Experimental Studies

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The state of S100-positive glia and the effect on it on the GABA-benzodiazepine receptor agonist, carbacetam, in diabetic retinopathy

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Purpose: *To determine the state of S100-positive glia and the effect on it of the gamma-aminobutyric acid (GABA)-benzodiazepine receptor agonist, carbacetam, in early experimental diabetic retinopathy (DR).*

Material and Methods: *DR was induced in 30 Wistar male rats (age, 3 months) by single intraperitoneal injection of 50 mg streptozotocin (STZ)/ kg body weight. These were assigned to two treatment groups (30 units of insulin only and 30 units of insulin plus carbacetam at a dose of 5 mg/ kg body weight in 0.5 mL of saline, respectively) and a control group of untreated diabetic rats, of 10 animals each. In addition, five intact animals were used to assess the retina at baseline conditions. Mouse anti-S100 protein monoclonal antibodies (Thermo Fisher Scientific) were used for immunohistochemical studies.*

Results: *In the intact retina, there was S100-positive staining in multiple polymorphous cells in the inner nuclear layer (INL) and glial fibers surrounding ganglion neurons like a collar. The intensity of S100 staining of Müller cells significantly increased, and their long radial processes were clearly visualized in early untreated DR. Particularly, intensive staining was observed in the outer nuclear layer (ONL), at the junction of this layer with the rod and cone layer, and in the ganglion layer. On day 28 of the experiment, microaneurysms were observed in the inner retina, with high S100 staining intensity in fibers which adhered to them. Animal treatment with insulin only resulted in a reduction in S100 staining intensity, whereas animal treatment with insulin plus carbacetam inhibited S100 protein expression and prevented the development of retinal microaneurysms, with only solitary Müller cells in the INL and the plexus in the ganglion cell layer exhibiting weak staining.*

Conclusion: *The GABA-benzodiazepine receptor agonist, carbacetam, was found to have an inhibiting effect on the expression of S100 protein and formation of retinal microaneurysms in early DR associated with STZ-induced diabetes in rats.*

Keywords

diabetic retinopathy, neurodegeneration, benzodiazepine receptors, immunohistochemistry, streptozotocin, Müller cells, S100 protein, retina

Introduction

Diabetes mellitus (DM), when poorly controlled or untreated, can lead to numerous serious diabetic complications, such as diabetic retinopathy (DR), neuropathy, nephropathy, etc. [1]. DR affects almost one-third of diabetics and remains the leading cause of blindness in working-age adults [2].

Classically, the disease refers to progressive retinal microvascular changes leading to tissue ischemia, increased permeability, neovascularization, and macular edema [3]. According to recent studies, DR is not only a diabetic microvascular complication, but also a neurodegenerative disease with a complex multifactorial pathogenesis [4]. Early Treatment Diabetic Retinopathy (ETDRS) grading is based on the microvascular changes in the posterior pole as imaged by seven-field color fundus photography and does not take into account neurodegenerative lesions that can develop before the onset of vasculopathy [5]. Impaired axonal transmission and merabolic pathways, loss of energy due to neuronal mitochondrial damage, protein misfolding, neuroinflammation, viral mutations, DNA mutations and other factors have been reported to facilitate the development of neurodegenerative disorders [6]. Neuronal dysfunction in DR is an independent patophysiological mechanism that evolves simultaneously with angiopathy [7].

The excitotoxicity of excessive glutamate, the most important neurochemical mechanism in DR, increases metabolism and induces apoptosis via activation of the caspase cascade by overloading cells with calcium [8]. Glutamate-mediated excitatory synaptic transmission and gamma-aminobutyric acid (GABA)-mediated inhibitory synaptic transmission are important for maintaining the excitatory/inhibitory balance in the nervous system [9]. GABA receptor subunits have been found in all retinal neurons, including amacrine and ganglion cells [10]. Reduced activity of retinal GABAergic receptors was found under diabetic conditions, and GABA-A receptor agonist induced retinal ganglion cell death [11].

S-100 calcium-binding proteins are members of a heterogenous family of proteins able to buffer intracellular Ca2+ ion concentration [12]. They regulate proliferation, differentiation, apoptosis, inflammation and migration of immune cells. Thus, S-100 proteins enhance and macrophage infiltration in a mouse model of DR. In addition, the findings of a study by Lim and colleagues [13] suggested that plasma A100A8 and S100A9 levels are correlated with the progression of DR in T2DM patients.

Given the established deficit of GABA-ergic mediation in DR, it seems promising to utilize GABA receptor agonists (e.g., benzodiazepines) to remedy this deficit in DR [14]. Carbacetam, a β-carboline derivative and a carboline isostere (1-oxo-3,3,6-trimethyl-1,2,3,4-tetra-hydro-indolo[2,3-c] quinolin), is a benzodiazepine which was developed at the Institute of Physical Organic and Coal Chemistry of the National Academy of Sciences of Ukraine. The preparation has anti-amnestic, anti-xiolytic, anti-hypoxic, anti-edematous and anti-shock effects and may be considered a promising neuroprotector [15, 16].

The purpose of the study was to determine the state of S100-positive glia and the effect on it of the GABA-benzodiazepine receptor agonist carbacetam in early experimental DR.

Material and Methods

All animal experiments were performed in compliance with EU 2010/63 Directive, Helsinki Declaration, and the Law of Ukraine on Protection of Animals from Cruel Treatment No. 3447-IV dated February 21, 2006, as amended on August 8, 2021. Animals were maintained under vivarium conditions and fed on a standard diet.

DR was induced in 30 Wistar male rats (age, 3 months; weight, 140-160 g) by single intraperitoneal injection of 50 mg streptozotocin (STZ, Sigma-Aldrich, Shanghai, China)/ kg body weight [17]. These were assigned to two treatment groups and a control group of untreated diabetic rats, of 10 animals each. The controls received 0.5 mL of saline, treatment group 1 received 30 units of insulin (Actrapid HM Penfill, Novo Nordisk A/S, Bagsvaerd, Denmark) only, and treatment group 2 received 30 units of insulin plus carbacetam at a dose of 5 mg/ kg body weight in 0.5 mL of saline. Insulin only or insulin plus carbacetam were administered intraperitoneally beginning on day 7 after STZ injection and continuing every other day for 28 days. In addition, five intact animals were used to assess the retina at baseline conditions.

Carbacetam was synthesized at Department of Biologically Active Compounds, the Litvinenko Institute of Physical Organic and Coal Chemistry of the National Academy of Sceinces of Ukraine, headed by S.L. Bogza, Dr Sc (Chem), Senior Researcher.

Tail vein blood samples were taken to assess fasting blood glucose levels using Accu-Chek Instant Test Strips (Accu-Chek, Roche, Mannheim, Germany) and an Accu-Chek Instant blood glucose meter. In all groups, blood glucose levels were consistently high throughout the follow-up period. At day 28, mean blood glucose levels for the controls and treatment groups 1 and 2 were 29.32 ± 1.25 mmol/L, 17.02±1.03 mmol/L and 14.38±1.25 mmol/L, respectively, with no significant difference ($p < 0.05$) between the treatment groups and controls.

Animals were euthanized with an overdose of thiopental (75 mg/ kg body weight) and decapitated on days 7, 14 and 28 after STZ injection. The globes were fixed with 10% neutral buffered formalin and paraffinized. Paraffin blocks were sectioned serially at a thickness of 2-3 μm on a rotary microtome HM 325 (Thermo Shandon, Runcorn, UK). Mouse anti-S100 protein monoclonal antibodies (Thermo Fisher Scientific, Waltham, MA) were used for immunohistochemical studies. Sections were stained with hematoxylin. Microscopy was performed and images were collected on an optical microscope (ZEISS Axio Imager A2; Carl Zeiss Microscopy Deutschland GmbH, Oberboken, Germany). Investigated slides were assigned a score of 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining) [18].

Statistical analyses were conducted using Statistica 10.0 software (StatSoft, Tulsa, OK, USA). Data are presented as mean and standard deviation. Exact Fisher test and analysis of variance (ANOVA) were used to comparisons of mean sample values. The level of significance $p < 0.05$ was assumed.

Results

The morphology of the intact retina was significant for a clear distinction between layers and immunospecific staining of S100-positive cells and cell processes, with these cells and processes having a staining score of 2-3 (Fig. 1; Fig 2. a, b). The polymorphic cells of the retinal inner nuclear layer (white arrows in Fig. 1) were the most intensively stained and morphologically similar to Müller cells, amacrine and bipolar cells, and horizontal neurons [12]. Only the proximal portions of the processes of these cells were positively stained. Glial cells surrounding ganglion neuron bodies (black arrows in Fig. 1) like a collar were also intensively stained.

At day 7 in the control group, intensive positive staining was still present only in the cells located in the inner nuclear layer, mostly in the middle of this layer (white arrows in Fig. 2c). These cells were morphologically similar to Müller cells. In addition, staining was marginal

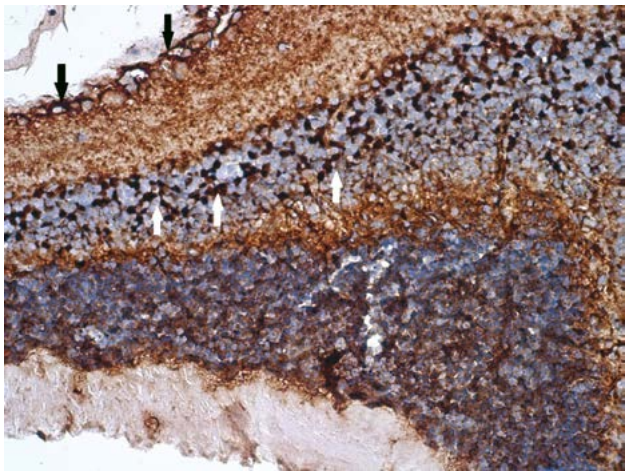

Fig. 1. Photomicrograph of representative results of immunohistochemistry staining for S100 protein in an intact rat retina preparation. Additional hematoxylin staining. Microscopic magnification, x400.

Note immunopositive staining of glial fibers in the ganglion cell layer (black arrows) and multiple polymorphous S100-positive cells in the inner nuclear layer.

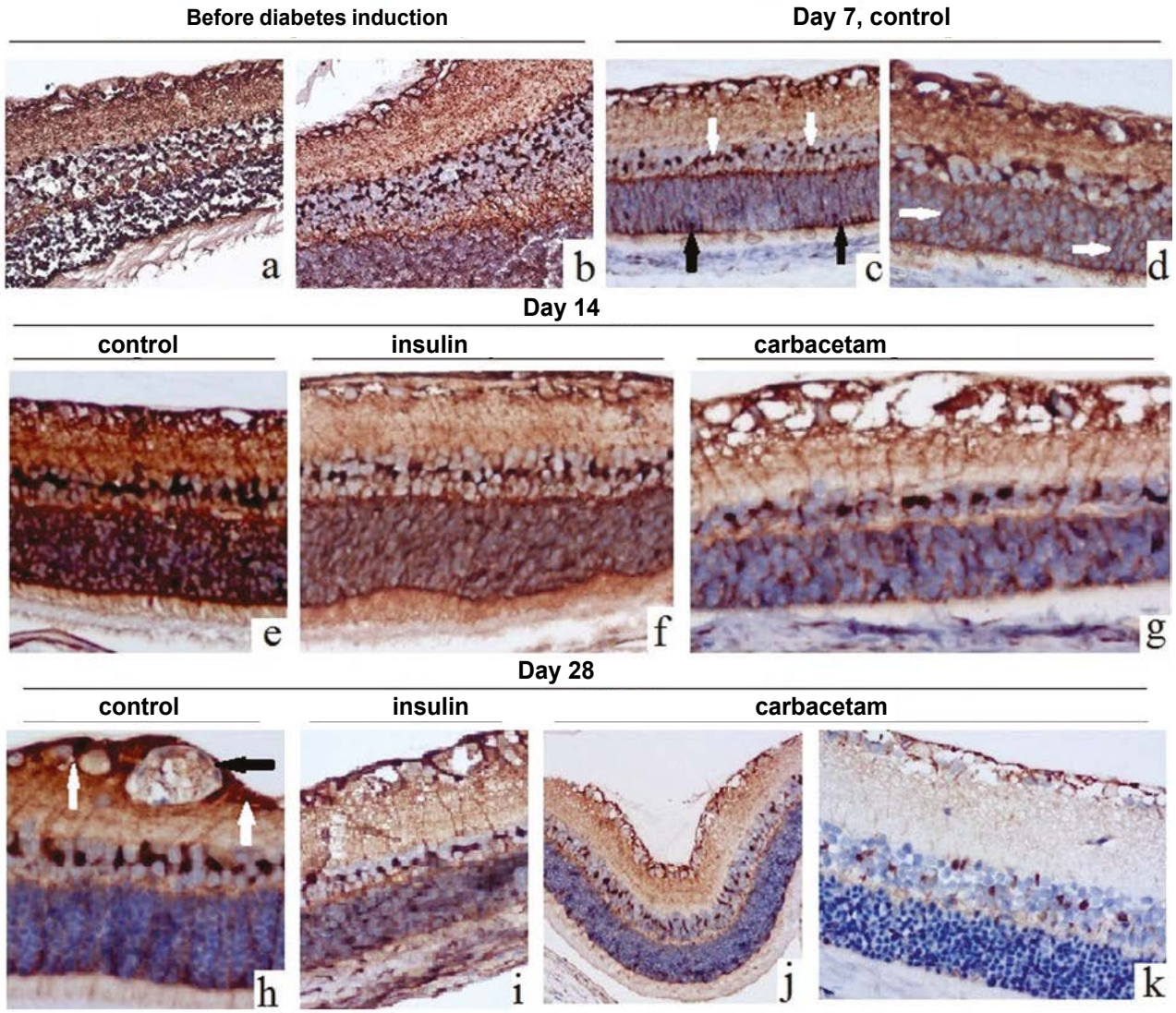

Fig. 2. Photomicrographs of representative results of immunohistochemistry staining for S100 protein in rat retina preparations made before diabetes induction (a, b) and on day 7 (c, d), day 14 (e, f, g) and day 28 (h, i, j, k) . Additional hematoxylin staining. Magnifications, x200 (a, c, j) and x400 (b, d, e, f, g, h, i, k). Note S100-positive cells in the inner nuclear layer (white arrows) and S100-positive portions of retinal pigment epithelial cells extending to the outer nuclear layer (black cells) in c; radial glial fibers in the outer retinal layers (white arrows) in d; and microaneurysms on the inner retinal surface (black arrow) and swirling of S100-positive glial fibers around aneurysms (white arrows) in h

in portions of some retinal pigment epithelium (RPE) cells extending into the outer nuclear layer (black arrows in Fig. 2c). Intensive staining was present on Müller cell processes as they traversed the outer nuclear layer (white arrows in Fig. 2d).

At day 14 in the control group, the staining intensity was maximal, with the cells having a staining score as high as 4 (Fig. 2e). Intensive staining was observed in the outer nuclear layer, at the junction of this layer with the rod and cone layer, and in the ganglion layer. At this time point, a general reduction in staining intensity was characteristic for the retina of the animals treated with insulin only as well as those treated with insulin plus carbacetam (Fig. 2f,g). Some Müller cells, filaments and solitary polygonal cells with processes in the outer retina exhibited a staining score of 1 to 2.

At day 28, in the control group, pathological angiogenetic lesions, microaneurysms (black arrow in Fig. 2h) with enlarged vessels appeared, sometimes with multiple gaps between them, arranged closely and enclosed inside a thick perivascular membrane, which corresdponded to nonproliferative DR (microangiomas and punctuate hemorrhages [12]). Intensively stained S100-positive processes (white arrows in Fig. 2h) were located closely to microaneurysms. Staining was less intensive in the retina of animals treated with insulin (Fig. 2h) compared to controls, with the morphology generally similar to the previous time point: there were intensively stained large cells with processes in the inner nuclear layer, radial cell processes of the outer plexiform layer, and solitary cells and twisted filaments in the outer nuclear layer.

The least intensive staining was in the retina of animals treated with carbacepam (Fig. 2j,k). Solitary Müller cells in the inner nuclear layer and fibers in the plexus in the ganglion cell layer exhibited a staining score of 1 to 2. In addition, no aneurysms were seen in the inner retina of animals treated with carbacepam.

The calculation of the distribution of inner nuclear layer cells with different S100 staining intensity scores on day 28 confirmed the obtained results (Fig. 3).

Most (85 ± 6.4%) inner nuclear cells in the retina of control animals had a staining score of 2 to 3, whereas most (66 ± 4.8%) inner nuclear cells in the retina of animals treated with insulin had a staining score of 1 to 2, and most (57 ± 3.6%) inner nuclear cells in the retina of animals treated with carbacetam had a staining score of 0 to 1 ($p < 0.05$).

Therefore, the development of early DR is characterized by an increase in the intensity of staining for S100 protein in Müller cells in the inner nuclear layer, the nerve fiber plexus in the ganglion cell layer, and radial fibers in the outer retinal layer and RPE layer. The use of insulin only reduced these manifestations in diabetic rat retina, whereas

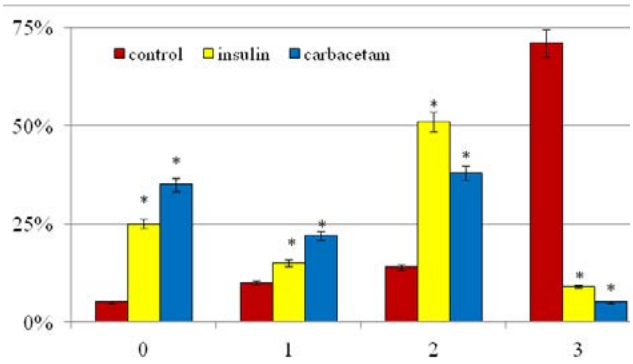

Fig. 3. Histogram for the distribution of inner nuclear layer cells with different S100 staining intensity scores in the control group versus groups treated with insulin only and insulin plus carbacetam on day 28.

Note: *, significant difference compared with controls ($p < 0.05$)

the use of insulin plus carbacetam prevented the activation of S100-positive retinal glia in diabetic rat retina.

Discussion

Chronic hyperglycemia, oxidative stress and accumulation of advanced glycation end products (AGEs) and glutamate are major mechanisms of retinal nerve cell damage leading to the development of diabetic retinal neurodegeneration [5, 19]. The hallmarks of diabetes-induced neuroglial degeneration, which include reactive gliosis, diminished retinal neuronal function and neural-cell apoptosis, have been observed to occur before overt microangiopathy in experimental models of diabetic retinopathy and in the retina of diabetic donors [20]. When assessed with eletroretinography, neurons of all retinal layers (ganglion, bipolar, amacrine and photoreceptor cells) exhibited functional changes well before retinal microangiopathy was detected by fundus photography [20].

Cellular pathology in DR involves at least nine types of retinal cells, including photoreceptors, horizontal and bipolar cells, amacrine cells, retinal ganglion cells, glial cells (Müller cells, astrocytes, and microglia), endothelial cells, pericytes, and RPE cells [4]. Of the several mechanisms associated with glial damage, the most important is glutamate excitotoxicity, which increases metabolism and induces apoptosis via activation of the caspase cascade. A trigger of this damage is the accumulation of intracellular calcium, which allows to consider the activation of expression of calcium-binding proteins as a reflection of protective mechanisms as well as a marker of calcium overload of neural cells [21].

In the current rat study, we found specific features of the distribution of S100 protein expression in the retina at baseline (before inducing diabetes). The intensive staining of multiple polymorphous cells in the inner nuclear layer confirmed the constitutive nature of the expression of S100, the protein which is involved in the regulation of neural cell functions [12]. Only the proximal portions of

the processes of these cells were positively stained, which indicated mostly somatic localization of S100.

It is likely that the intensively stained nerve fibers that formed a collar surrounding ganglion cells were of astrocyte origin. In response to metabolic stress under hyperglycemic conditions, astrocytes produce proinflammatory interleukins, chemokines, cyclooxigenase-2, and multiple growth factors which are involved in the activation of microglia and recruiting monocytes/macrophages, T-cells and dendritic cells, thus contributing to inflammation [22]. Retinal astrocytes normally express glial fibrillary acidic protein (GFAP), and GFAP expression in astrocytes is significantly higher than in Müller cells [23].

In our previous rat study on STZ-induced early DR [24], at baseline, both individual GFAP-positive cells and their processes were found only in the retinal ganglion cell layer and retinal nerve fiber layer, and individual scattered GFAP-positive cells (likely, Müller cells) alone were found in the retinal inner nuclear layer. With DR progression, clear GFAP-positive radial fibers appeared, which were seen running through the retinal inner plexiform layer to the outer nuclear layer, were numerous GFAP-positive cells with processes were seen. It is likely that this description reflects the changes in the involvement of Müller cells: normally, GFAP is weakly expressed in these cells, but its expression in them increases in diabetes [25].

Unlike GFAP, the S100 protein had a rather high level of expression in retinal neural cells in normal rats. Subsequent DR development was accompanied by overexpression of both GFAP and S100 in the retinal inner layers. This reflected their excessive activation and, given that they likely produced proinflammatory and growth factors (an overexpression of vascular endothelial-derived growth factor under conditions of this study has been reported previously [26]), it was possible that this was the cause of the early development of microaneurysms on the inner retinal surface found in the control group at day 28.

Throughout the experiment, in the control group, an increase in S100 expression was most pronounced in Müller cells, large neurons in the retinal inner nuclear layer and their clearly seen transretinal processes. The spread of S100-positive staining on long radial processes indicated a significant calcium overload and corresponded to the activation of retinal apoptosis [24].

The increase in S100-positive staining in Müller cells can be explained also by models of retinal glutamate excitotoxicity in diabetes [27]. DM alters the glutamate-glutamine balance between glian cells and neurons through reduced activity of glutamine synthetase in Müller cells, which not only hampers the capacity of these cells to convert the excess of glutamate into glutamine, but also impairs glutamate oxidation to alpha-katoglutarate, leading to extracellular glutamate accumulation. Glutamate transporters are involved in maintaining extracellular glutamate at a low level to protect neurons from excitotocic damage. Glutamate/aspartate transporter (GLAST), the main glutamate transporter expressed by Müller cells, accounts for at least 50% of glutamate uptake in the mammalian retina [28].

Of note is the disappearance of multiple polymorphic S100-positive cells in the inner nuclear layer in the development of DR. It has been reported on focal degeneration of amacrine cells with reduced amplitude in electroretinographic oscillatory potentials in diabetes [29]. We have previously demonstrated the activation of apoptosis of the cells in this retinal layer [30]. Therefore, reduced activity and possibly death of these cells occurred already in early stages of experimental DR.

Upregulation of glutamate receptors' [N-methyl-D-aspartate receptor (NMAR)1 and GluE2/3] immunoreactivities was observed in the gangion, amacrine and bipolar cells as well as in the inner and outer plexiforma layers in STZ-induced diabetes in rats [31]. Immunoreactivity of calcium-binding proteins (calbindin and parvablumin) was also concomitantly increased.

This could explain the activation of S100 expression in the form of marginal staining of the RPE cells extending to the junction of the outer nuclear layer and rod and cone layer. It is likely that the activation of S100 expression in the RPE cells reflected a compensatory response to calcium accumulation in photoreceptors.

It was demonstrated in the current study that the restoration of GABA-ergic mediation of the state of S100-positive glia through the use of the benzodiazepine receptor agonist, carbacetam, can be utilized as an option for pathogenetic correction of diabetic neuronal dysfunction. The use of both insulin and carbacetam reduced S100 expression in the cells of the inner nuclear layer and radial processes of Müller cells.

The protective effect of carbacetam was most pronounced at day 28, with solitary Müller cells in the outer nuclear layer and nerve fiber plexus in the ganglion cell layer exhibiting a staining score of 1 to 2. Apparently, the use of carbacetam prevented pathogenetic processes leading to neurodegeneration in DR. This was indicated by the absence of aneurysms in the inner retinal layer in the eyes of animals treated with carbacetam. These findings add to our previous reports [24, 26], highlighting the value of S100-positive glia and demonstrating the positive effect of carbacetam in diabetes.

The use of GABA analogs has been acknowledged as an approach for the prevention of diabetic neuropathy: pregabalin, a structural derivative of GABA, was the first drug to receive Food and Drug Administration (FDA) approval for treating diabetic neuropathic pain [32]. A study by Ali and colleagues [32] demonstrated that pregabalin improved GABAergic control and alleviated retinal neuroinflammation, apoptosis and oxidative stress. In our study, carbacetam demonstrated the properties similar to pregabalin. Therefore, the restoration of GABA-ergic mediation is a sound pathogenetic approach to the management of diabetic retinal neuronal dysfunction.

By this way of reasoning, we conclude that, in early DR under conditions of STZ-induced diabetes, S100 staining

intensity significantly increased in Müller cells, with clear visualization of their long radial processes. At day 28 of the experiment, microaneurysms were observed in the inner retina, with high S100 staining intensity in fibers which adhered to them. Animal treatment with insulin only resulted in a reduction in S100 staining intensity, whereas animal treatment with insulin plus carbacetam inhibited S100 protein expression and prevented the development of retinal microaneurysms.

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Disclosures

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Disclaimer. *The opinions presented in this article are those of the authors and do not necessarily represent those of their institutions.*

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