https://doi.org/10.31288/oftalmolzh202514753

Effect of receptor protein kinase inhibition on the morphogenesis of experimental diabetic retinopathy

Usenko K. O.

Bogomolets National Medical University

Kyiv (Ukraine)

Purpose: *To assess the effect of receptor protein kinase inhibition with the multi-target kinase inhibitor sorafenib on the morphogenesis of experimental diabetic retinopathy (DR).*

Material and Methods: *Type 1 diabetes mellitus (DM) was induced in 60 Wistar male rats (age, 3 months) by single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, China) at a dose of 50 mg/kg body weight. Rats were divided into 3 groups 20 rats each: group 1 or controls (no treatment), group 2 (30 units of insulin only (Novo Nordisk A/S, Bagsvaerd, Denmark) every other day), and group 3 (insulin combined with soranib (sorafenib, Cipla, India), 50 µg/kg body weight daily). Five intact rats were used to determine baseline parameters. Rats were euthanized on days 7, 14, 28 and month 3. Paraffin retinal sections were stained with hematoxylin and eosin or AZAN trichrome (BIOGNOST Ltd, Croatia) and visualized by light microscopy.*

Results: *On day 7 after STZ injection, general signs of diabetic damage to the retina (vascular abnormalities, retinal edema and ischemic areas) were observed. Specific signs of DR (microaneurysms along the inner retinal surface, ganglion cell degeneration, reactive gliosis, cell proliferation in the outer retinal layers and an increased inner limiting membrane thickness with accumulation of coarse basophilic fibers) manifested subsequently in the course of experimental type 1 DM. Insulin-only treatment contributed to a reduction in all manifestations of diabetic damage to the retina, whereas application of sorafenib as an adjunct to treatment reduced general signs and prevented specific manifestations of DR.*

Conclusion: *Inhibition of cellular protein kinases made it possible to prevent signs of DR, which prompts further research on opportunities for application of this approach in the treatment of DR.*

Keywords:

diabetic retinopathy, streptozotocin, microaneurysms, retinal degeneration, gliosis, insulin, sorafenib

Introduction

Diabetes mellitus (DM) is a major global public health problem [1]. The global diabetes prevalence in 20-79 year olds in 2021 was estimated to be 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045 [2]. Diabetic retinopathy (DR) is a common complication of DM, affects one-third of diabetes patients and remains the leading cause of blindness among working-age adults worldwide [1].

There are many pathological processes involved in the development of DR and these include hypoxia, oxidative stress with the accumulation of reactive oxygen species and peroxidation products, inflammation with the production of interleukins and growth factors (above all, vascular endothelial growth factor [VEGF]), and activation of protein kinase C and hexosamine pathway [3, 4]. These processes result in mitochondrial damage, cellular apoptosis, inflammation, lipid peroxidation, and structural and functional alterations in the retina, leading to microvascular abnormalities in the form of neovascularization and tissue edema [4].

Diabetic metabolic alterations cause specific vascular lesions that pertain to small vessels (microangiopathy) and involve precapillary arterioles, capillaries and small veins [5]. Pericyte loss, thickening of the basement membrane, and damage and proliferation of endothelial cells are observed. DR may be not only a microvascular disease, but a result of neuroretinal degeneration that appears structurally as neural apoptosis of amacrine and Muller cells, reactive gliosis and thickening of retinal layers [5].

Current pathogenesis-based approaches to the treatment of DR include the development of steroid and anti-VEGF slow-release systems [6]. Inhibiting the angiopoietin-2 pathway (e.g., by blocking Tie2 receptors) is believed to be a feasible adjunct to the above approaches [6].

Receptor protein kinase inhibitors appear to be a promising adjunct to conventional treatment strategies (involving anti-VEGF agents) for DR [6]. Imatinib, a tyrosine kinase inhibitor, has been shown to inhibit VEGF expression, the level of hypoxia inducible factor 1 alpha (HIF-1α) [7] and reduce the expression of caspase-3 (especially in retinal ganglion and Muller cells) and increase the level of antiapoptotic Bax and Bcl-xL proteins in the retina [8] in streptozotocin-induced diabetic rats.

We have demonstrated a persistent hypoglycemic effect of sorafenib, a protein multi-kinase inhibitor, in animal models of type 1 and type 2 diabetes using prolonged fat diet with low-dose streptozotocin [9]. The hypoglycemic effect of sorafenib may be exerted by the down-regulation of the expression of gluconeogenesis-related enzymes through blocking the ERK/c-MYC signaling pathway [10]. Sorafenib (Nexavar®, Bayer Corp, New Jersey, USA) is Federal Drug Agency (FDA) approved for patients with unresectable hepatocellular carcinoma (HCC) and renal cell carcinoma.

Sorafenib activates AMP-activated protein kinase (AMPK) in HCC culture cells under hyperglycemic conditions, which causes Warburg effect and promotes catabolism to restore energy homeostasis [11]. Low-dose sorafenib safely and effectively suppressed nonalcoholic steatohepatitis (NASH) progression through the induction of mild mitochondrial uncoupling and subsequent AMPK activation in both mice and monkeys [12].

The above data indicates the feasibility of conducting this pilot study for assessing the effects of protein kinase inhibitors on the development of morphological signs of experimental DR. One question that the first phase of the study had to answer is whether sorafenib impacts the morphogenesis of experimental DR.

The purpose of the study was to assess the effect of receptor protein kinase inhibition with the multi-target kinase inhibitor sorafenib on the morphogenesis of 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. The study was approved by the Bioethics Committee of the Bohomolets National Medical University. Animals were maintained in vivarium conditions and fed conventionally.

Sixty Wistar male rats (age, 3 months; weight, 140-160 g) were used in experiments. Experimental type 1 diabetes was induced by single intraperitoneal injection of streptozotocin (Sigma-Aldrich, Shanghai, China) at a dose of 50 mg/kg body weight, freshly dissolved in 0.1 cold M citrate buffer of pH 4.5. 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 once in three days. Three days after injection, the blood glucose level in rats that received streptozotocin was 15 mMol/l or higher. Throughout the experiment, animals exhibited marked polydipsia, polyuria, ketonuria, glucosuria and substantially decreased body weight, which confirmed adequacy of the model used for reproducing insulin-dependent ketotic DM in rats. Follow-up duration was 3 months. Five intact rats were used to determine baseline parameters.

In seven days, rats with sustained hyperglycemia were randomly divided in three groups 20 animals each. Group 1 (controls) was composed of untreated hyperglycemic animals. Groups 2 and 3 animals were treated intraperitoneally with short-acting insulin (Actrapid HM Penfill, Novo Nordisk A/S, Bagsvaerd, Denmark) at a dose of 30 units every other day. In addition, group 3 animals were treated with soranib (sorafenib, 200 mg, Cipla Pvt Ltd, Mumbai India) per os at a dose of 50 μg/kg body weight daily.

Rats were euthanized with sodium thiopental (75 μg/kg intraperitoneally) and decapitated on days 7, 14, 28 and month 3 (5 animals at each time point). After decapitation, both eyes were enucleated and fixed in 10% buffered neutral formalin, embedded in paraffin and processed routinely for light microscopy. Paraffin blocks were sectioned onto 2-3-μm slides using a microtome (Shandon Finesse 325 Microtome, Thermo Shandon, Knutsford, Cheshire, UK). Sections were stained with hematoxylin and eosin and AZAN trichrome (BIOGNOST Ltd, Zagreb, Croatia) and visualized by light microscopy [13]. The stained nuclei appeared red, neural fibers and glial cells appeared in red shades, and collagen containing connective tissue cells appeared in blue shades. We failed to find any examples of the application of this dye for staining of diabetic rat retina in the available literature.

Pathohistological studies were conducted at the Department of Morphology, Clinical Pathology and Forensic Medicine, National Healthcare University of Ukraine (Chair, Prof. O.O. Diadyk). Zeiss (Zeiss, Oberkochen, Germany) light microscopes (Axio Imager A2, with a 10 x ocular and 5, 10, 20, and 40x objectives and Axiocam ERc 5s camera; and PrimoStar with Axiocam 105 color camera) and Olympus BX 40 microscope (Olympus, Tokyo, Japan) were utilized for microscopic studies and digital image archiving.

Results

The morphogenesis of DR over the period of follow-up is presented in Fig. 1. There was a decreased cell density in the retinal nuclear layers, with edema of all retinal layers (which was especially notable in the inner plexiform layer), and areas of diffused ischemic damage with vacuolated cytoplasm of neural cells. Retinal and choroidal vessels appeared dilated and occasionally showed signs of microthrombosis.

Numerous vascular abnormalities in the form of microaneurysms were seen in the retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL) (noted with black arrows in Fig. 1). Ganglion cell density decreased

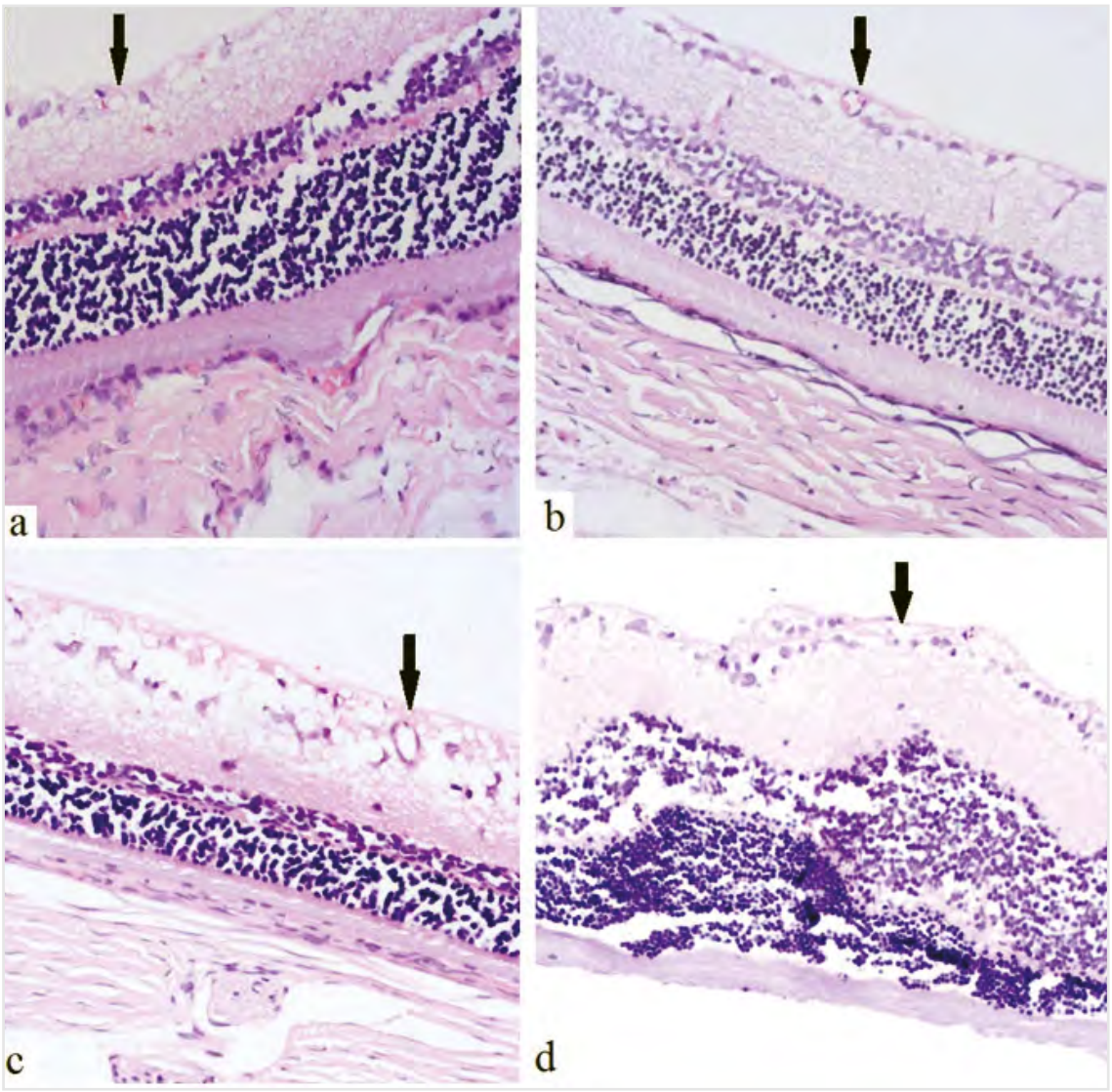

Fig. 1. Retinal preparations of untreated diabetic rats (group 1 or controls) on day 7 (a), day 14 (b), day 28 (c) and month 3 (d) of the follow-up. Hematoxylin and eosin staining. Original magnification, 200x. Note marked edema of all retinal layers, especially the inner plexiform layer, with numerous ischemic areas; low neuronal density and apparent edema in the nuclear layers; retinal venules and choroidal plexus are hyperemic and dilated (a). Further reduction in cell density and loosening of neural fibers; extracellular edema and degeneration of ganglion cells and thinning and structural loss of photoreceptor segments are seen (b, c). Foci of angiogenesis along the inner retinal surface (black arrow) and severe loss of structure in retinal layers (d).

with time, with cells showing signs of degeneration and pyknotic nuclei. These changes increased in severity from day 7 to month 3 (Fig. 1a-c). At the last time point, retinal layers showed severe loss of structure (Fig. 1d). This was caused by severe retinal edema and the development of cell proliferation in the form of dense aggregates of basophil cells in the retinal outer nuclear layer (ONL). Of note was a progressive in the density and thickness of the internal limiting membrane (ILM) with accumulation of coarse basophilic fibers which was clearly seen at large magnification (noted with a white star in Fig. 2c).

Dilated microvessels on the inner retinal surface were seen also in intact animals (noted with a black arrow in Fig. 2a), but they were of small diameter and had thin walls and one lumen. Microaneurysms, as opposed to microvessels, usually had several lumens enclosed within a dense membrane, and were more numerous than microvessels (noted with white arrows in Fig. 2b,c). Intercellular edema and extended ischemic areas were also clearly seen. Ganglion cell density was decreased compared to controls, with these cells showing signs of degeneration, whereas the number and size of astrocytes were increased similar to those observed in reactive gliosis.

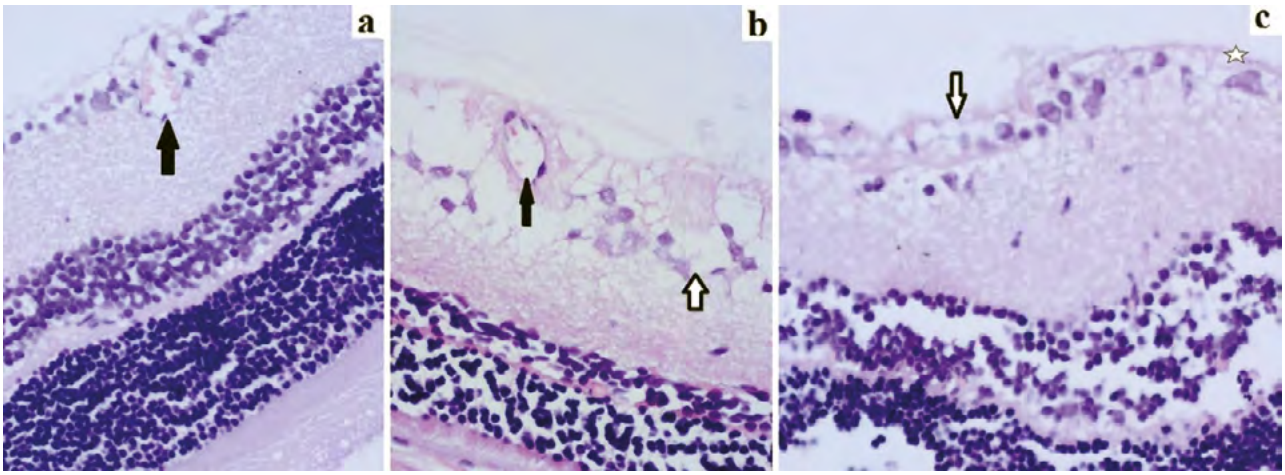

Fig. 2. Retinal preparations of intact rat (a) and untreated diabetic rats (group 1 or controls) on day 14 (b) and month 3 (c) of the follow-up. Hematoxylin and eosin staining. Original magnification, 400x. Retina of intact rat (a). Dilated capillary (black arrow), foci of angiogenesis (white arrow), apparent edema of the inner retina, and thickened internal limiting membrane (b). Long connections of newly formed capillaries on the inner retinal surface (white arrow); hypochromic, edematous or absent ganglion cells; numerous nuclei of large astrocytes (astrogliosis), and thickened retinal nerve fiber layer (white arrow) (c).

In the retina of the animals treated with insulin only (Fig. 3a) and insulin plus sorafenib (Fig. 3b), early signs of DR were less marked compared to untreated diabetic rats. In group 2, cell density in the retinal nuclear layers appeared to be the same as in intact rats, with practically no edema or ischemic areas in the retinal layers. Ganglion cell density, however, appeared to be decreased, and the outer retinal layers showed loss of structure (Fig. 3a).

In group 3, insulin combined with sorafenib prevented the development of early signs of DR (Fig. 3b). In addition, the retina in general had a normal structure, and no DR signs typical for the control group were seen. Moreover, not any specific early signs of DR (angiogenesis foci or cell proliferation) were noted.

In order to clarify the features of the retinal morphogenesis in diabetes, we performed an immunohistochemical study on retinal sections on slides using Azan trichrome staining which is commonly used for visual differentiation between neural cells [13]. In intact rats, ONL neurons (rod and cone neurons) appeared reddish yellow. Inner nuclear layer (INL) neurons and ganglion neurons showed polychromatophilic staining, and astrocytes in the RNFL appeared pink (Fig. 4a). Interestingly, the retinal plexiform layers and RNFL fibers showed blue staining. Inner photoreceptor segments (IPS) versus outer photoreceptor segments (OPS) were clearly differentiated by color (blue and pink, respectively).

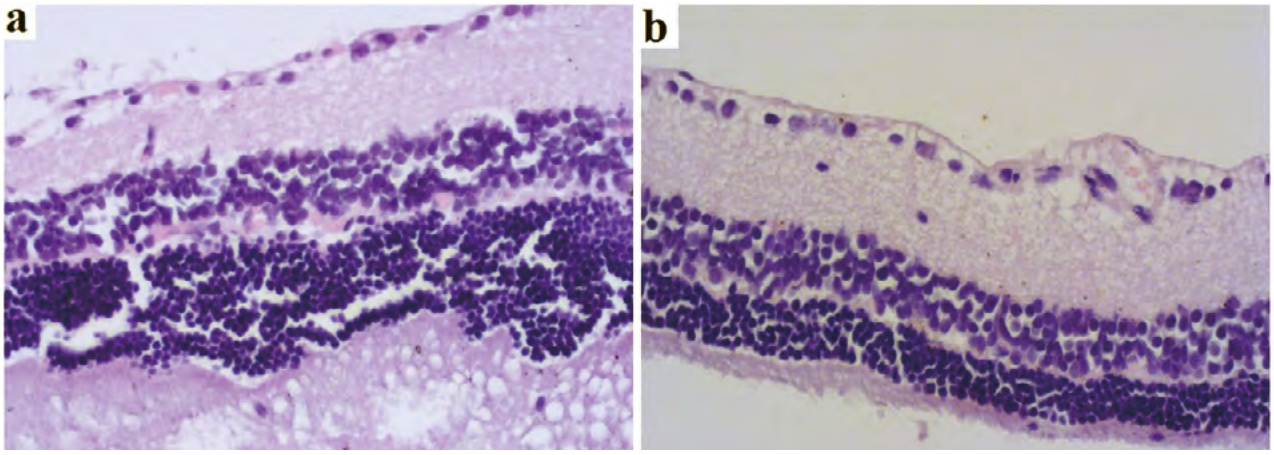

Fig. 3. Retinal preparations of diabetic rats treated with insulin only (group 2) (a) and insulin combined with sorafenib (group 3) (b) at month 3 of the follow-up. Hematoxylin and eosin staining. Original magnification, 400x. Reduction in nuclear layer cell density and neuronal degeneration signs are not apparent, no foci of angiogenesis are seen, and there is an increase in the cell density and thickness and loss of structure in the outer nuclear layer (a). Retinal structure appears well preserved, with no foci of angiogenesis in the inner layers or cell proliferation in the outer layers (b).

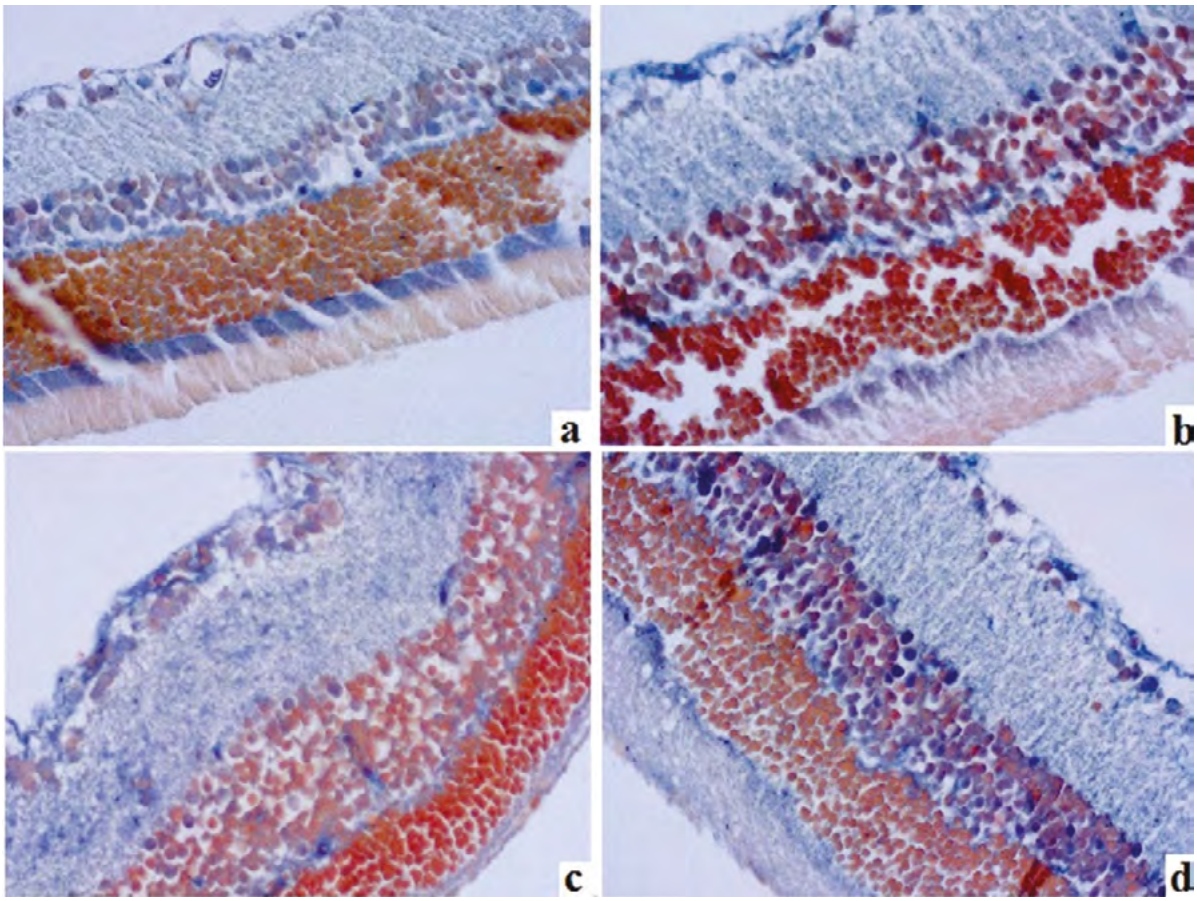

Fig. 4. Retinal preparations of intact rat (a), untreated diabetic rat (b) and diabetic rats treated with insulin only (group 2) (c) and insulin combined with sorafenib (group 3) (d) at month 3 of the follow-up. Azan trichrome staining. Original magnification, 400x.

At 3 months, in the presence of signs of DR in untreated diabetic animals, ONL cells showed intensive red staining, especially in the areas of high cell density and proliferation (Fig. 4b). In the INL, there were a large proportion of variously stained cells: large round cells appeared blue, most cells were polygonal in shape and showed polychromatophilic staining, and a small proportion of cells showed terra cotta staining. More distinctive blue staining was seen in plexiform layer plexuses and along the inner retinal surface. Staining was, however, significantly less intensive in the photoreceptor layer.

Diabetic rats that received insulin-only treatment showed less apparent general and specific signs of DR compared to those untreated, with a reduction in the intensity of red staining in the nuclear layers (Fig. 4c), but still rather high blue intensity of the RNFL. Diabetic rats that received insulin plus sorafenib showed a comparatively normal structure of the retinal layers, with no increase in the density or conglomeration of fibers along the inner retinal surface (Fig. 4d). This suggests that sorafenib inhibits reactive astrocyte gliosis in the presence of DR.

Therefore, type 1 diabetes rats showed general signs of DR, like reduced neural cell density, tissue edema, ischemic regions and dilated retinal vessels. Specific signs of DR included microaneurysms on the inner retinal surface, ganglion cell degeneration, reactive gliosis, cell proliferation in the outer retinal layers and an increased ILM thickness with accumulation of coarse basophilic fibers. Insulin-only treatment resulted in the attenuation of all signs of diabetic damage to the retina, whereas sorafenib reduced general signs and prevented the development of specific signs of DR. Azan thichrome staining enabled clear differentiation between the inner photoreceptor layer and the outer photoreceptor layers; cells in the nuclear layers; and neural fibers on the inner retinal surface.

Discussion

Diabetic retinal microangiopathy is accompanied by ischemia, neovascularization, increased vascular permeability and edema in the retina [14]. In early DR, metabolic abnormalities and oxidative stress result the accumulation of toxins in the retina, thus impairing neurovascular relationships. This is manifested by hyperemia of the retinal venular bed, microthrombus formation, retinal edema and ischemia [15]. In our opinion, acute and persistent hyperemia that developed in early days after streptozotocin injection caused initial toxic events (hyperemia and dilation of venules in the outer plexiform layer), which were found on day 7.

Subsequent development of secondary mechanisms of damage (mostly hypoxic and inflammatory damage) was accompanied by the progression of vascular changes with microaneurysm formation. In addition, DR progression has been reported to be accompanied by microhemorrhages, cotton-wool spots (an ischemic lesion of the RNFL), accumulation of exudates, intraretinal microvascular abnormalities (acellular capillaries and capillary non-perfusion) and increased vascular permeability [16].

We also noted signs of neurodegeneration like reduced thickness and cell density of the nuclear layers, ganglion cell death and reactive gliosis. Degeneration of ganglion cell bodies and axons was especially important. They are believed to be the retinal cells most susceptible to degeneration under diabetic conditions [17]. We observed changes of this type in untreated diabetic animals throughout the 3-month follow-up.

Formation of microaneurysms and development of cell proliferation were among the specific signs of DR found in the current study. Microaneurysms are a manifestation of diabetic microangiopathy; their mechanism involves retinal microglial and mactoglial activation (reactive gliosis) by products of abnormal glucose metabolism [5, 15, 17]. Activated glial cells are the source of numerous pro-inflammatory cytokines and growth factors (above all, VEGF, which contributes to increased vascular permeability, pericyte and endothelial apoptosis, and retinal neovascularization) [5, 16]. In the current study, cell proliferation developed in the inner retinal layers in densely packed round cells with intense staining of basophilic cytoplasm. We believe that Muller glial–mesenchymal transition (GMT) is a potential mechanism of this cell proliferation. GMT has been reported as the fibrogenic mechanism of DR [18]. Muller cell transdifferentiation into myofibroblasts might be triggered by excessive levels of VEGF and transforming growth factor-beta in the retina in DR.

Treatment of diabetic rats with insulin only resulted in an expected reduction in the intensity of diabetic retinal damage, but did not eliminate the general or specific signs of DR. The effect of sorafenib was characterized by the prevention of all manifestations of DR, which could be explained by the inhibition of protein kinases, particularly of the Ras/Raf-1/MEK/ERK cascade that is activated in DR [19]. The development of diabetic retinal neurodegeneration was also prevented through the inhibition of the p38MAPK pathway with Raf-1 kinase inhibitory protein [20]. It is noteworthy that, under diabetic conditions, the Ras/Raf/MEK/ERK cascade has an important role in the activation of VEGF and retinal matrix metalloproteinase-9 (MMP-9) resulting in the apoptosis of retinal capillary cells [19].

The effects of sorafenib reported in the current and other studies can be explained by molecular mechanisms. Sorafenib is a multi-target kinase inhibitor of cell proliferation in vitro, which inhibits intracellular signal kinases, particularly, the Raf/MEK/ERK pathway and receptor tyrosine kinases [21]. Deactivation of phosphorylation of ERK protein is one of the mechanisms of sorafenib inhibiting gastric cancer [22]. Therefore, inhibition of cellular protein kinases through inhibition of kinase cascades can slow down the impact of excessive levels of pro-inflammatory and growth factors in DR. This explains well the prevention of abnormal cell proliferation and microaneurysms in diabetic rats treated with sorafenib.

Therefore, the results of this study suggest that inhibition of cellular protein kinases is a promising pathogenesis-based approach to the therapeutic treatment of DR. Studies are underway to assess their efficacy in combination with anti-VEGF therapy for DR [6]. The results of the current study, however, indicate the presence of an independent effect of sorafenib which prevented manifestations of DR. This may be especially important in the treatment of early DR in which anti-VEGF therapy is not indicated yet. This hypothesis prompts further research on (1) the molecular mechanisms underlying the effect of sorafenib in experimental DR and (2) the impact of protein kinase inhibitors in experimental type 2 DM.

Conclusion

General signs of damage to the retina (vascular abnormalities, retinal edema and ischemic regions) were found already at early stages, whereas specific signs of DR (microaneurysms on the inner retinal surface, ganglion cell degeneration, reactive gliosis, cell proliferation in the outer retinal layers and an increased ILM thickness with accumulation of coarse basophilic fibers) manifested subsequently in the course of experimental type 1 DM. Insulin-only treatment contributed to a reduction in manifestations of diabetic damage to the retina, whereas application of sorafenib as an adjunct to treatment contributed to maintenance of cell density in the retinal nuclear layers and prevented retinal edema and ischemia, dilation of retinal vessels, microaneurysms, ganglion cell degeneration, reactive gliosis, and retinal cell proliferation.

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Disclosures

Received: 12.10.2024

Accepted: 12.02.2025

Corresponding author: *Kateryna O. Usenko, Cand Sc (Med), Ophthalmologist, Ophthalmology Department, Institute of Post-graduate Education, Bogomolets National Medical University (Kyiv, Ukraine), E-mail: usenko1205@gmail.com*

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Conflict of interest: *No conflict of interest is declared.*