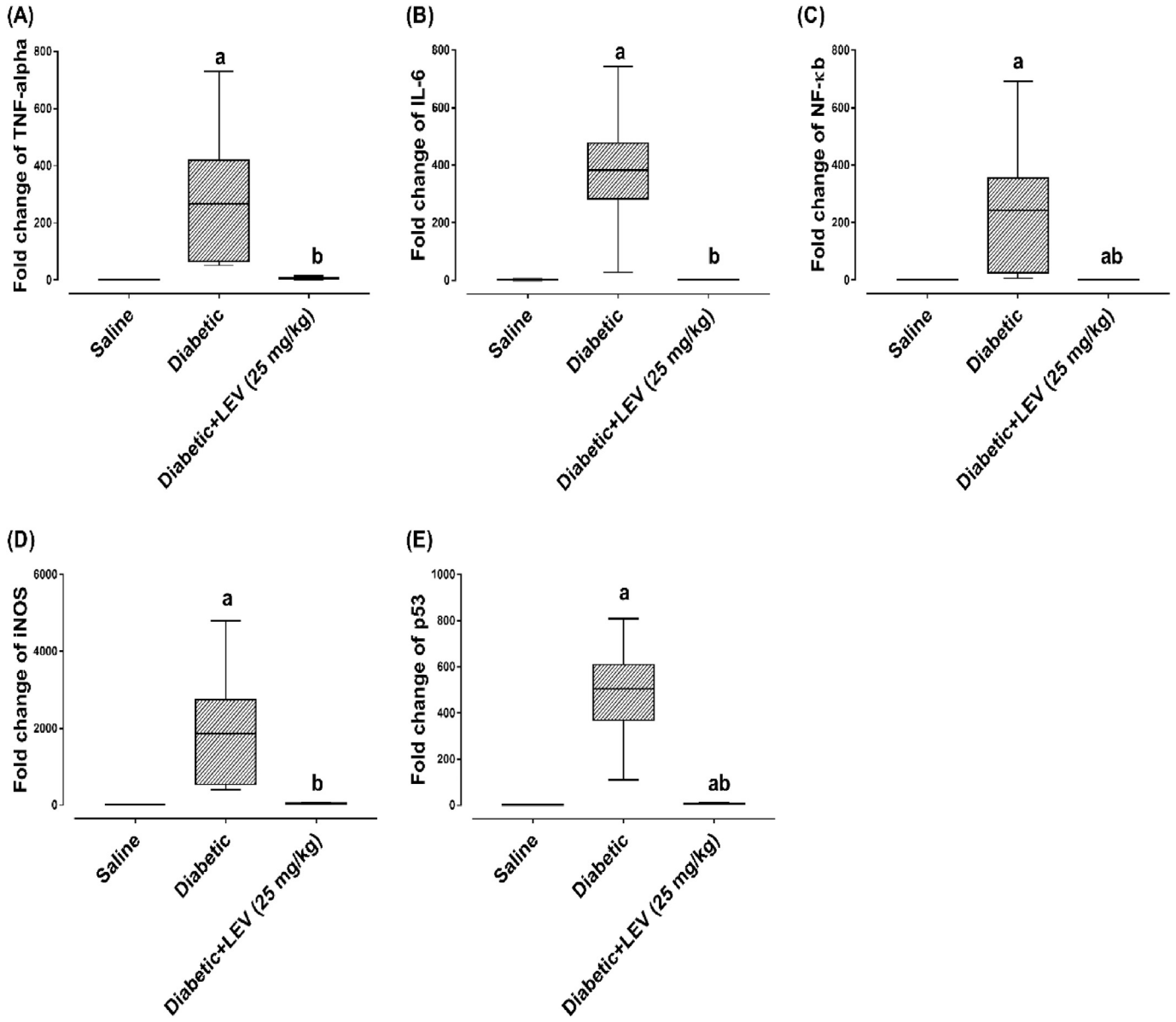

Fig. 5. Polymerase chain reaction for cytotoxic markers in the retina of mice. A: TNF-α. B: IL-6, C: NFκB, D: iNOS and E: Tp53 in each group. Data are medians in box-plots and analysis was done using Kruskal-Wallis ANOVA and a post-hoc test. $p < 0.05$ was the accepted level of significance. *Versus saline, #versus diabetic.

retinae, implicating a pathogenic role for GFAP in DR. Similar results were obtained in animal models of DR, and downregulation of GFAP was considered a protective mechanism [88–90]. Our data also showed the upregulation of Tp53, which is an anti-apoptotic transcription factor induced in retinal cells that preserves glial cells for immune-mediated clearance of tissue debris [15–19,71]. Additionally, our immunohistochemistry staining indicated an inflammatory response mediated by NF-κB, IL6 and TNF-α. The pivotal role played by inflammation in the pathogenesis and deterioration of DR has been exploited in clinical studies [72,91]. DM increases the release of inflammatory cytokines, including TNF-α and IL-1β, in brain tissues and plasma [92,93]. In general, inflammatory factors contribute to accelerated neurodegeneration [94–99]. The increased levels of IL-1β and TNF-α can lead to the initiation and maintenance of numerous harmful brain changes, with prominent changes in the synaptic architectures and functionalities [100–104].

Using levetiracetam in DR in the current model study showed an improved cytokine profile with amelioration of the inflammatory status. Levetiracetam possesses anti-inflammatory activity mainly via reducing the expression of inflammatory cytokines IL6, TNF-α and IL-1β [105]. Recently, Abed El-Gaphar et al. reported that levetiracetam's anti-inflammatory activities are mediated via inhibition of the JAK/STAT pathway [106]. Concurrently, the expression of the apoptotic marker Tp53 and iNOS are also downregulated in levetiracetam-treated mice. This change was obviously reflected by the enhanced and regenerated histopathology of different retinal layers, especially the GCL and INL.

Furthermore, levetiracetam downregulated the neuronal stress indicator and microglial activation marker GFAP [107]. Nonetheless, levetiracetam upregulated the neuronal regeneration marker GAP43 [82]. Additionally, in a previous study, levetiracetam exerted neuroprotective effects that were characterized and reported in the context of diabetic neuropathy [40]. GFAP and GAP43 serve as markers of efficacy for DR therapeutics in different studies [73,108,108].

Levetiracetam acts primarily through the inhibition of SV2A. SV2A is a 12-transmembrane protein with sequence homology to GLUT at its N-terminal domains [38]. Our data showed downregulation of GLUT1 expression in the retinae of mice treated with levetiracetam. This

finding may highlight an additional role of levetiracetam in retinal glucose levels and render the retina almost normoglycaemic, but this assumption still requires further investigation.

In conclusion, our findings show the promising potential of levetiracetam in cases of DR. The paucity of non-invasive therapeutic modalities may emphasize the importance of further investigating the clinical efficacy and safety of levetiracetam in cases of diabetic microvascular complications. The effect of levetiracetam in this experimental model of DR was mainly exerted through its anti-inflammatory and neuroprotective role.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

None.

Acknowledgments

Levetiracetam was kindly provided by Multi Apex Pharmaceutical Company in Cairo, Egypt. In addition, the authors acknowledge the Egyptian Knowledge Bank (EKB), which supported manuscript language revision through Springer-Nature language-editing services.

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